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Changes in bacterial metabolism as a response to dissolved organic matter modification during protozoan grazing in coastal Cantabrian and Mediterranean waters

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Summary

We explored how marine dissolved organic matter (DOM) altered by bacterial growth and protozoan grazing modify the metabolism of Southeastern Cantabrian Sea (CS) and NW Mediterranean Sea (MS) coastal bacterial communities. Major metabolic features were measured in treatments with half of the natural water replaced by water with different DOM quality, characterized by fluorescent DOM analysis and collected from key times of the predator–prey curve. In both ecosystems, protozoan-altered DOM led to similar increases in bacterial carbon demand (238% and 213%) and decreases in bacterial growth efficiency (BGE: 56% for the CS and 46% for the MS). These low BGEs were caused by similar bacterial production but much higher bacterial respiration rates, which in turn were positively related to aminopeptidase activity. However, in the CS bacterial community dominated by *Bacteroidetes* (41%), the enhanced hydrolytic activity was produced at a lower metabolic cost than in the MS, dominated by SAR11 (47%), which suggests a better adaptation of *Bacteroidetes* to the DOM altered during protozoan grazing. These results highlight protozoan grazing as a relevant factor influencing BGE in coastal ecosystems, and relate bacterial community composition to the major metabolic processes that result after a change in the quality of marine DOM.

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Introduction

Dissolved organic matter (DOM) represents a major fraction of total organic matter in the ocean (Sharp, 2002), and heterotrophic bacteria and archaea are the main planktonic organisms involved in its consumption (Pomeroy, 1974). The availability of DOM to heterotrophic bacteria depends on its biochemical composition and molecular size, and therefore, any structural or compositional transformation of DOM will affect several aspects of bacterial metabolism, including carbon consumption, growth rate, respiration rate, enzymatic degradation and demand for inorganic nutrients (Findlay, 2003). Resource-mediated changes in bacterial activity have the potential to alter ecosystem processes, such as the balance between the fraction of organic matter that is respired and the fraction which remains available to be transferred to higher trophic levels (Smith and Kemp, 2003; Lennon and Cottingham, 2008). Bacterial growth efficiency (BGE) is a variable that reflects this balance and therefore estimates the fate of organic carbon inputs, determining whether bacteria act as a link (recyclers) or sink (mineralizers) in aquatic ecosystems (Sherr and Sherr, 1996; del Giorgio and Cole, 1998; Carlson *et al.*, 2007).

Marine DOM undergoes continuous transformations both by the action of abiotic factors such as ultraviolet light (Moran and Zepp, 1997; Schmitt-Kolpin *et al.*, 1998), and also by the biological processes carried out by bacteria, protozoa and viruses (Nagata, 2000; Ogawa *et al.*, 2001; Motegi *et al.*, 2009). The changes in the biochemical characteristics and the quality of DOM can be linked to its optical properties (Stedmon *et al.*, 2003), as fluorescence provides reliable information about the redox state, quality (humic-like or protein-like materials) and biological and photochemical reactivity of DOM (Miller *et al.*, 2006; Fellman *et al.*, 2010). Marine fluorescent dissolved organic matter (FDOM) has been mainly considered a by-product of the bacterial metabolism (Chen and Bada, 1992; Kramer and Herndl, 2004; Nieto-Cid *et al.*, 2006; Yamashita and Tanoue, 2008), but some recent studies have shown that it can also be produced by eukaryotic cells (Steinberg *et al.*, 2004; Urban-Rich *et al.*, 2006; Ortega-Retuerta *et al.*,

2009; Romera-Castillo *et al*., 2010). Several molecular species derived from bacteria including porins (Yamada and Tanoue, 2003), D-amino acids (McCarthy *et al*., 1998; Kaiser and Benner, 2008), muramic acid (Benner and Kaiser, 2003; Kaiser and Benner, 2008) and lipopolysaccharides (Wakeham *et al*., 2003) have been detected in marine refractory DOM and might be released by direct exudation during bacterial growth (Jiao *et al*., 2010) and be relatively unavailable to other bacteria. Protozoan grazing and lysis by viruses, which usually follow periods of intense bacterial growth, may also have a relevant impact on DOM composition (Kujawinski *et al*., 2004; Gruber *et al*., 2006; Boras *et al*., 2010). The grazing activity of bacterivorous protozoa release undigested components as well as high molecular weight (HMW) and low molecular weight (LMW) dissolved organic compounds (Taylor *et al*., 1985), which produce changes in the chemical composition of the DOM (Nagata and Kirchman, 1991; Strom *et al*., 1997; Nagata, 2000; Carlson, 2002). Among other compounds, researchers have identified dissolved free and combined amino acids (Nagata and Kirchman, 1991), colloidal material (Koike *et al*., 1990) and enzymes (Nagata, 2000). In addition, the release of altered DOM will be enhanced in microenvironments where there is strong bacterioplankton growth, intense grazing pressure and viral lysis (Artolozaga *et al*., 2002). This altered DOM enhancement might result in microheterogeneity in the lability of the sources of C and is expected to have an impact on the metabolism and physiological behaviour of other bacteria that approach these microenvironments. But to our knowledge, the influence of protozoan grazing on bacterial BGE mediated by DOM alteration has not yet been studied.

At short time scales, any alteration in the quality or quantity of the carbon available to bacteria is likely to cause bacterial phenotypic changes mediated by the regulation of genetic expression, but in the long term, the presence of microbially altered DOM may also cause changes in community composition (Findlay *et al*., 2003). It is well known that different groups of bacteria are responsible for mineralizing different fractions of DOM (Cottrell and Kirchman, 2000; Elifantz *et al*., 2007), and thus, the metabolic response of a given bacterial community to new and altered DOM will depend on its species composition. In this sense, Comte and del Giorgio (2011) postulated that the determinant factor for this phenotypic response might be the intrinsic properties of the communities, specifically those related to the level of metabolic and functional plasticity of the dominant phylotypes.

The aim of this work was to determine how DOM alteration caused by bacterial growth and during protozoan grazing affects BGE and consequently net ecosystem DOM retention. Bacterial respiration, bacterial production and BGE were measured in coastal bacterial assemblages which had part of its DOM replaced by DOM derived from different moments of the predator–prey coexistence. We hypothesize that the phenotypic changes resulting from the use of DOMs of different qualities generate a large metabolic cost to bacteria, which will be reflected in decreasing BGEs. In order to determine the role that community composition plays in these phenotypic responses, we conducted this study in two coastal ecosystems dominated by different groups of bacteria, Armintza Station [Southeastern (SE) Cantabrian Sea (CS)] and the Blanes Bay Microbial Observatory [Northwestern (NW) Mediterranean Sea (MS)].

