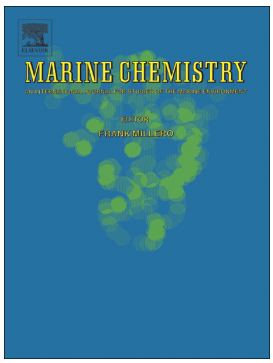

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Major differences in dissolved organic matter characteristics and bacterial processing over an extensive brackish water gradient, the Baltic Sea

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Abstract

Dissolved organic matter (DOM) in marine waters is a complex mixture of compounds and elements that contribute substantially to the global carbon cycle. The large reservoir of dissolved organic carbon (DOC) represents a vital resource for heterotrophic bacteria. Bacteria can utilise, produce, recycle and transform components of the DOM pool, and the physicochemical characteristics of this pool can directly influence bacterial activity; with consequences for nutrient cycling and primary productivity. In the present study we explored bacterial transformation of naturally occurring DOM across an extensive brackish water gradient in the Baltic Sea. Highest DOC utilisation (indicated by decreased DOC concentration) was recorded in the more saline southerly region where waters are characterised by more autochthonous DOM. These sites expressed the lowest bacterial growth efficiency (BGE), whereas in northerly regions, characterised by higher terrestrial and allochthonous DOM, the DOC utilisation was low and BGE was highest. Bacterial processing of the DOM pool in the south resulted in larger molecular weight compounds and compounds associated with secondary terrestrial humic matter being degraded, and a processed DOM pool that was more aromatic in nature and contributed more strongly to water colour; while the opposite was true in the north. Nutrient concentration and stoichiometry and DOM characteristics affected bacterial sctivity, including metabolic status (BGE), which influenced DOM transformations. Our study highlights dramatic differences in DOM characteristics and microbial carbon cycling in sub-basins of the Baltic Sea. These findings are critical for our understanding of carbon and nutrient biogeochemistry, particularly in light of climate change scenarios.

Keywords: Dissolved organic matter, DOC utilization, DOM fluorescence, bacterial growth efficiency, bacterial production, Baltic Sea.

Highlights

- Clear spatial differences were seen in DOM characteristics and bacterial response.
- Bacterial growth and metabolic status have a dual role influencing the DOM pool.
- Physicochemical and biological processes interact, influencing the carbon cycle.

1. Introduction

The dissolved organic matter (DOM) pool is a complex mixture of molecules of disparate structure and of diverse origin. The DOM pool incorporates various forms of elements that are vital for microbial growth, such as: carbon (C), nitrogen (N) and phosphorus (P). In marine ecosystems the DOM pool, particularly the dissolved organic carbon (DOC) fraction, represents an important resource for heterotrophic bacteria (Ducklow et al., 1986; Sherr and Sherr, 1988). Bacteria are in turn fundamental for the recycling of key nutrients (Hansell and Carlson, 2002).

DOM in marine waters is in copious supply (Hedges, 1992; Benner and Amon, 2015). While DOM in open water marine systems is dominantly derived from autochthonous processes (i.e. phytoplankton primary production and related processes: Nagata, 2000), allochthonous terrestrial organic matter can also be an important contributor to the DOM pool. This latter scenario can be especially pertinent in enclosed or coastal waters (Ask et al., 2009; Deutsch et al., 2012; Fleming-Lehtinen et al., 2015). The characteristics of the DOM pool are influenced by its origin (e.g. autochthonous, allochthonous, land use, catchment composition) and these attributes in turn control its bioavailability and fate. These factors influence its potential importance in the ecosystem (Asmala et al., 2013; Boyd and Osburn, 2004; Stedmon et al., 2003). The concentration and properties of the DOM pool can directly influence heterotrophic processes at the base of the food web. Supplementary DOC and allochthonous nutrients may enable bacteria to outcompete autotrophic primary producers (Fandino et al., 2001; Lignell et al., 2008; Sandberg et al., 2004; Smith et al., 1995). Furthermore, DOM can catalyse other concurrent changes, such as controlling the penetration of UV and visible solar radiation in the surface ocean (Dupont and

Aksnes, 2013; Nelson and Siegel, 2013). Thus, any modification of the DOM pool may result in changes in the balance of basal production (heterotrophic bacterial and autotrophic algal production) or changes in food web structure. The outcome of such changes have the potential to influence ecosystem function (Azam et al., 1983; Azam, 1998; Sandberg et al., 2004; Hansson et al., 2013; Lefébure et al., 2013) and the global carbon cycle (Jiao et al., 2010).

Since only a limited portion of the DOC pool is available to bacteria (Hoikkala et al., 2015; Søndergaard and Middelboe, 1995) carbon limitation of bacterioplankton growth is common (e.g. Carlson and Ducklow 1996; Kirchman and Rich, 1997). To understand the fate of DOM in marine systems it is therefore important to combine bacterial utilisation studies with detailed characterisation of the prevailing DOM pool. By examining DOM absorbance and fluorescence properties it is possible to gain or infer some important quantitative (e.g. concentrations of chromophoric dissolved organic matter (CDOM) or humic substances) and qualitative insights, such as: estimates of molecular weight (Amon and Benner, 1996; Asmala et al., 2013; Wallin et al., 2015), aromaticity (Weishaar et al., 2003), and DOM origin (e.g. terrestrial, marine produced or catchment land use). Characteristics of the DOM pool have been linked to DOC concentration, the potential bioavailability of the DOM, bacterial growth efficiency (BGE), and biological breakdown and production processes (Asmala et al., 2013; Benner and Amon, 2015; Fichot and Benner, 2012; Trabelsi and Rassoulzadegan, 2011). Consequently, knowledge about the characteristics of the DOM pool, its bioavailability and the efficiency of bacterial utilisation (Asmala et al., 2013; Dinasquet et al., 2013; Figueroa et al., 2016) is critical for understanding ecosystem function (Sandberg et al., 2004) and carbon cycling (Bianchi et al., 2013;

Jiao et al., 2010). Obtaining such insights appears especially pertinent when considering climate change predictions (Andersson et al., 2015; Jiao et al., 2010), particularly those for enclosed water bodies such as the Baltic Sea (Andersson et al., 2015).

In this study we examined the bioavailability of DOC in open-sea waters of the three major basins of the Baltic Sea, and assessed the bacterial-DOM interactions ongoing. Environmental sampling was combined with DOC utilisation experiments at four stations in each basin. We explored the influence of DOC concentration and optical DOM characteristics on bacterial growth and DOC utilisation. We aimed to determine if: 1) spatial differences in DOC concentration and DOM characteristics occurred along this latitudinal gradient, 2) differences in DOM influenced the efficiency with which DOC was utilised, 3) nutrient limitation resulted in decreased DOC utilisation, and 4) altered DOC utilisation has potential consequences for the Baltic Sea carbon cycle. We discuss our findings in the context of wider ecosystem function, global elemental cycles and climate change.

2. Materials and Methods

2.1. Study system and rationale. The Baltic Sea is a semi-enclosed sea that is strongly influenced by an extensive catchment area. DOC concentrations in Baltic Sea open waters do not differ strongly between the three major basins (Hoikkala et al., 2015; Ripszam et al., 2015). However, the northern basins are highly influenced by river discharges of DOC-rich waters (Stepanauskas et al., 2002, Hoikkala et al., 2015, Fleming-Lehtinen et al., 2015; Reader et al., 2014; Räike et al., 2012), and the salinity

and N and P concentrations generally increase in a southerly direction (Andersson et al., 2015; Hoikkala et al., 2015). These factors are strong drivers of the ecological gradients that occur in the Baltic Sea.