Results

Phase I: Collection and characterization of altered DOM

Growth of the microbial communities showed the expected evolution from a typical predator–prey system (Fig. 1). The bacterial community began to grow exponentially in the first hours of incubation, with a mean growth rate of 1.45 ± 0.28 d^{-1} in CS and ranging from 0.55 to 0.82 d^{-1} in MS waters, achieving their maximum abundance in the first 28–40 h ($4.9 \times 10^6 \pm 9.0 \times 10^5$ cell ml^{-1} in the CS and ranging from 1.4×10^6 to 1.5×10^6 cell ml^{-1} in the MS). At the point of maximal bacterial abundance, before the protozoan abundance was maximal, DOM-Bact was collected. In response to the increase in the number of prey, protozoa started growing exponentially up to 56–70 h of incubation, at which time DOM-Prot was collected. Protozoa showed specific growth rates of 1.65 ± 0.49 d^{-1} in the CS and of 0.72 d^{-1} in the MS (range 0.63–0.80 d^{-1}), and they reached densities of $1.4 \times 10^4 \pm 1.8 \times 10^3$ cell ml^{-1} in the CS and ranging from 3.7×10^3 to 7.0×10^3 cell ml^{-1} in the MS experiments. Finally, the DOM-End was collected when no growth was detected in bacteria or protozoa, after 96–120 h of incubation.

To characterize the DOM, peak-T (to identify protein-like substances), peak-C and peak-M (as indicators of humic-like substances) fluorescences were analysed. Because the fluorescence of peak-C and peak-M maintained the same trend and were not significantly different from each other (data not shown), humic-like substances were analysed by adding the two peaks. The initial DOM of CS microcosms had a higher amount of humic-like and protein-like substances than the initial DOM of MS microcosms (Table 1A). However, in both ecosystems, the altered DOMs collected in phase I after protozoan grazing contained on average a greater amount of humic-like substances and protein-like substances than the initial DOMs (Wilcoxon test, $P = 0.028$ for both). The concentration of humic compounds increased during bacterial growth and subsequently continued to rise up

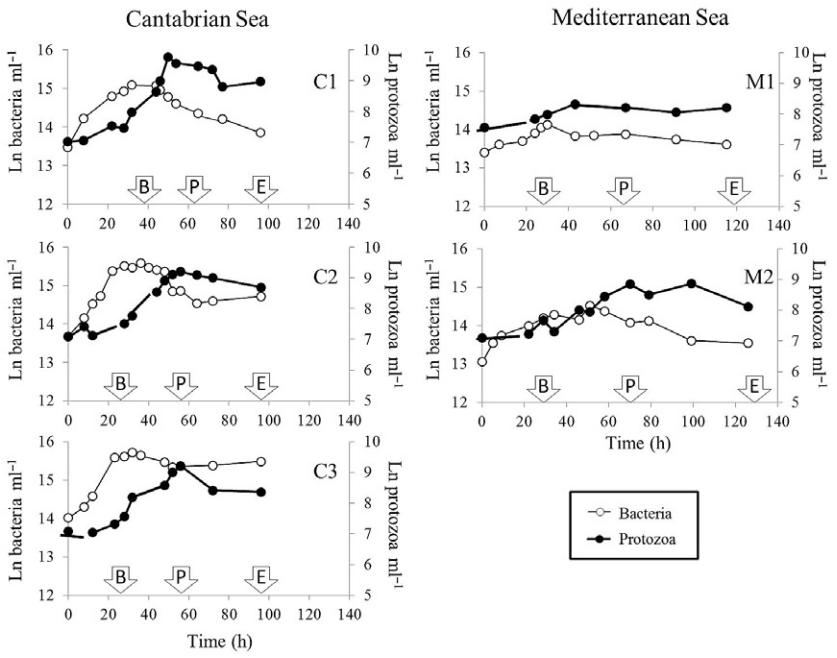

Fig. 1. Time course of bacterial and protozoan communities in the phase I microcosms in the three experiments carried out in the Cantabrian Sea (C1, C2 and C3) and in the two experiments carried out in the Mediterranean Sea (M1 and M2). Arrows indicate when DOM-Bact (B), DOM-Prot (P) and DOM-End (E) samples were taken.

until the moment of maximum abundance of protozoa, with this increase being far higher in the experiments of the MS: the increase of humic-like substances with respect to the initial values were on average 4.8% and 5.1% in DOM-Bact and DOM-Prot in the CS and 21.3% and 72.1% in the MS. The concentration of protein-like substances (Coble's peak-T) also increased during the first 70 h of incubation, especially during protozoan growth (on average 26.2% in the CS and 24.9% in the MS). Thus, DOM-Prot was characterized by a greater amount of humic-like and protein-like substances than DOM-Bact and DOM-End in both ecosystems.

Table 1. (A) Humic-like (peak-M + peak-C) and protein-like (peak-T) substances (quinine sulfate units, QSU) detected in the Cantabrian Sea and the Mediterranean Sea phase I microcosms in the initial time, in the maximum of bacterial abundance (DOM-Bact), in the maximum of protozoan abundance (DOM-Prot) and in the final time of the microcosms (DOM-End). (B) Relative (in %) consumption (–) or production (+) of humic-like (peak-M + peak-C) and protein-like (peak-T) substances in the phase II treatments in the two marine systems.

A) FDOM characterization of the altered DOMs (phase I microcosms)

	Cantabrian Sea ($n = 3$)				Mediterranean Sea ($n = 2$)			
	Initial	DOM-Bact	DOM-Prot	DOM-End	Initial	DOM-Bact	DOM-Prot	DOM-End
Humic-like (QSU)	3.74 ± 1.51 [2.77–6.41]	3.92 ± 1.20 [2.86–5.83]	3.93 ± 1.64 [2.99–6.84]	n.d.	1.22 [1.03–1.40]	1.48 [1.40–1.55]	2.1 [1.92–2.28]	1.75 [1.43–2.06]
Protein-like (QSU)	1.95 ± 0.36 [1.48–2.31]	2.23 ± 0.45 [1.56–2.50]	2.46 ± 0.68 [1.69–3.32]	n.d.	1.81 [1.15–2.48]	1.37 [1.09–1.65]	3.38 [1.76–5.01]	2.26 [1.17–3.34]
Peak-T/Peak-(M + C)	0.52	0.57	0.62	n.d.	1.48	0.93	1.61	1.29

B) FDOM consumption (–) or production (+) (phase II treatments)

	Cantabrian Sea ($n = 3$)				Mediterranean Sea ($n = 2$)			
	Control	DOM-Bact	DOM-Prot	DOM-End	Control	DOM-Bact	DOM-Prot	DOM-End
Δ Humic-like (%)	74.3 ± 33.8 [50.4–98.2]	n.d.	96.3 ± 0.0 [96.3–n.d.]	n.d.	40.4 [30.1–50.6]	83.7 [10.0–157.4]	42.9 [33.1–52.6]	72.8 [30.1–115.5]
Δ Protein-like (%)	–25.4 ± 8.1 [(–31.1)–(–19.6)]	n.d.	–49.3 ± 0.0 [(–49.3)–n.d.]	n.d.	104.9 [18.8–191.0]	78.1 [33.4–122.8]	121.7 [118.4–125.0]	134.9 [114.4–155.4]

Data are given as mean ± standard deviation and range of values for the Cantabrian Sea (sampled three times) and as mean and range of values for the Mediterranean Sea (sampled two times)
n.d., no data.