2.2. Sampling and water collection (in-situ). Sampling was carried out in July 2011 at four stations in each of the three major basins of the Baltic Sea (Fig. 1). Two trips were made, one in the Baltic Proper (July 5th) and one in the Gulf of Bothnia (Bothnian Sea and Bothnian Bay, July 19th - 21st). Water was collected from a depth of 2 m using Niskin bottles and salinity, temperature, pH, total nitrogen (TN), total phosphorus (TP) and dissolved organic carbon (DOC) were measured, as described below.

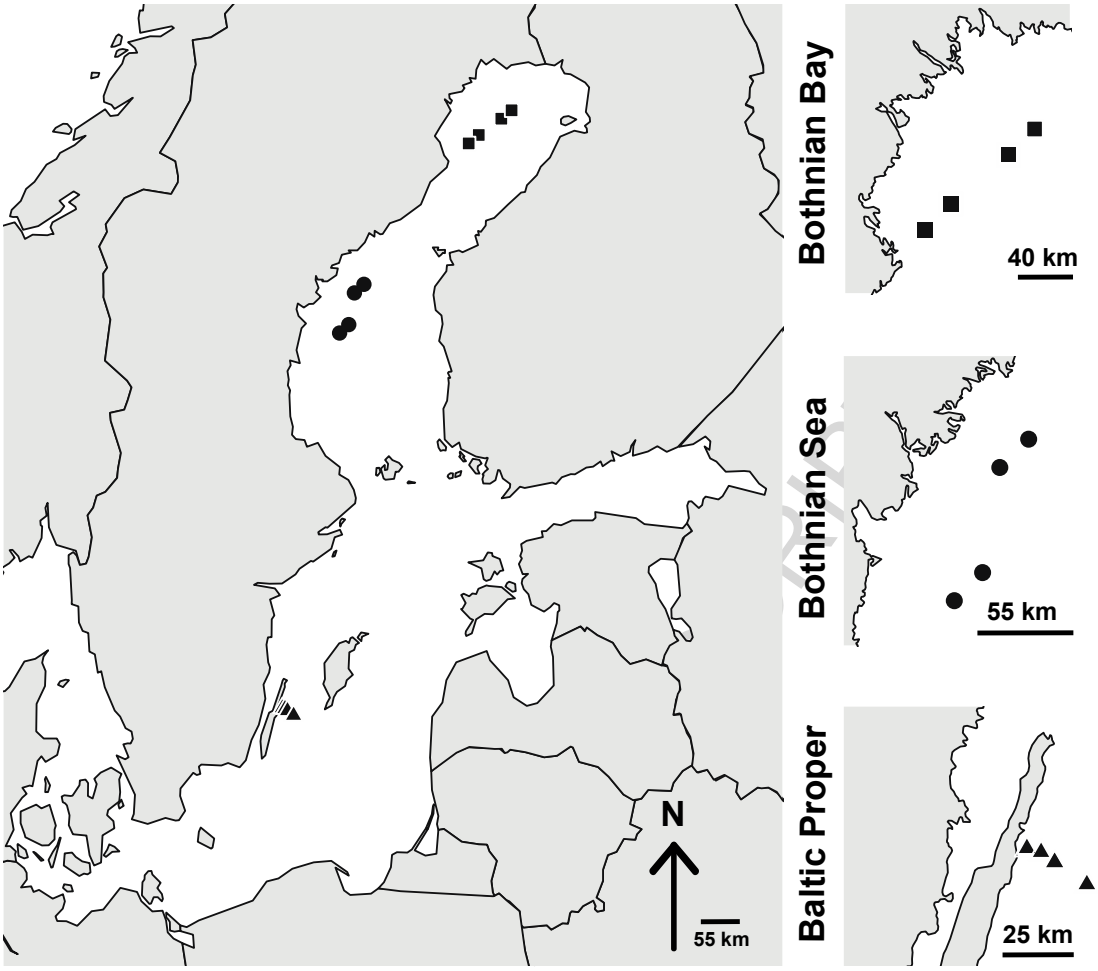

Figure. 1. Map of the Baltic Sea showing sampling locations of the four open sea stations sampled in each of the three major basins (Baltic Proper, Bothnian Sea and Bothnian Bay).

2.3. Preparation of experimental study. To remove larger organisms 10 L of water was passed through a 0.45 µm capsule filter (Millipak-40. Millipore) using gravity filtration. The filter capsule was rinsed with ~1 L of sample water prior to use, and 0.45 µm filtrate was used to rinse the recipient acid-washed plastic carboy. The final 6 L of water passing through the 0.45 µm filter was collected, from here onwards referred to as 'filtrate 1'. Circa 2 L of water was also gravity filtered through a pre-combusted 47 mm GF/F filter, referred to as 'filtrate 2'. This process was repeated for each station using a fresh filter capsule and fresh acid washed containers on each

occasion, and the process was completed within ~4 hours. Filtration through a combusted GF/F filter has been shown to decrease bacterial numbers (Nayar and Chou, 2003) and this was observed in this study. For example bacterial numbers in microcosm start waters (a combination of filtrate 1 and 2) were 52 % (SD 7, n = 4) lower than the *in situ* waters of the Baltic Proper samples (not tested in other basins). It is possible that the filtration procedure removed larger members of the bacterial community, possibly altering the natural size distribution at the start of the experiment.

2.4. Microcosm setup and sampling. At each station six 1 L polycarbonate bottles (microcosm units) were filled with a combination of 900 mL of filtrate 1 and 100 mL of filtrate 2. Filtrate 1 and 2 waters were only combined for their respective stations. A filter-sterilised solution consisting of nitrate, ammonia and phosphate (additions of 20 µM N and 3 µM P, in MilliQ water) was added to three of the microcosm units per station (+NP treatment) to preclude N or P limitation (as used similarly in Degerman et al., 2013). In standard microcosm units 200 µL of filter sterile MilliQ water was added, a volume corresponding to the solution of nutrients added above. Microcosm units were run in triplicate for each station, making six microcosms per station (three standard and three +NP treatment), twenty-four microcosms per basin and a total of seventy-two microcosms units. Acid washed and sterile equipment was used for all filtration, storage, preparation, incubation and sampling stages.

Preparation of microcosms was completed within ~6 hours of initial water collection. All experimental units were immediately incubated in the dark and maintained at 15 °C (Gulf of Bothnia) or 18 °C (Baltic Proper, Table 1). Experimental units were

sampled on day 0, 1, 3, 5 and 10 of incubation (removing circa 50 ml on each occasion). The Day 0 sample, taken from initial bulk combinations of filtrate 1 and filtrate 2 waters (i.e. mixture prior to addition to individual microcosm units), was a single sample per station and used to represent the starting values for all treatments (i.e. both standard and +NP treatments). Start and end concentrations of TN and TP were measured using a Bran & Luebbe TRAACS 800 autoanalyser according to Grasshoff *et al.* (1983), following the process described in Traving et al., (2017). Due to the nature of the field sampling during which the experiment was carried out, it was not possible to monitor inorganic and organic nutrient concentrations. Start C (DOC), N (TN) and P (TP) stoichiometric ratios were calculated.

Table 1. Mean values (standard deviation) of in-situ physicochemical variables (n = 4 independent stations per basin). Nutrient stoichiometry values represent waters from standard microcosm at the start of the experiment, expressed as basin mean values (*n* = 12).

	Temperature (°C)	pH	Salinity	DOC (µmol C L^{-1})	TP (µmol L^{-1})	TN (µmol L^{-1})	C:N	N:P	C:P
Baltic Proper	17.6 (0.2)	8.5 (0.1)	6.8 (0.1)	708 (58)	0.21 (0.04)	16.55 (0.91)	31.6 (4.9)	16.6 (3.0)	527.0 (144.2)
Bothnian Sea	14.4 (0.2)	8.3 (0.1)	5.2 (0.1)	466 (42)	0.18 (0.03)	16.16 (1.14)	21.3 (3.1)	18.9 (2.7)	402.6 (78.5)
Bothnian Bay	15.5 (0.1)	8.1 (0.1)	2.8 (0.1)	416 (42)	0.08 (0.01)	13.28 (0.61)	23.1 (1.4)	33.8 (6.5)	780.7 (157.0)

The following variables were measured on every sampling day and in every experimental microcosm unit.

2.5. Bacterial abundance and production. Bacterial abundance (BA) samples (1.5 mL) were taken in duplicate 2 mL cryovials, fixed with 0.2 µm filtered glutaraldehyde (1% final concentration) and flash frozen in liquid nitrogen prior to storage at -80 °C. Samples were stained with SybrGreen (Invitrogen) and cells were counted on a FACSCantoII flow cytometer (BD Biosciences), as previously described (Gasol and del Giorgio, 2000). Fluorescent beads (True count beads, Becton Dickinson) were used to calibrate the flow rate. Bacterial production (BP) was measured by [3H]-

thymidine incorporation (Fuhrman & Azam 1982), as modified for microcentrifugation (Smith and Azam 1992). Triplicate 1.7 ml aliquots were incubated for 1 hour with [methyl-3H]-thymidine in sterile 2.0 ml capacity polypropylene tubes at in situ temperature. Saturation curves were used to determine suitable thymidine concentrations in the Baltic Proper and Gulf of Bothnia regions separately (20 and 24 nM final concentration, respectively, and a specific activity of 73.4 Ci $mmol^{-1}$) and analysed with a Beckman 6500 scintillation counter. A single sample per microcosm, killed by adding 5% trichloracetic acid prior to the addition of thymidine, served as a blank. Thymidine incorporation was converted to cell production using 1.4×10^{18} cells $mole^{-1}$ (Wikner and Hagström 1999) and 20.4 fg C $cell^{-1}$ (Lee and Fuhrman, 1987) to estimate carbon biomass production.

2.6. DOC concentration and DOM characteristics. Duplicate 12 mL samples were filtered through pre-combusted GF/F filters into 15 ml acid washed polypropylene tubes, acidified with 120 µL of 2 M HCl, and stored at 4°C until analysis. DOC samples were analysed using high temperature catalytic oxidation (Shimadzu TOC-5000), as detailed in Traving et al., (2017). DOM fluorescence samples were prepared by collecting a single 40 mL sample that was filtered at low pressure through a pre-combusted GF/F filter into a 50 mL tube and immediately frozen (-20°C) until processing. It should be noted that freezing is not optimal as it may alter DOM fluorescence (e.g. Fellman et al., 2008), potentially in a random manner (Spencer et al., 2007). However the extensive gradient studied and field sampling carried out gave no viable alternative. Since all samples in the present study were treated identically we infer that the observed trends are valid for the direct comparisons carried out. Nevertheless, comparisons of specific values between this and other studies should be

done with caution. Samples were acclimated to room temperature on a Horiba Aqualog spectrofluorometer (Horiba Scientific) in a 1 cm quartz cuvette. This instrument simultaneously measures absorption (from 240 nm to 600 nm) and fluorescence (at excitation and emission wavelengths 240 nm to 600 nm) at 3 nm intervals. Correction, calibration and calculation of informative variables were carried out (Asmala et al., 2013; Murphy et al., 2010; Stedmon et al., 2000). The following variables were extracted or calculated: 1. the ratio between $a_{CDOM(254)}$ and $a_{CDOM(365)}$ (referred to as: a254:a365), 2. a slope of the spectra for wavelengths 275-295 nm (slope coefficient, S275-295); both indicators of DOM molecular weight (Asmala et al., 2013; Fichot and Benner, 2012; Helms et al., 2008; Wallin et al., 2015), 3. absorbance at 440 nm ($a_{CDOM(440)}$), referred to as chromophoric dissolved organic matter (CDOM) and indicative of water colour (Harvey et al., 2015), 4. $SUVA_{254}$, indicative of DOM aromaticity (Ripszam et al., 2015; Weishaar et al., 2003), 5. fluorescence peak C (peak C, Ex/Em of 350/420-480 nm), a secondary humic peaks associated with terrestrial origin (Cammack et al., 2004; Coble, 1996; Stedmon and Markager, 2005), 6. fluorescence peaks B (peak B, Ex/Em of 275/310 nm) and T (peak T, Ex/Em of 275/340 nm), protein-like peaks of similar structural composition to tyrosine and tryptophan, respectively (Coble, 1996), 7. fluorescence peaks A (peak A, Ex/Em of 260/380-460 nm) and M (peak M, Ex/Em of 312/380-420 nm), primary dissolved humic substances and marine humic associated compounds, respectively (Coble, 1996), and 8. the fluorescent peaks summed together as total humic-like or total amino-like peaks.

2.7. DOC utilisation, BGE and fluctuation of variables. Calculations of change (increase or decrease, Δ) were carried out between days 0 and 5 ($\Delta_{0\text{-}5}$) and between

days 0 and 10 ($\Delta_{0\text{-}10}$), the latter being the full length of microcosm incubations. Trends were generally similar for both incubation time periods examined. However, only data for $\Delta_{0\text{-}5}$ are presented as this represented the more active period of the incubation (see results). Variables for which Δ data are calculated include: BA, DOC, a254:a365, S275-295, $SUVA_{254}$, peak B, peak C, and peak T. Lastly, ΔDOC (or DOC utilisation) was calculated between days 1 and 5 due to missing DOC data at some stations on day 0. Where DOC data was present on day 0 there was no marked decrease in DOC between days 0 and 1. Other calculations reliant on ΔDOC (e.g. BGE) were also calculated using requisite data from the corresponding time period. BGE (%) was calculated as the integrated cumulative bacterial production during days 1-5 ($BP_{cum1\text{-}5}$) divided by the ΔDOC between days 1 and 5 ($\Delta DOC_{1\text{-}5}$), multiplied by 100 (Figueroa et al., 2016).

2.8. Statistical analyses. A Kendall-Tau correlation analysis was carried out on in-situ physicochemical data. A Principal component analysis (PCA) was performed to examine the similarity and separation of stations within and between the three different basins. No pre-processing of the data was undertaken. A one-way analysis of variance (ANOVA) with Tukey's HSD (honest significant differences) post hoc analysis was also carried out on in-situ data.

Cumulative bacterial production, BGE and Δ data were analysed with a two-way ANOVA to examine the effects of basin and treatment (+/- NP), and any interaction between these.

A Kendall-Tau correlation analysis was performed on the raw data from the experimental microcosms. All variables measured, on all sampling days, in all treatments, and from all stations were included. Missing data values (3.5% of all data values) were imputed as means of replicates. A repeated measures-multivariate analysis of variance (RM-MANOVA) was performed to examine significant changes over the duration of the experiment and the influence of treatment and basin. Data used in the RM-MANOVA analysis did not conform to normality and did not improve with transformation, however these methods have been shown to be resilient to violations in normality (Finch, 2005) and have been successfully applied elsewhere (e.g. Ferrari et al., 2014). A PCA analysis was carried out on the above variables from standard microcosm data only (i.e. +NP microcosms excluded).