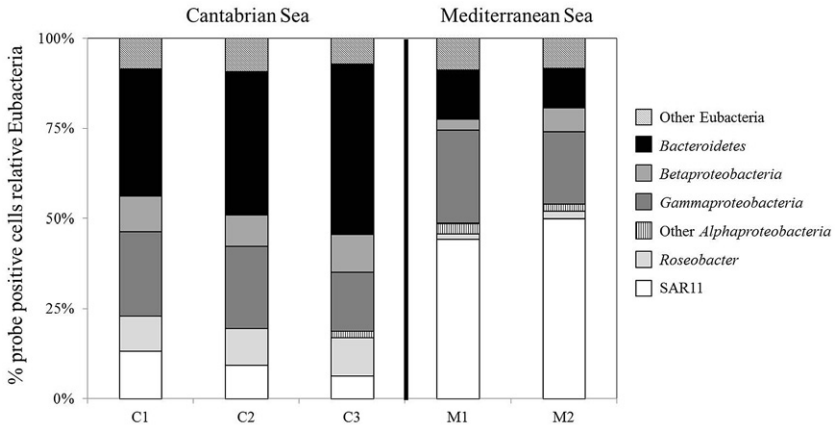

Fig. 2. Bacterial community composition measured by CARD-FISH in samples taken for phase II treatments in the Cantabrian Sea (C1, C2 and C3) and in the Mediterranean Sea (M1 and M2).

Phase II treatments: Characteristics of the bacterial communities in the CS and the MS

No significant differences were found in either abundance or relative proportion of the major bacterial groups between the natural community and the controls. After concentration by filtration and subsequent dilution of the community, the bacterial densities were $2.4 \times 10^6 \pm 9.2 \times 10^5$ cell ml^{-1} in the CS and ranged from 5.4×10^5 to 5.9×10^5 cell ml^{-1} in the MS (recovery levels of $80.3 \pm 10.8\%$ and $94.8 \pm 5.4\%$ respectively). Relevant differences in the initial bacterial community composition (BCC) were detected between the two ecosystems (Fig. 2). Whereas in the CS, the dominant phylogroup was *Bacteroidetes* bacteria ($41.0 \pm 3.3\%$), with a low contribution of SAR11 ($9.7 \pm 2.0\%$); in the MS, SAR11 was the most abundant phylogroup ($47.0 \pm 2.8\%$ of the Eubacteria).

Results on bacterial activities are summarized in Table 2. In the controls, the cell-specific bacterial respirations (BRs) were on average 4.5 times lower in the CS than in the MS, while cell-specific bacterial production (BP) were on average 2.8 times higher in the CS. As a result, cell-specific bacterial carbon demand (BCD) was lower in the CS than in the MS, but BGE was on average 10.3 times higher in the CS (28.9%) than in the MS (2.8%). However, and because of the low number of samples analysed (three for the CS and two for the MS), no significant differences were observed with a confidence interval of 95% for any of the three parameters (Mann–Whitney *U*-test, $P = 0.083$ in the three).

The aminopeptidase (AMP) activity was the dominant extracellular enzymatic activity in both ecosystems. On average cell-specific AMP was 1.6 times higher in CS ($181.6 \times 10^{-8} \pm 87.7$ nmol cell^{-1} d^{-1}) than in MS waters (115.3×10^{-8} nmol cell^{-1} d^{-1}, ranging from 106.2 nmol cell^{-1} d^{-1} to 124.3 nmol cell^{-1} d^{-1}). In contrast, the sum of cell-specific glucosidase activities was 1.3 times lower in CS than in MS waters. As a result, the AMP : Glu ratio measured was around two times higher in the CS (20.9) than in the MS (10.1).

The dissolved organic carbon (DOC) concentration was on average 1.3 times higher in the CS (146.7 ± 39.0 μM) than in the MS (109.2 μM, range 100.9–117.4 μM) although the differences were not statistically significant (Table 3).

Phase II treatments: Effect of biologically altered DOM on bacterial metabolic activity

The comparison of the mean values for cell-specific BR, BP, BCD and BGE between each treatment and its corresponding control for the two systems is shown in Fig. 3. Analysis of the detected differences suggests the occurrence of significant metabolic changes in bacteria as a response to the presence of altered DOM. A common feature for the bacterial communities from both ecosystems was that their cell-specific BRs were significantly higher in the treatments with altered DOM than in the controls (Wilcoxon test, $P < 0.05$, for the three treatments using data standardized to the control). It was remarkable that the highest differences were detected in the treatments, which contained DOM-Prot (2.7 and 2.4 times higher than the control in CS and MS waters respectively). These communities, however, did not significantly change their cell-specific BPs to the same extent: the values obtained in the treatments were only slightly higher or lower than in their controls. The three altered DOMs caused greater cell-specific BCD and lower BGE in the two ecosystems, with the only exception of the BGE in the DOM-Bact treatment in the MS. However, significant differences in cell-specific BCD were only found in DOM-Prot treatment (Wilcoxon test, $P < 0.05$) and in BGE in DOM-Prot and DOM-End (Wilcoxon test, $P < 0.05$) with data standardized to the control. The lower BGEs observed

Table 2. Bacterial activity data from phase II treatments (DOM-Bact, DOM-Prot and DOM-End) and controls. Cell-specific bacterial respiration (BR), cell-specific bacterial production (BP), cell-specific bacterial carbon demand (BCD), bacterial growth efficiency (BGE), cell-specific aminopeptidase activity (AMP), cell-specific α-glucosidase activity (α-Glu) and cell-specific β-glucosidase activity (β-Glu).

	Cantabrian Sea ($n = 3$)				Mediterranean Sea ($n = 2$)			
	Control	DOM-Bact	DOM-Prot	DOM-End	Control	DOM-Bact	DOM-Prot	DOM-End
BR ($\times 10^{-9}$ µg C cell^{-1} d^{-1})	42.1 ± 20.5 [18.9–57.7]	83.6 ± 69.6 [34.5–163.2]	121.5 ± 92.0 [41.1–221.8]	77.2 ± 71.2 [20.2–162.5]	188.5 [133.5–243.6]	234.5 [217.8–251.2]	406.9 [389.0–424.8]	338.4 [264.6–412.2]
BP ($\times 10^{-9}$ µg C cell^{-1} d^{-1})	14.8 ± 2.3 [13.1–17.4]	12.2 ± 2.8 [9.8–15.3]	14.1 ± 5.1 [8.2–16.6]	18.5 ± 10.0 [8.2–28.2]	5.3 [4.9–5.6]	6.1 [4.7–7.5]	6.1 [5.8–6.4]	6.3 [6.0–6.6]
BCD ($\times 10^{-9}$ µg C cell^{-1} d^{-1})	56.9 ± 21.1 [32.6–70.8]	95.8 ± 72.1 [45.9–178.5]	135.5 ± 95.7 [49.3–238.5]	95.7 ± 76.9 [28.3–181.7]	193.5 [138.5–248.4]	241.1 [226.4–255.9]	413.0 [395.4–430.6]	344.9 [270.9–418.8]
BGE (%)	28.9 ± 12.1 [18.6–42.2]	16.4 ± 8.2 [8.6–25.0]	12.8 ± 5.1 [7.0–16.6]	25.3 ± 9.5 [10.6–28.8]	2.8 [2.0–3.6]	2.8 [1.8–3.8]	1.5 [1.3–1.6]	2.0 [1.6–2.3]
AMP ($\times 10^{-8}$ nmol cell^{-1} d^{-1})	181.6 ± 87.7 [80.4–235.1]	185.8 ± 54.0 [148.5–247.7]	306.1 ± 206.6 [158.1–542.1]	204.4 ± 48.2 [153.4–249.2]	115.3 [106.2–124.3]	118.1 [113.9–122.2]	145.3 [111.4–179.2]	128.8 [118.9–138.6]
α-Glu ($\times 10^{-8}$ nmol cell^{-1} d^{-1})	3.4 ± 2.0 [2.0–4.8]	1.6 ± 2.2 [0.0–3.1]	3.1 ± 0.3 [2.9–3.3]	4.3 ± 1.9 [3.0–5.6]	4.0 [3.4–4.6]	3.7 [2.1–5.3]	6.4 [6.2–6.6]	4.5 [3.0–6.0]
β-Glu ($\times 10^{-8}$ nmol cell^{-1} d^{-1})	5.3 ± 5.3 [1.5–9.1]	1.5 ± 1.1 [0.7–2.3]	3.0 ± 0.2 [2.9–3.1]	2.8 ± 0.2 [2.6–2.9]	7.4 [5.7–9.0]	7.2 [4.5–9.9]	7.2 [5.4–8.9]	8.0 [5.5–10.5]
AMP/*Glu	20.9	59.9	50.2	28.8	10.1	10.8	10.7	10.3

Data are given as mean ± standard deviation and range of values for the Cantabrian Sea (sampled three times) and as mean and range of values for the Mediterranean Sea (sampled two times).
*Glu refers to the sum of α and β -glucosidase activities.

were due to their higher cell-specific BR in the presence of the new and microbially altered DOM.

The presence and use of the different altered DOM also accounted for changes in bacterial extracellular enzyme activities (EEA), although they were different in the two coastal marine ecosystems (Table 2). In both systems, we observed a noticeable increase in cell-specific AMP activity in the presence of the DOM-Prot: 68.5% increase respect to the controls in CS and 26% in MS waters. However, the sum of cell-specific glucosidase activities was reduced by 30% in the CS, while in the MS, it increased by 20%. Thus, in CS waters, the AMP : Glu ratio of the bacterial communities under DOM altered by grazing increased 2.4 times, but in MS waters, this ratio remained quite similar to what was observed in the controls.

Despite these changes in enzymatic activities, bacteria did not seem to be able to consume humic-like substances, or at least, these substances were not consumed at a greater speed than they were produced because they accumulated in the two ecosystems, both in controls and in treatments (see Table 1B). With regard to protein-like substances, whereas in the MS they accumulated in the treatments containing DOM-Prot and DOM-End, in the CS the bacterial communities were able to consume these substances efficiently: as much as the 25.4 and 49.3% of the protein-like substances present in the control and DOM-Prot treatments were consumed within 24 h of incubation.

In both ecosystems, DOC content in the treatments containing DOM-Bact was lower than in the controls, on average 5.5% less in the CS and 5.9% less in the MS (Table 3). The DOC concentrations were similar in DOM-Bact and DOM-Prot treatments, whereas in the DOM-End treatments, a slight decrease was observed in both ecosystems in relation to DOM-Prot (4.2% and 9.9% in the CS and in the MS respectively).

Discussion

We clearly show that, in both marine ecosystems, microbial activity (i.e. bacterial growth but particularly the subsequent protozoan growth and feeding on bacteria) causes alterations in the composition of the marine DOM, which in turn trigger major metabolic changes in the bacterial communities. The use of microbially altered DOMs, and especially of DOM altered during the predatory action of protozoa (DOM-Prot), led to an increase in cell-specific BCD of up to 238% and 213% as well as to a decrease in the growth efficiency of up to 56% and 46% for the bacterial communities of the CS and the MS respectively. These decreases in BGE were not due to lower production rates, which remained fairly similar, but to much higher respiration rates, which explained the enhanced

Table 3. DOC concentration (μM) measurements from phase II treatments (DOM-Bact, DOM-Prot and DOM-End) and controls, of the experiments in the Cantabrian Sea (C1, C2, C3) and experiments in the Mediterranean Sea (M1, M2).

	Cantabrian sea					Mediterranean sea			
	Control	DOM-Bact	DOM-Prot	DOM-End		Control	DOM-Bact	DOM-Prot	DOM-End
C1	112.6 ± 0.6	105.7 ± 0.5	103.7 ± 0.3	97.9 ± 0.5	M1	117.4 ± 0.2	103.4 ± 0.0	102.8 ± 0.5	89.0 ± 0.5
C2	138.2 ± 0.3	130.3 ± 0.5	132.1 ± 0.4	132.0 ± 0.2	M2	100.9 ± 1.1	84.8 ± 0.4	88.8 ± 0.5	82.9 ± 0.5
C3	135.2 ± 0.2	128.7 ± 0.4	127.1 ± 0.5	120.4 ± 0.3					

Data are given as mean ± standard error of three replicates.

carbon consumptions. The DOC concentrations in the treatments was slightly lower than that available in the controls, presumably due to it consumption and transformation into microbial biomass and CO_2 during the phase I of the experiments (Table 3). However, no clear relationship was found between these small differences in the DOC concentration and the changes in BGE. In this way, in the MS, the differences in DOC concentration between the control and DOM-Bact were not related to differences in BGE. By contrast, the BGE in DOM-Prot treatment was significantly lower than that in DOM-Bact, although no differences were found in the DOC concentration. Similar absence of relationship was found in the experiments carried out in the CS. Thus, we consider that the effects observed in bacterial metabolism were mainly attributable to the quality and not to the quantity of the added DOM. On the other hand, any nutrient limitation in the treatments that could mask the metabolic changes provoked by altered DOM was tested out by incorporating inorganic nutrients to one additional treatment which contained DOM-Prot; no significant differences were found in BR, BP or BGE with respect to the same treatment without nutrients (data not shown).