To explore drivers of specific changes or trends recorded, correlations were carried out between a selected experimental variables, cumulative data (e.g. cumulative BP), nutrient stoichiometric ratios (e.g. C:N or C:P), and Δ data (e.g. BA or BGE). Some data were normalised (0-1 scale) and others were transformed (ln). In all cases where such transformations were applied it is defined where the results are presented.

Statistical analyses and figure production were mainly performed in R Core Team (2013) using the packages: Rcmdr, prcomp, ggplot2, maps, mapdata and ggbiplot. The RM-MANOVA was performed in SPSS (IBM SPSS Statistics software version 22.0.0.0).

3. Results

3.1. Station similarity and basin differentiation. In-situ physicochemical variables indicated lower nutrient concentrations (TN and TP), salinity, pH and DOC in the northerly reaches of the Baltic Sea (the Bothnian Bay), as compared to more southerly stations (Table 1). Surface water temperature was also lower in the Gulf of Bothnia as compared to the Baltic Proper. However, during our specific sampling program temperature was higher in the Bothnian Bay, than the Bothnian Sea. Strong and significant ($p < 0.0001$) correlations were found between salinity and TP ($r = 0.7404$), salinity and pH ($r = 0.8722$), TN and TP ($r = 0.7176$), and TP and pH ($r = 0.7837$). The stations within each basin clustered together closely in the PCA analysis, and clear separation between the three basins was observed (Fig. S1). The global ANOVA indicated significant differences between the three basins for most in-situ physicochemical variables measured (Table S1). Stations are thus considered as replicates within each basin during analysis of the microcosm study.

3.1.1. Initial conditions. Clear variation in optical DOM characteristic variables were observed between basins at the start of the microcosm incubation. The a254:a365 ratio was higher in the Baltic Proper and decreased in a northerly direction. $SUVA_{254}$ and CDOM showed the opposite trend, being highest in the Bothnian Bay (Fig. S2). Values for peak B, peak C, and peak T were generally higher in the Bothnian Bay or similar across all basins at the start of the incubations (Fig. S3).

3.2. DOC utilisation, bacterial abundance and bacterial production. DOC was utilised and decreased particularly between days 1 and 5 of the incubation. Mean

decreases in DOC were 233 µmol L^{-1}, 58 µmol L^{-1} and 17 µmol L^{-1} (by day 5) in the Baltic Proper, Bothnian Sea and Bothnian Bay microcosms, respectively (Fig. 2).

Initial BA and BP rates were similar in all microcosms, however the trends during incubation differed with basin (Fig. 2). These spatial differences (basin effects) were significant for most variables, including over the course of the incubation period (Table 2). BP and BA generally peaked during days 1-5 of the incubation period, although the correlation between the two measured variables was generally poor. The highest BA values were recorded in the Baltic Proper microcosms whereas the highest rates of BP occurred in Bothnian Sea microcosms (Fig. 2). Between days 5 and 10, BP rates (and BA) generally decreased or plateaued. The initial period of high BA and BP rates (days 0-5) corresponded with the phase during which DOC decreased. Lower rates of BP by day 10 coincided with a general increase in DOC at this stage (Fig. 2).

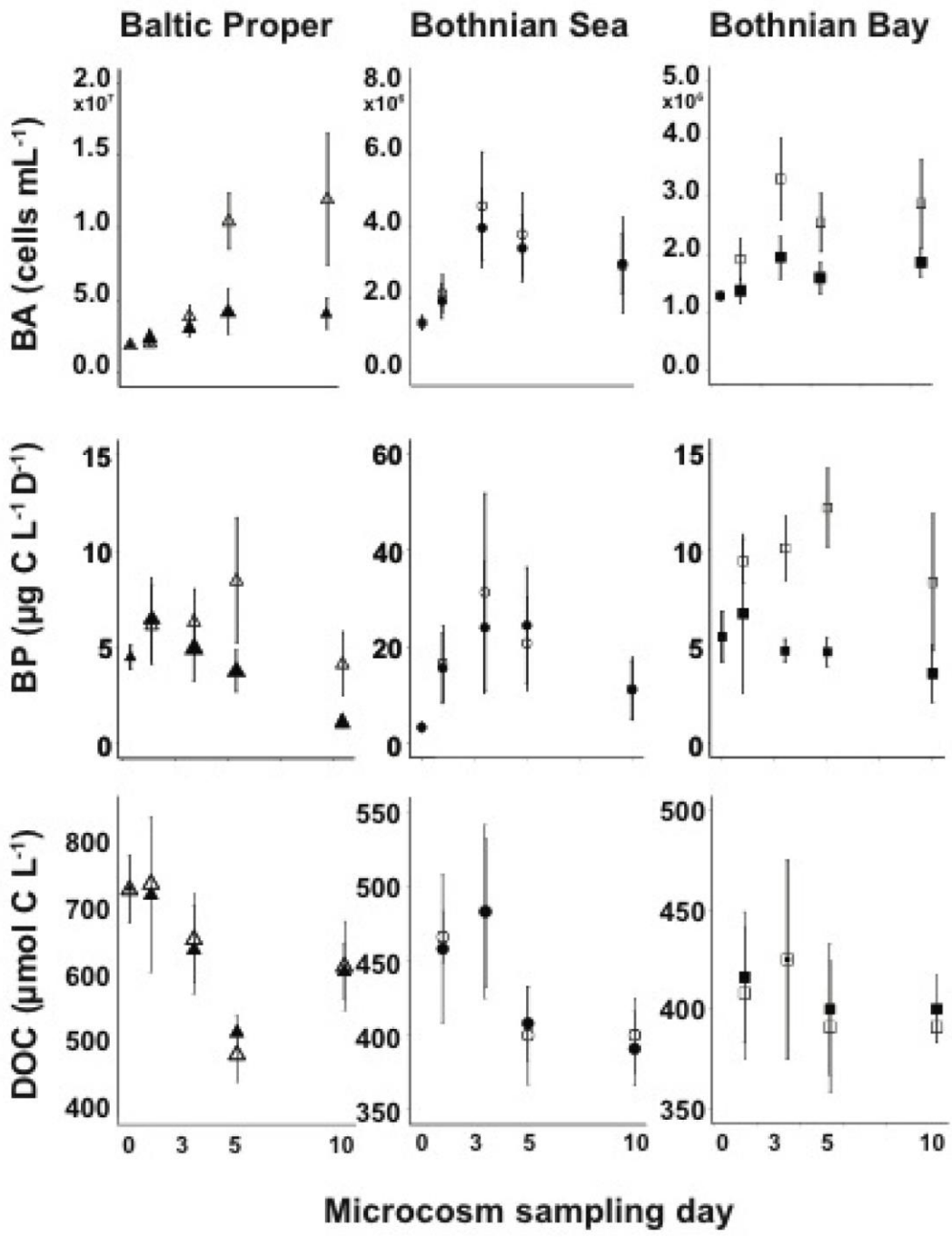

Figure 2. Temporal trends in mean values for bacterial abundance (BA), bacterial production (BP) and dissolved organic carbon (DOC) in microcosm experiments. Data are presented by basin (Baltic Proper, triangles; Bothnian Sea, circles; and Bothnian Bay, squares), with standard (filled symbols) and +NP treatments (open symbols) shown. Standard deviation is indicated by error bars where $n = 12$. **Note axis scales are not identical and vary between basins for a single variable.**

Table 2. Between and within subject contrasts from RM-MANOVA carried out on microcosm experiment. Statistically significant ($p < 0.05$) are indicated by bold text.