The high similarity of these metabolic responses in the two systems was remarkable because the bacterial communities of the controls from the CS and the MS were completely different, both in composition and in metabolic activity. The bacteria from the CS were slightly more numerous, dominated by *Bacteroidetes*, and as much as 10 times more efficient in processing carbon (higher BGE) than their counterparts of the MS. In contrast, MS bacterial communities were dominated by SAR11, which

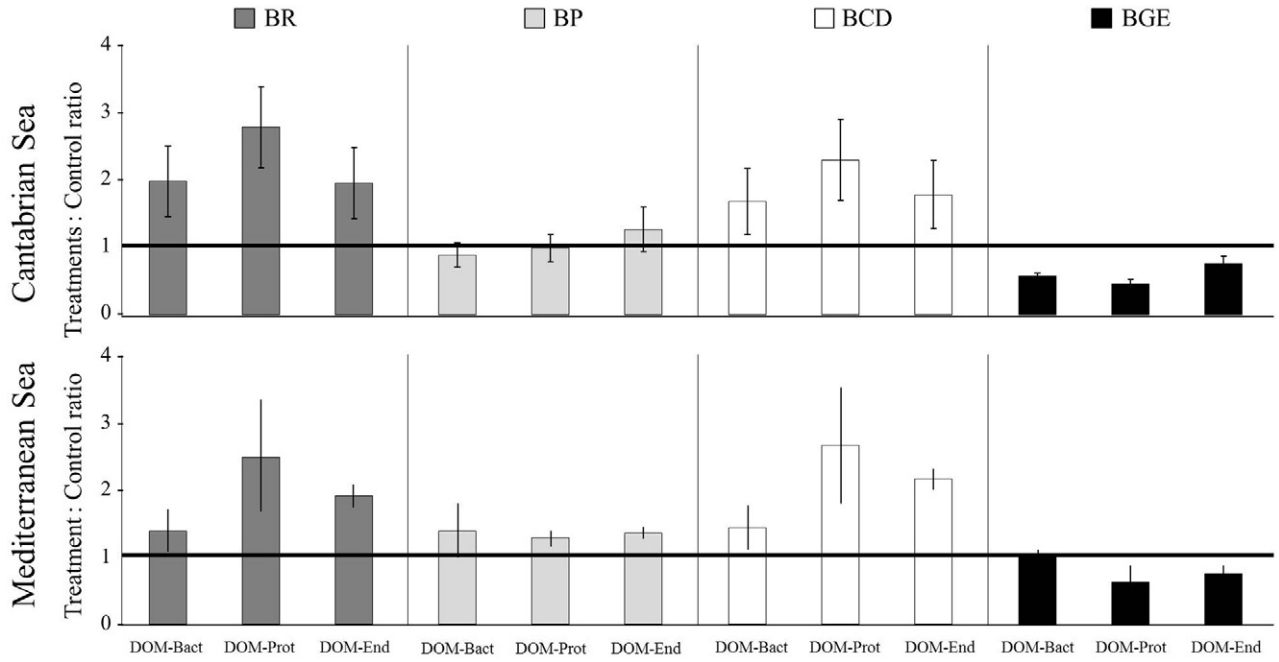

Fig. 3. Ratio between treatments and controls of cell-specific bacterial respiration (BR), cell-specific bacterial production (BP), cell-specific bacterial carbon demand (BCD) and bacterial growth efficiency (BGE) in presence of microbially altered DOMs. Data are given as mean ± standard deviation for the Cantabrian Sea (sampled three times) and as mean and range of values (vertical bar) for the Mediterranean Sea (sampled two times). The horizontal line indicates equal value for treatments and the respective control. Columns below the horizontal line indicate that the value for treatment was lower than for control, and columns above the line indicate that the value for the treatment was higher than for the control.

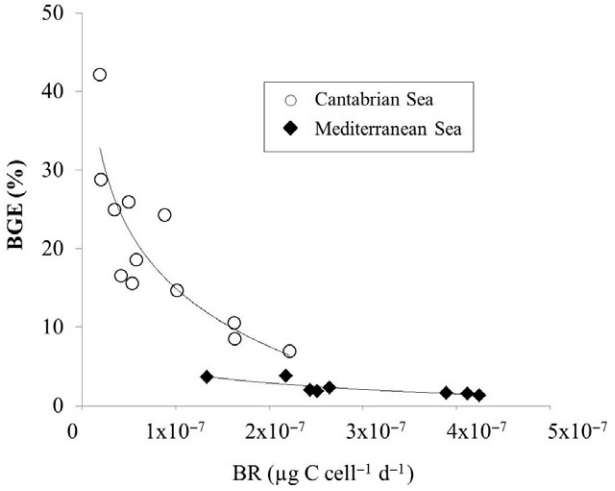

Fig. 4. Relationship between cell-specific bacterial respiration (BR) and bacterial growth efficiency (BGE) in the phase II treatments of the Cantabrian Sea [BGE = –10.71 ln (cell-specific BR) – 157.61; $r^2 = 0.80$, $P < 0.001$] and the Mediterranean Sea [BGE = 2.01 ln (cell-specific BR) – 28.07; $r^2 = 0.75$, $P = 0.006$].

accounted for almost 50% of total bacteria, consumed three times more carbon, produced almost three times less carbon and respired four and a half times more carbon than the CS bacteria. All these differences led to an average BGE of 28.9% for the CS, 10 times higher than the BGE of 2.8% obtained for the bacterial community of the MS. Cottrell and Kirchman (2003) found substantial changes in the percentage of active cells (in leucine and thymidine incorporation) within different bacterial groups across a gradient in the Delaware estuary, where *Alphaproteobacteria* accounted for assimilation of ^{3}H-leucine threefold higher than *Bacteroidetes*. Even so, we found that ^{3}H-leucine incorporation was three times higher in the CS than in the MS. Changes in bacterial assemblage structure and relative abundances of the major groups that have been detected year-round, both in the CS (Z. Baña, A. Uranga, N. Abad, I. Artolozaga, I. Azua, M. Unanue, B. Ayo and J. Iriberri, unpublished) and in the MS (Schauer *et al.*, 2003; Alonso-Sáez *et al.*, 2007), could involve more severe differences in the carbon processing and BGE between these two ecosystems.