	Time		**Basin**		**Treatment**		**Time *Basin**		**Basin *Treatment**		**Time *Treatment**		**Time *Basin**	
df	**1**		**2**		**1**		**2**		**2**		**1**		**2**	
Variable	*F*	p	*F*	p	*F*	p	*F*	p	*F*	p	*F*	p	*F*	p
BP	24.04	**<0.001**	47.26	**<0.001**	4.96	**0.029**	35.09	**<0.001**	0.89	0.416	6.60	**0.012**	4.43	0.160
BA	340.27	**<0.001**	142.06	**<0.001**	105.64	**0.0001**	112.73	**<0.001**	39.84	**<0.001**	86.92	**<0.001**	66.22	**<0.001**
DOC	230.79	**<0.001**	481.67	**<0.001**	0.16	0.691	57.67	**<0.001**	0.38	0.687	0.38	0.538	0.13	0.877
a254:365	0.02	0.963	154.07	**<0.001**	0.34	0.563	0.42	0.660	0.05	0.954	0.92	0.763	0.91	0.409
$SUVA_{254}$	42.90	**<0.001**	3569.4	**<0.001**	0.027	0.869	37.79	**<0.001**	0.02	0.980	0.05	0.832	0.26	0.773
peak B	0.17	0.681	91.17	**<0.001**	3.96	0.051	5.71	**0.005**	2.20	0.118	0.36	0.553	0.44	0.645
peak C	75.01	**<0.001**	598.51	**<0.001**	0.07	0.787	30.58	**0.003**	0.04	0.960	0.01	0.929	0.62	0.539
peak T	8.03	**0.006**	2.34	0.104	0.22	0.642	6.54	**<0.00**	5.81	**0.005**	0.25	0.620	1.00	0.374

3.2.1. BGE and relative DOC utilisation. Since BP rates and BA were generally highest during the first five days of microcosm incubation (and declined between days 5-10) we present BGE for this active part of the experiment (i.e. till day 5). Relative DOC utilisation was highest in the Baltic Proper (~30 % utilised by day 5) and decreased in a northerly direction, ~15 % utilisation in the Bothnian Sea and <5 % utilisation in the Bothnian Bay (Table 3). Conversely, BGE showed a clear increase in a northerly direction with values of ~1.5, 16 and 26 % for the Baltic Proper, Bothnian Sea and Bothnian Bay, respectively (Table 3).

Table 3. Mean relative change (Δ, %) during the active phase of incubation (standard error). DOC utilisation (ΔDOC), cumulative bacterial production (BP_{cum}, µg C L^{-1}) and bacterial growth efficiency (BGE) between days 1 and 5. For all values n = 7-12.

Basin	Baltic Proper (BP)		Bothnian Sea		Bothnian Bay	
Treatment	-NP	+NP	-NP	+NP	-NP	+NP
BGE_{1-5}	1.4 (0.4)	1.6 (0.4)	16.9 (6.6)	16.0 (4.1)	20.8 (6.7)	30.8 (4.2)
$BP_{cum\ 1-5}$	25.4 (2.4)	32.7 (2.4)	97.5 (2.7)	109.9 (17.1)	27.1 (1.8)	49.2 (1.1)
ΔBA_{0-5}	118.4 (19.9)	444.1 (20.6)	150.6 (13.2)	177.5 (16.6)	18.3 (6.7)	88.6 (11.0)
ΔDOC_{1-5}	-27.6 (3.9)	-33.1 (2.3)	-10.7 (3.9)	-14.2 (2.1)	-3.9 (1.2)	-3.8 (0.5)
$\Delta a254{:}a365_{0-5}$	8.1 (14.8)	12.5 (15.5)	-2.1 (2.1)	-2.7 (2.1)	-6.9 (3.6)	-4.1 (4.3)
$\Delta S275{:}295_{0-5}$	4.2	4.6	-5.5	-5.5	-4.3	-4.2

	(2.9)	(3.0)	(0.6)	(0.6)	(1.6)	(2.0)
$\Delta SUVA_{0-5}$	44.1 (3.8)	53.9 (3.9)	2.4 (1.0)	3.8 (2.1)	-1.7 (2.4)	-2.9 (2.7)
$\Delta peak\ B_{0-5}$	1.2 (1.7)	8.2 (2.0)	25.3 (10.2)	11.3 (7.6)	-4.2 (8.7)	-7.7 (9.7)
$\Delta peak\ C_{0-5}$	-4.2 (0.9)	-2.0 (0.8)	8.0 (1.1)	8.1 (1.3)	11.39 (0.8)	10.0 (0.6)
$\Delta peak\ T_{0-5}$	-7.0 (1.6)	8.8 (1.0)	1.6 (6.7)	-2.7 (5.9)	-16.4 (6.5)	-16.3 (7.1)

3.3 Changes in TN and TP

As expected, TN and TP concentrations were elevated in the +NP treatment. However, over the duration of the experiment no marked changes in microcosm TN and TP concentrations were observed in either the standard or +NP treatments (Table S2).

3.3.1. Effect of nutrient addition, +NP. In general nutrient addition increased BA and BP rate in the Baltic Proper and Bothnian Bay microcosms, as compared to their respective standard microcosms. However, this effect was only seen in the latter stages of the overall incubation period (Fig. 2), increasing the respective integrated cumulative BP (BP_{cum}) value (Table 3). No such effect was seen in the Bothnian Sea microcosms (Fig. 2 and Table 3). Nutrient addition slightly increased the mean percentage of DOC utilised (~3-5 %) in more southerly basins, although no effect on DOC utilisation was seen in the Bothnian Bay microcosms (Table 3). Only in the Bothnian Bay did changes due to nutrient addition translate into increased mean BGE

(Table 3). Addition of nutrients had little impact on the optical DOM characteristic variables measured (Table 3). With the exception of changes in BA and BP, changes due to the addition of nutrients were not significant (Table 2).

3.4 Trends and associations during incubation. Certain variables in the raw data were strongly and significantly correlated and therefore removed from the RM-ANOVA analysis to prevent biasing the result. The variables retained include: BP, BA, DOC, a254:a365, $SUVA_{254}$, peak B, peak C, and peak T. With the exception of $SUVA_{254}$-DOC (r = -0.68) and $SUVA_{254}$-a254:a365 (r = -0.61), correlations between the retained variables was relatively low (r = < +/-0.55).

During incubation the response of DOM characteristics differed between basins. The Bothnian Bay exhibited relatively higher levels of peak C than the other two basins at the start and while it remained relatively constant in the Baltic Proper incubations it increased markedly during the active phase of incubation (up to day 5) in the Bothnian Sea and the Bothnian Bay microcosms (Fig. S3). Fluorescence peaks B and T fluctuated during the incubation period but clear trends were not present (Fig. S3). The a254:a365 ratio and S275-295 were highest in the southern basin and lowest in the northern most basin with a minor increase recorded during incubations from the Baltic Proper and a minor decrease observed during incubation in the northern basin incubations (Fig. S2). $SUVA_{254}$ values increased during the active phase of the microcosm incubation in the Baltic Proper, however decreased during this phase in the more northerly basins. A similar trend was observed with CDOM, except for the Bothnian Sea microcosms in which it fluctuated and appeared to increase, rather than

decrease, during the same phase (Fig. S2). Changes over time in the incubations were significant for the majority of variables (Table 2).

S275-295 correlated spatially with CDOM, with higher CDOM values corresponding to lower S275-295 values. The same trend was seen during the microcosm experiment within each individual basin, suggesting that changes in CDOM during incubation also correlated with changes in S275-295 (n = 54-60; R^2 = 0.64, 0.66 and 0.74 for Baltic Proper, Bothnian Sea and Bothnian Bay, respectively). A similar spatial correlation was seen between lnDOC concentration and ln$SUVA_{254}$ values (ALL, n = 131, R^2 = 0.79, p = <0.001), however, the correlation only remained substantial in the Baltic Proper when exploring this trend for microcosm units in each separate basin (n = 47-51; R^2 = 0.68, 0.39 and 0.22 for Baltic Proper, Bothnian Sea and Bothnian Bay, respectively).

3.4.1. Relative changes (relative Δ values, %, till day 5) in measured variables.

During the active part of the experiment (i.e. till day 5), relative increases in $SUVA_{254}$, S275-295 and a254:a365 were recorded in the Baltic Proper microcosms. Marginal relative increases or relative decreases were recorded in the Bothnian Sea microcosms, and relative decreases in the Bothnian Bay microcosms (Table 3). Relative decreases in peak B and peak T were strongest in the Bothnian Bay microcosms, while a relative increase in peak C was detected in the Bothnian Bay and Bothnian Sea compared to a relative decreased in the Baltic Proper (Table 3). Changes (Δ %) were generally significantly different between basins (Table S3).

3.4.2. Significance and interaction (time-basin-treatment). The RM-MANOVA indicated that basin, treatment and time all contributed to significant differences in the experimental microcosms (Time*Basin*Treatment: $F_{64,72} = 4.678$, p = <0.001). However, the effects of time, basin and time*basin exhibited higher *F* values and were more significant than any treatment effects (i.e. addition of nutrients, +NP). Treatment effects (and interactions) were generally only significant for BA (Table 2), indicating that time (i.e. changes during microcosm incubation) and basin (i.e. origin of water used in experimental microcosms) were stronger drivers of the significant differences seen. Mean differences of individual variables between basins and their significance (post hoc Bonferroni tests) are shown in Table S4.

3.4.3. Associations between measured variables. Since the addition of nutrients had a limited effect, the following data only encompass the standard microcosm incubations (without nutrient addition). Other correlations are shown in supplementary results.

Higher starting DOC concentrations correlated with higher ΔDOC values (DOC utilisation) during the active phase of the microcosm incubation (DOC v ΔDOC_{1-5}, $n = 28$, $R^2 = 0.91$, p = <0.001) and with lower BGE (BGE_{1-5} v DOC, $n = 23$, $R^2 = 0.79$, p = <0.001). However, high DOC utilization correlated with low BGE (BGE_{1-5} v ΔDOC_{1-5}, $n = 52$, $R^2 = 0.78$, p = <0.001).

Nutrient concentrations and nutrient stoichiometry at the start of the incubations varied between basins (Table 1). Higher starting concentrations of TN and TP corresponded with larger increases in BA during the active incubation period (TN v

$\Delta BA_{0\text{-}5}$, n = 33, R^2 = 0.65, p = <0.001 and TP v $\Delta BA_{0\text{-}5}$, n = 33, R^2 = 0.64, p = <0.001). Lower starting C:N ratios had a positive effect on BGE (lnC:N v $lnBGE_{1\text{-}5}$, n = 23, R^2 = 0.71, p = <0.001), with the highest BGE recorded at C:N ratios of ~23. However, at higher C:N ratios DOC utilisation was larger (C:N v $\Delta DOC_{0\text{-}5}$, n = 28, R^2 = 0.76, p = <0.001).

Microcosm units exhibiting low BGE values exhibited larger relative increases in $SUVA_{254}$ ($lnBGE_{1\text{-}5}$ v ln normalised % $\Delta SUVA_{0\text{-}5}$, n = 23, R^2 = 0.47, p = <0.001), while those exhibiting higher BGE showed smaller increases in $SUVA_{254}$ or even decreases. The opposite trend was observed for peak C, with relative decreases in peak C at lower BGE values ($lnBGE_{1\text{-}5}$ v ln normalised % Δpeak $C_{0\text{-}5}$, n = 23, R^2 = 0.63, p = <0.001). Furthermore, with higher starting DOC concentrations the production of peak C was lesser, and at the higher end of DOC concentrations peak C decreased (DOC v Δpeak $C_{0\text{-}5}$, n = 36, R^2 = 0.66, p = <0.001).

The PCA analysis indicated clear clustering of samples from each basin, and clear separation between samples from each basin (Fig. 3). Moreover, there was a clear difference in the association of the measured variables to the different basins.