The experimental procedure used in this study allowed to determine, in the short time (less than 6 h), the metabolic responses of a given bacterial community to different types of DOM. In spite of the great compositional and functional differences found between the bacterial communities of the two ecosystems, they showed similar major metabolic changes after being supplemented with DOM altered by bacterial growth and by the subsequent protozoan growth and feeding on bacteria. In all cases, the most striking response was generated by the DOM collected during and after protozoan grazing, and the most relevant change was the increase in cell-specific BR, which in turn affected BGE. In fact, a negative semi-logarithmic relationship (Fig. 4) between cell-specific BR and BGE was observed in both the CS [BGE = –10.71 ln (cell-specific BR) – 157.61; $r^2 = 0.80$, $P < 0.001$] and the MS [BGE = –2.01 ln (cell-specific BR] – 28.07; $r^2 = 0.75$, $P = 0.006$). These relationships indicate that the increased cell-specific BR would explain > 70% of the decrease in growth efficiency of the two bacterial communities. Nevertheless, the slopes of the curves and the ranges of cell-specific BR values were very different in the two marine systems. If BGE is assumed to indicate the degree of resource optimization, these results would indicate that the CS community, dominated by *Bacteroidetes*, would be better prepared to face changes implying variations in respiration rates, and these adaptations would be reflected in the changing BGE values. However, the MS community, dominated by SAR11, underwent highly variable cell-specific BR rates corresponding to not-very variable low bacterial growth efficiencies. These results are consistent with the known pattern of higher BGE in more eutrophic systems (del Giorgio and Cole, 1998) and higher contribution of *Bacteroidetes* to community composition in these environments (Rappé *et al.*, 2002; Lefort and Gasol, 2013). In addition, it should be considered that we have measured bacterial production from the incorporation of ^{3}H-leucine. Even taking into account the minor contribution of bacterial production to the BGE values, the differences found in the capacity to assimilate ^{3}H-leucine between *Bacteroidetes* and SAR11 (Cottrell and Kirchman, 2003) could contribute to increase the differences found in this study in BGE of the CS and the MS bacterial communities.

Our results also showed increased cell-specific AMP activities for the two communities when they were in the presence of altered DOMs, which in a time-course experiment, we assume to be associated to higher enzyme production rates. The synthesis of enzymes is costly for bacteria (Middelboe and Søndergaard, 1993; Sinsabaugh *et al.*, 1997; Carlson *et al.*, 2007), and when other influencing variables such as temperature or inorganic nutrients can be considered to be constant or not limiting as in this case, cell-specific enzymatic activities should relate to cell-specific BR (del Giorgio and Cole, 1998; Baltar *et al.*, 2009). Indeed, we found a positive relationship between the cell-specific activity of the dominant extracellular enzyme (AMP) and the cell-specific BR of the bacterial communities in both coastal ecosystems (Fig. 5), but again with differences between them: the slope was more than six times lower for the CS communities than for the MS communities and also the ranges of values were very different for both communities.

The wider range of cell-specific AMP values, together with the low slopes found for the bacterial community in

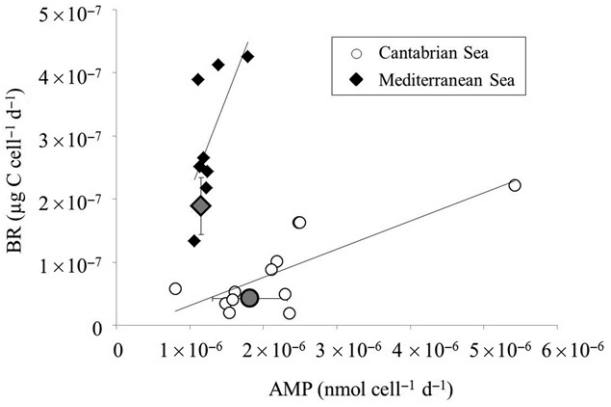

Fig. 5. Relationship between cell-specific aminopeptidase activity (AMP) and cell-specific bacterial respiration (BR) in phase II treatments (BR = 0.044AMP – 2×10^{-8}, $r^2 = 0.564$, in Cantabrian Sea and BR = 0.265AMP – 8×10^{-8}, $r^2 = 0.383$, in Mediterranean Sea waters). The larger and shaded points indicate the mean values of the controls in the Cantabrian Sea (round) and in the Mediterranean Sea (diamond).

the CS, dominated by *Bacteroidetes*, indicate a good adaptation of these bacteria towards a changing environment. In comparison, the high slope (change in cell-specific BR for a certain change in cell-specific AMP) found in the MS suggests that this community, dominated by SAR11, would need an extra metabolic cost to hydrolyse a certain amount of polymeric substrate, which would be reflected in low BGEs. Middelboe and Søndergaard (1993) suggested that the energy demand associated to extracellular enzymatic activity implies a decrease in the efficiency of carbon utilization. As the changes in extracellular enzymatic activities occur as fast as cell turnover times (Findlay and Sinsabaugh, 2003), their effect can be immediately reflected in BGE.

Therefore, the need to produce hydrolytic enzymes may be a significant factor explaining the high energetic demand observed in the two bacterial communities. In addition to the physicochemical conditions of the marine ecosystem, the major factors influencing the expression of a given profile of extracellular enzymes are the BCC because it determines the genetic potential or capacity of the community, and the organic matter composition as a regulator of the expression or synthesis of the hydrolytic enzymes (Chróst and Siuda, 2002; Hoppe *et al*., 2002). With regard to BCC, the lower energetic cost associated to the higher expression of AMP activity by the CS bacteria (see mean values for the controls in Fig. 5) may be related to the dominance of *Bacteroidetes* bacteria in summer in this area. *Bacteroidetes* are considered as specialist bacteria that prefer HMW organic matter (Cottrell and Kirchman, 2000; Elifantz *et al*., 2005), which contains a significant amount of proteins and polypeptides (Coffin, 1989; Rosenstock and Simon, 1993; Kroer *et al*., 1994; Keil and Kirchman, 1999). Thus, in the short term, the CS community would be well prepared to process the organic matter resulting from bacterial growth and protozoan grazing. In contrast, the BCC of the MS coast in summer was noticeably different: *Bacteroidetes* were scarce (approximately 11% on average), while the dominant group was SAR11 (47% on average) which are bacteria known to prefer LMW compounds such as glucose (Alonso and Pernthaler, 2006; Alonso-Sáez *et al*., 2012) or dissolved free amino acids and dimethylsulfoniopropionate (Malmstrom *et al*., 2004). This BCC in the MS fits well with the higher energetic costs associated to the expression of AMP activity (Fig. 5). Another difference in the enzyme activity profiles of the two communities is the ratio of aminopeptidases to the sum of glucosidases (α-glucosidase plus β-glucosidase), which was twice as high for the CS (20.9) than for the MS (10.1) bacterial communities (Table 2). It is well known that different groups of bacteria can express different levels of hydrolytic enzyme activities (Martinez *et al*., 1996; Pinhassi *et al*., 1999). In this way, these different ratios might be caused by the community composition aspect as they also varied in different ways when the communities were in the presence of the altered DOMs. Whereas the ratio increased in the CS community (between 1.4 and 2.9 times), it remained stable in the MS community.