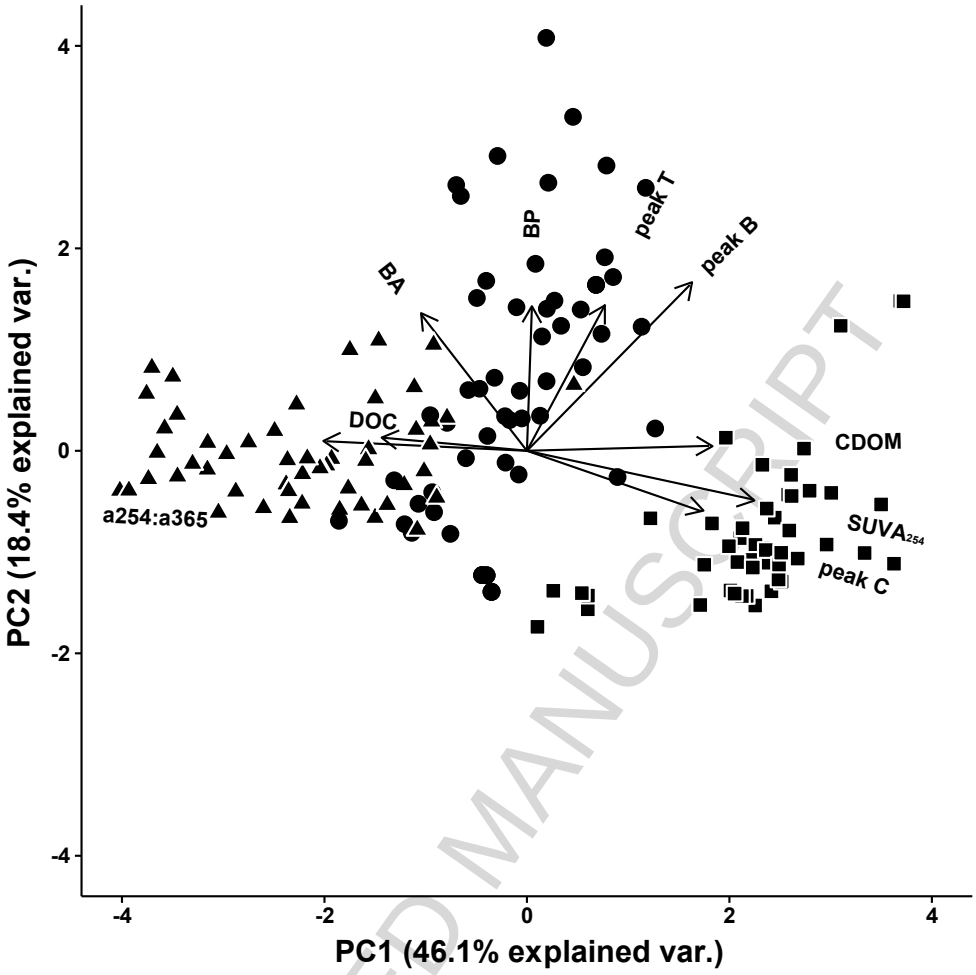

Figure 3. Principal component analysis (PCA) of bacterial and DOM characteristic variables from all standard (+NP excluded) microcosm units and all sampling occasions (Baltic Proper, triangles; Bothnian Sea, circles; and Bothnian Bay, squares). PC1 and PC2 encompass 64.5 % of the cumulative variance in the data set. PC1 (46 % of variance) was most strongly loaded by $SUVA_{254}$ (+0.46), CDOM (0.42), peak C (0.40), a254:a365 (-0.39) and DOC (-0.36). PC2 (18 % of variance) was most strongly loaded by peak T (0.53), peak B (0.51), BP (0.45) and BA (0.42).

4. Discussion

Seawater contains a vast pool of carbon and the concentrations, characteristics, and bioavailability of this matter can differ seasonally and spatially as it is continuously altered by degradative and formative physicochemical and biological processes (Benner and Amon, 2015; Jiao et al., 2010; Nagata 2000). In this study we find that spatial differences in the nutrient status and DOM characteristics play an important role in controlling the bacterial utilisation of DOC, thus controlling the BGE and influencing the DOM pool itself.

4.1. Spatial variation and within basin similarity. The unique hydrology and extensive latitudinal expanse of the Baltic Sea maintains a high degree of spatial and seasonal physicochemical variation. Clear differences in biological communities and processes also exist, including at the basal microbial level (e.g. Andersson et al., 2015; Herlemann et al., 2011). Within the bounds of each of the three major basins studied, the sampled stations showed clear physicochemical similarities (Fig. S1, Table 1) and were in general significantly different from other basins (Table S1). This affirms spatial physicochemical gradients (Table 1) and validates the consideration of offshore water-bodies within each basin as single entities for the purpose of this, and similarly designed studies.

In contrast to other studies (compiled in Hoikkala et al., 2015) we recorded higher DOC concentrations at the southerly Baltic Proper stations. This was likely due to the dual effect of the relative closeness to land of the southern stations sampled and the presence of an extensive phytoplankton bloom at the time of sampling (Hansson and Öberg, 2011). Importantly, our data show that the composition of the DOM pool

differed strongly between the studied basins (Fig. S2 and S3) and this is particularly germane for such studies, as these characteristics influence DOM bioavailability or reactivity (Asmala et al., 2013; Autio et al., 2015; Benner and Amon, 2015). Water colour (CDOM, Harvey et al., 2015), DOM aromaticity ($SUVA_{254}$, Weishaar et al., 2003) and levels of secondary humic material of terrestrial origin (peak C, Cammack et al., 2004; Stedmon and Markager, 2005) were all highest in the northern Bothnian Bay basin and lower in the Baltic Proper. On the other hand S275-295 and a254:a365 were highest in the Baltic Proper, both inversely related to the DOM molecular weight (Asmala et al., 2013; Fichot and Benner, 2012; Helms et al., 2008; Wallin et al., 2015). Taken together these data indicate clear spatial trends that are in accordance with the strong terrestrial influence in the northerly basins (Alling et al., 2008; Deutsch et al., 2012; Harvey et al., 2015; Stedmon et al., 2007) and are indicative of more autochthonous DOM sources in the southerly Baltic Proper (Andersson et al., 2015; Hoikkala et al., 2015; Maciejewska and Pempkowiak, 2014).

4.2. Bacterial growth, DOC utilisation and BGE. BA in all microcosms generally reached highest levels by day three or five before it plateaued or decreased. Despite similar starting rates on day zero BP differed strongly between basins, with highest rates recorded in the Bothnian Sea microcosms. It is possible that this is due to a more suitable stoichiometric balance of nutrients in the Bothnian Sea (Table 1). This active phase of the incubation (day 0-5) corresponded with the phase during which DOC utilisation also took place. During this phase, largest mean DOC utilisation was recorded in the southerly Baltic Proper basin (~30%) and decreased in a northerly direction (Bothnian Sea ~12% and Bothnian Bay ~4%), with values being in a similar range to previous studies (Asmala et al., 2013; Hoikkala, 2015; Zweifel et al., 1993).

Highest DOC utilisation occurred in the region with higher starting DOC concentrations, as Søndergaard and Middelboe (1995) found in a large cross-system analysis. However, the clear regional differences in the DOM pool characteristics indicate that the control of bacterial DOC utilisation is a more complex process. The prevailing conditions resulted in BGE values that were comparable with similar studies (Asmala et al., 2013; Attermeyer et al., 2014; Figueroa et al., 2016). However, BGE was negatively correlated with DOC utilisation. BGE values were highest in the Bothnian Bay basin (~25 %) and decreased in a southerly direction (~16 and ~2 %, Bothnian Sea and Baltic Proper, respectively). Similar relationships have been reported recently where higher BGE levels were found in river waters strongly influenced by humic matter or forested soils, supporting the notion that DOM characteristics influence bacterial metabolism (Autio et al., 2015; Berggren and del Giorgio, 2015).

4.3. Influence of nutrients on bacterial activity. The addition of N and P (+NP) resulted in significantly elevated BA and BP rates in the Baltic Proper and Bothnian Bay microcosms (Table 2). In essence nutrient addition sustained a longer period of elevated BA and BP (Fig. 2, and BP_{cum} Table 3). However, little effect was seen on DOC utilisation and only in the Bothnian Bay did it result in a markedly different basin mean BGE (Table 3). This strong increase in BGE in the Bothnian Bay may relate to the adjusted C:N:P stoichiometric ratios that aligned all basin ratios more closely in the +NP treatments (basin mean C:N:P = 19-34:2:1), in particular reducing the C:P ratios that were at their most extreme in the Bothnian Bay natural waters (Table 1). While stoichiometric ratios of these vital nutrients have been shown to be important in marine systems (Thingstad et al., 2008; Andersson et al., 2013) the

addition of P would likely have alleviated the major limiting nutrient in the Bothnian Bay (Tamminen and Andersen, 2007; Andersson et al., 2015). Furthermore, nutrient addition did not induce significant changes in DOM characteristics (Table 3), which showed stronger and significant changes spatially and over the time period of the incubation (Table 2). The lack of change in DOM degradation may indicate that nutrient addition did not strongly alter the bacterial community composition, that functional redundancy within the local bacterial community strongly determines the outcome, or that a common pool of generalist bacteria drove the degradation of DOM at each site (Allison and Martiny, 2008; Attermeyer et al., 2014; Dinasquet et al., 2013). However specific studies would be required to clarify these issue since our measurements generally encompass bulk values and net changes during the experiment.