The composition of the organic matter also has a major influence in shaping the extracellular enzymatic expression of a bacterial community (Chróst and Siuda, 2006), in the subsequent uptake of organic compounds (Apple and del Giorgio, 2007) and in the corresponding energetic cost. In this study, DOC characterization relied on the quantification of both the humic-like and the protein-like compounds of the FDOM, which can account for as much as 70% of DOC in coastal ecosystems (Chen and Bada, 1992; Chen *et al*., 2002; Kowalczuk *et al*., 2010). Although the initial composition of the organic matter in the predator–prey microcosms developed in phase I was different, they evolved similarly in the two ecosystems. CS seawater was slightly richer in humic-like compounds and slightly poorer in protein-like compounds [ratio PL/HL (Protein-like/Humic-like) 0.52] than MS seawater (ratio 1.48, see Table 1A). However and most interestingly, a progressive accumulation of both humic-like and protein-like compounds while bacteria were growing and protozoa were grazing was detected in both ecosystems, although to a greater extent in the MS. Large stocks of humic-like compounds can result from their release by bacteria, both when they grow exponentially and when they remain in the stationary and senescent phases (Romera-Castillo *et al*., 2011). In the case of protein-like compounds, their concentrations have been reported to increase during the exponential growth phase of bacteria and to decrease after bacterial populations reached the stationary growth phase (Kawasaki and Benner, 2006; Romera-Castillo *et al*.,

2011). Therefore, the accumulation of humic-like and protein-like compounds would mainly be derived from egestion and excretion processes performed by the protozoa. On the basis of the aforementioned previous studies, we would have expected higher increases in humic-like and protein-like compounds in the case of the CS microcosms as compared with the MS microcosms because in the former, both bacteria and protozoa grew faster and reached higher cell densities, and the ratio protozoa/bacteria, used as an indicator of grazing pressure, was also higher (4.42×10^{-3} vs. 1.81×10^{-3}) than in the latter. In this sense, it should be considered that the accumulation is the result of an unbalance between release and uptake because a fraction of the continuously released organic matter is being continuously consumed by the bacterial community. In this respect, when both communities were in the presence of the altered DOMs (Table 1B), the CS community was able to consume at least the protein-like fraction of the FDOM both in the controls and in the presence of the DOM-Prot, whereas in the case of the MS community, only an accumulation of organic compounds was observed, which confirms our previous suggestion. As different bacterial groups have different strategies for carbon utilization (e.g. Alonso-Sáez and Gasol, 2007), BCC is a key factor in the regulation of the production and dynamics of the refractory DOM.

In summary, this study shows that bacterial growth, but particularly the subsequent protozoan growth and feeding on bacteria, causes alterations in marine DOM, which triggers major metabolic changes in the bacterial community. In the short term, this metabolic response consists of an increase in extracellular enzymatic activity that implies an energetic cost, an increase in carbon consumption not related to bacterial production and a relevant decrease in BGE as a final consequence. The rate and extent of these processes seem to be related to, and to depend on, the phylogenetic composition of the bacterial community.

Experimental procedures

Sampling and strategy

We ran five experiments with surface seawater (0.5 m depth) from two coastal marine environments: three experiments were carried out in the SE CS (Armintza Station, 43°26'2.68' 'N, 2°54'2.21' 'W) during May (26th, C1) and June (24th, C2 and 30th, C3) 2007, and two experiments were carried out in the NW MS (Blanes Bay Microbial Observatory, 41°40'13.48' 'N, 2°48'0.59' 'W) during July (2nd, M1) 2008 and July (9th, M2) 2009. The experimental set-up (Fig. S1) was performed in two phases, the first one to obtain DOM altered by the microbial communities (phase I), and the second one to describe the effects of that DOM on marine bacterioplankton (phase II).

Phase I: seawater was prefiltered through 8 μm-pore-size polycarbonate membrane filters, in order to remove micro- and mesozooplankton. Five litre microcosms were established for each experiment and incubated at 20°C to facilitate the growth of both bacteria and protozoa. Bacterial and protozoan abundances were monitored every 4 h during 96–120 h (Fig. 1). Through the incubation, DOM was obtained at the points of (i) maximum bacterial abundance (which we label as DOM-Bact), (ii) maximum protozoan abundance (labelled as DOM-Prot) and (iii) the decline of the system (labelled DOM-End). These DOMs were obtained by filtration of 500 ml through precombusted Whatman 0.22 μm pore-size mixed cellulose ester membrane filters (Madrid, Spain) and preservation of the filtrate at −20°C. The quality and variability of these three altered DOMs, together with that of the initial DOM, were characterized by the determination of FDOM.

Phase II: as soon as possible (between 2 and 4 days after the last altered DOM was collected), surface seawater was collected again from the sampling station. Samples were taken to analyse microbial abundances and BCC. Fresh seawater was divided into three treatments, in which it was supplemented with the DOM-Bact, DOM-Prot and DOM-End collected in phase I. Seawater was prefiltered through Millipore AP25 glass fibre prefilters (0.9 μm nominal pore-size; Madrid, Spain), in order to separate bacteria from the rest of the planktonic community. Bacterioplankton was concentrated by gravity filtration on a precombusted Whatman 0.22 μm pore-size mixed cellulose membrane filter. Five hundred millilitre of the bacterial concentrate were diluted with 500 ml of either DOM-Bact, DOM-Prot or DOM-End, finally obtaining approximately the same bacterial abundance as in the initial untouched sample. A control was also prepared by diluting the concentrated bacterioplankton community with undisturbed DOM, obtained from the same initial sample used in phase II (prefiltered through precombusted Whatman 0.22 μm pore-size mixed cellulose ester membrane filters). Aliquots for DOC quantification and FDOM determination were taken immediately after DOM replacement, and in the case of FDOM, also after 24 h of incubation. Inorganic nutrients (0.5 μM NO_3^-, 0.05 μM PO_4^- and 0.5 μM NH_4^+) were incorporated into an additional treatment that contained also a mix of bacterial concentrate and DOM-Prot (DOM-nut) in order to check any nutrient limitation. Bacterial activity measurements (BR, BP and EEA) were determined immediately after the DOM replacements were done in all treatments and controls.

Microbial community abundance

Enumeration of the microbial community was carried out by direct counting as in Porter and Feig (1980). Samples were preserved with 0.2 μm-filtered borax-buffered formalin (2% v/v final concentration) for bacteria and with sodium tetraborate-buffered formalin (2% v/v final concentration) for protozoa, and stored at 4°C in the dark. Known volumes of subsamples were 4'6'-diamidino-2-phenylindole (DAPI)-stained (2 μg ml^{-1} final concentration) for 10 min and filtered through Whatman 0.22 μm or 0.8 μm polycarbonate black filters for bacterial and protozoan counts respectively. The filters were mounted on a glass slide with non-fluorescent immersion oil. Samples were analysed in duplicate, and randomly selected fields (20 for bacteria and 100 for

protozoa) were counted of each filter on a Nikon Optiphot with EF-D epifluorescence equipment (magnification 1250×, Nikon, Tokyo, Japan).