Despite the relatively unaltered DOM processing due to nutrient supplementation, ambient starting nutrient concentrations (and stoichiometric ratios) correlated closely with changes in BA (standard microcosms only). High starting concentrations of TN and TP, plus low C:P and N:P stoichiometric ratios resulted in larger increases in BA. However, no corresponding correlation was found with DOC. While the concentrations and stoichiometric ratios of these elements at the start of the incubation are important, and have the potential to limit bacterial growth (Degerman et al., 2013; Zweifel et al., 1993), the minimal number of close correlations with BP, BGE or changes in DOM characteristic variables indicate that there are clear differences between the influence of nutrients on growth (i.e. BA) and the physiological processes taking place (Guillemette and del Giorgio, 2012). This further supports the reasoning that changes seen here relate to the physiological capacity of stable local bacterial

communities. However, high C:N starting ratios correlated with largest decreases in DOC during the active phase of the experiment, and with the lowest BGE values. This supports previous suggestions that in addition to the DOM characteristics and the total BA or BP capacity, the metabolic balance (i.e. BGE) of the bacterial community is also vital (Guillemette and del Giorgio, 2012).

4.4. DOM characteristics and bacterial interaction. Clear differences in DOM characteristics were recorded across the studied gradient, including support for the hypothesis that DOM would be more strongly autochthonous in the south. However, during the active period of incubation the molecular weight of the DOM pool (as defined by the S275-295 proxy) decreased in the Baltic Proper, whereas it increased in microcosms from the two more northerly basins (Table 3). In the Baltic Proper microcosms a clear increase in CDOM was also observed during incubation (Fig. S2). This would suggest that larger molecular weight constituents within the Baltic Proper DOM pool were broken down, whereas DOM components of a larger size became relatively more dominant in the DOM pool of the northerly basins. Concurrently, bacterial activity contributed to the production of CDOM in Baltic Proper microcosms, as reported from other systems (Kramer and Herndl, 2004; Nelson et al., 2004; Yamashita and Tanoue, 2004). However, the exact nature of this processed portion of the DOM pool, and its interaction with resident biological communities, is complex. The Baltic Proper DOM pool became increasingly aromatic in nature during incubation (Table 3), with the relative change in DOC (i.e. utilisation) and change in aromaticity being associated, and the highest levels of DOC utilisation corresponding to highest levels of aromaticity increase. Thus, bacterial activity in the Baltic Proper decreased DOC concentrations, breaking down larger

molecular weight compounds and the processed DOM pool was more aromatic and contributed to increasing water colour. This appears to relate to functional aspects of the local bacterial community and is not at odds with an earlier study that found bacteria from the Baltic Proper grew well, if not better than the native bacteria, in Bothnian Sea water containing natural DOM (Lindh et al., 2015). However, the high initial DOC concentrations recorded in the Baltic Proper, due mainly to a contemporary phytoplankton bloom, would also likely have contributed to this trend (and to the low BGE recorded in this region). This pool of autochthonous DOC would have been readily available and respired, resulting in extensive carbon losses (Berggren and del Giorgio, 2015).

Changes to the intrinsic nature of the DOM pool will influence its subsequent bioavailability, and have the potential to result in carbon limitation (Carlson and Ducklow 1996; Figueroa et al., 2016; Kirchman and Rich, 1997). Such carbon limitation scenarios are likely to contribute to the similar temporal patterns of BP and BA seen in our experimental microcosms, including the apparently limited influence of nutrients. It may be that viral lysis also played a role (e.g. Middelboe and Jørgensen, 2006), though this can not be ascertained directly. In experimental systems where concurrent physicochemical alteration of a finite DOM pool is limited, and the bacterial community remains constrained by the starting inoculum, limitation may appear particularly pronounced. However, in the natural environment the dynamic nature of these interactions will undoubtedly change this perspective. In the Baltic Sea, where waters generally transfer between basins in a southerly direction due to the net freshwater influx in the north, the DOM pool is exposed to an extensive continuum of biological and physicochemical action. Thus, the patterns of DOM

characteristics (and changes) detailed here could conceivably indicate that the DOM pool, in addition to being altered by bacterial activity, is also a formative driver of local bacterial community structure (Herlemann et al., 2013; Judd et al., 2006; Lindh et al, 2015; Logue et al., 2016).

Samples with high aromaticity or high molecular weight (i.e. from more northerly basins) generally expressed higher levels of secondary humic matter of terrestrial origin (peak C: Cammack et al., 2004; Coble, 1996; Stedmon and Markager, 2005). However, during the microcosm incubation these variables responded very differently between basins (Table 3). Largest relative increases in aromaticity generally corresponding with largest decreases in secondary humic matter. Additionally, during microcosm incubation mean basin changes in DOM molecular weight and secondary humic matter of terrestrial origin followed latitudinal patterns that were opposite to each other (Table 3). In the Baltic Proper bacterial activity depleted secondary humic material of terrestrial origin, resulting in smaller molecular weight DOM that was more aromatic in nature. Such processes have been observed in the dark ocean where heterotrophic production was significant (Jørgensen et al., 2011). On the other hand, in the two more northerly basins the DOM pool became less/less strongly aromatic and the relative contribution of higher molecular weight secondary humic matter increased (Table 3). Furthermore, the trends in secondary humic matter correlated with BGE, where microcosms expressing high BGE showed largest increases in secondary humic material, whereas microcosms with low BGE expressed smaller increases or decreases. This is in keeping with a study in lakes, where largest increases in secondary humic peaks were found in incubations dominated by anabolic (i.e. high BGE), rather than catabolic (low BGE) processes (Guillemette and del

Giorgio, 2012), leading the authors to conclude such factors would also have importance for the transfer of energy and nutrients within the food web.

Protein-like peaks (peak B and peak T: Coble, 1996), however, responded quite differently to bacterial activity, and although changes were often significant (Table 2) the patterns did not follow linearly across the latitudinal gradient studied (Table 3). We recorded the largest decreases in protein-like fluorescent peaks in the Bothnian Bay (Table 3), a pattern that has also been observed in lakes (Guillemette and del Giorgio, 2012). However, in the mid-gradient Bothnian Sea microcosm these two peaks appeared to be produced, particularly strongly in the case of peak B. It appears that a different process controls the production or utilisation of protein-like compounds in this study, with production associated to the BA and BP variables (Fig. 3 and Table 3), potentially representing cell wall proteins (Kawasaki and Benner 2006; Stoderegger and Herndl, 1998; Tanoue et al., 1995) or other structural components (Kaiser and Benner, 2008; Ogawa et al., 2001).

5. Conclusion. The dual role of bacteria in both utilising and producing DOM, and the interplay between DOM characteristics, nutrient status, and bacterial metabolism all determine the fate of DOM and thus the composition of the bulk DOM pool. In this study we addressed the net balance of these complex processes. Our study suggests that spatial differences in DOM characteristics, nutrient levels and nutrient stoichiometric ratios are important factors controlling bacterial growth and BGE, and that these processes in turn influence the DOM pool. Markedly different DOM-bacterial interactions were observed in each region of the studied gradient, catalysing different consequences for the DOM pool. It is clear that bacterial growth and

metabolism (e.g. BGE) can alter the characteristics and properties of the DOM pool and that these modifications can influence bioavailability, have repercussions for long term carbon sequestration (Brophy and Carlsson 1989; Jiao et al., 2010; Ogawaa et al., 2001), and can influence the global carbon cycle (Benner and Amon, 2015; Jiao et al., 2010). Furthermore, climate change scenarios indicate that surface water warming, elevated rainfall and terrestrial run off, and altered nutrient status within the studied system are expected (Eilola, 2013; Graham, 2004; Wikner and Andersson, 2012). This will influence the complex DOM-nutrient-bacterial interactions that currently exist and thereby influence the passage of nutrients and energy to higher trophic levels.

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Graphical abstract