BP

Bacterial production rates were estimated from the uptake of tritiated leucine (40 nM final concentration) according to the method of Kirchman and colleagues (1985). Triplicated 5 ml subsamples plus two Trichloroacetic Acid (TCA)-killed blanks were incubated at in-situ temperature in the dark for 1–2 h with the isotope. Incubations were stopped by adding TCA at 5% final concentration and placing them in ice for 5 min. Subsequently, the samples were filtered through 0.22 μm Sartorius cellulose acetate membrane filters and rinsed five times with 5 ml 5% TCA. The filters were let to dry, introduced into scintillation vials and dissolved in 500 μl of ethyl acetate for 20 min. Afterwards, 4 ml scintillation cocktail (Ultima Gold, Perkin Elmer, Walham, Massachusetts) was added, and the radioactivity was assessed on a Perkin Elmer Tri-Carb 2900TR scintillation counter using external standards for quenching correction. Rates of leucine incorporation were converted into carbon production with a theoretical conversion factor of 1.55 kg C mol leu^{-1} which assumes no isotope dilution (Simon and Azam, 1989).

BR

Two subsamples from each treatment and control were placed in 4 ml sealed glass chambers designed to prevent oxygen exchange with the outside. The decrease in oxygen concentration inside the chambers was monitored by using a microrespiration system (Unisense, Aarhus, Denmark) which allows continuous measurements, with an oxygen microsensor (Briand *et al*., 2004). As small temperature changes influence oxygen determinations by oxygen microprobes, all glass chambers were introduced in a temperature-controlled bath in order to keep the temperature constant during the whole incubation. The rate of respiration of the bacterial community in the sample was determined from the slope of the regression between oxygen concentration and time in periods shorter than 6 h. Respiration rates were transformed into carbon by using a respiratory quotient of 1 (del Giorgio *et al*., 2006).

BGE and BCD

BGE was calculated as the ratio of BP to BCD, where BCD was calculated as the sum of BP and BR, and expressed as a percentage (BGE = BP/(BP + BR)*100). Both BP and BR were measured at the same time scales (incubation periods < 6 h).

EEA

The potential activities of three extracellular enzymes, alpha-glucosidase (α-Glu), beta-glucosidase (β-Glu) and leucine-AMP, were estimated by measuring the hydrolysis of the fluorogenic substrate analogues 4-methylumbelliferyl-α-D-glucoside (MUF-α-Glu), 4-methylumbelliferyl-β-D-glucoside (MUF-β-Glu) and L-leucine-7-amido-4-methyl-coumarin (MCA-Leu) respectively (Hoppe, 1983). Appropriate stocks of the chemicals were prepared in methyl cellosolve and added at saturating concentrations (250 μM MUF-α-Glu and MUF-β-Glu and 350 μM MCA-Leu) to 3 ml triplicate subsamples from the treatments and control. The enzyme activities were detected using a Perkin Elmer spectrofluorometer (LS50B in the CS and LS55B in the MS experiments) at an Ex/Em wavelength of 342 nm/453 nm for glucosidases and 360 nm/445 nm for aminopeptidase activity. The initial linear increase in relative fluorescence was transformed to cleavage activity using a standard curve established with different concentrations of the fluorochromes MUF (glucosidase activities) and MCA (aminopeptidase activity).

BCC

BCC was determined by the CARD-FISH (Catalysed reporter deposition Fluorescence In Situ Hybridization) technique as described by Pernthaler and colleagues (2002), using HRP (Horseradish Peroxidase)-labelled oligonucleotide probes and designed to target specifically the domain Bacteria [mixture of EUB I (Amann *et al*., 1990) and EUB II-III (Daims *et al*., 1999)] and four of its main phylogenetic groups: ALF968 (Neef, 1997) for *Alphaproteobacteria*, including ROS537 (Eilers *et al*., 2001) and SAR11-441R (Morris *et al*., 2002) for the *Rhodobacteraceae* and SAR11 groups respectively; BET42a (Manz *et al*., 1992) for *Betaproteobacteria*; GAM42a (Manz *et al*., 1992) for *Gammaproteobacteria*; and CF319a (Manz *et al*., 1996) for *Bacteroidetes*. The Eubacteria antisense control probe NON338 (Wallner *et al*., 1993) was used as negative control. All hybridizations were conducted during 2 h at 35°C, followed by 15 min amplification with tyramide-Alexa488. Formamide concentration in hybridization buffer was 55% excepting for probes ALF968 and SAR11-441R (45%) and NON338 (20%). Filter sections were DAPI stained for subsequent enumeration of the bacteria in the epifluorescence microscope.

Determination of DOC

Triplicate of 10–15 ml of water sample was collected in precombusted glass ampoules for DOC analysis. Ortophosphoric acid was added to acidify the sample to ~ pH 2, and the ampoules were heat-sealed and stored in the dark at 4°C until analysis. DOC was measured with a Shimadzu TOC-CVS organic carbon analyser (Izasa S.A, Madrid, Spain). The system was standardized daily with a potassium hydrogen phthalate standard. The concentration of DOC was determined by subtracting the instrument blank area from the average peak area and dividing by the slope of the standard curve. The precision of the equipment was ± 0.7 μmol l^{-1}.

Determination of FDOM

Samples of initial DOM, DOM-Bact, DOM-Prot and DOM-End from phase I, as well as treatments and controls

from phase II, were measured following the method of Nieto-Cid and colleagues (2006). Single measurements of the collected samples were performed with a Perkin Elmer spectrofluorometer (LS50B in the CS and LS55B in the MS experiments) in a 1 cm quartz fluorescence cell. Slit widths were 10.0 nm for the excitation and emission wavelengths and scan speed was 250 nm min^{-1}. The Ex/Em wavelengths used for single measurements were those established by Coble (1996): Ex/Em 280 nm/350 nm (peak-T) as indicator of protein-like substances, Ex/Em 340 nm/440 nm (peak-C) and Ex/Em 320 nm/410 nm (peak-M) as indicators of humic-like substances. Fluorescence measurements were transformed to quinine sulfate units (QSU), using quinine sulphate dihydrate made up in 0.05 mol l^{-1} sulfuric acid, with standard curves at Ex/Em 340 nm/440 nm, where 1 QSU was equivalent to the fluorescence emission of 1 μg l^{-1}. The blank (Milli-Q; Merck, Madrid, Spain) fluorescence was subtracted from all samples. In order to detect consumption or production of different fluorescent compounds in phase II of the experiments, the treatments were incubated during 24 h, and FDOM was determined at the beginning and at the end of the incubation.

Statistical analysis

Nonparametric Mann–Whitney *U*-tests and paired Wilcoxon tests were used to identify significant differences between ecosystems and related samples or treatments. The statistical analyses were performed using SPSS Statistics Version 19 (IBM, Madrid, Spain).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Detailed diagram of the experimental set-up carried out in the SE Cantabrian Sea and in the NW Mediterranean Sea and performed in two phases: obtaining DOM altered by the microbial communities (phase I) and altered DOM replacement (phase II).