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Gradients in microbial methanol uptake: productive coastal upwelling waters to oligotrophic gyres in the Atlantic Ocean

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Methanol biogeochemistry and its importance as a carbon source in seawater is relatively unexplored. We report the first microbial methanol carbon assimilation rates (k) in productive coastal upwelling waters of up to 0.117 ± 0.002 d^{-1} (~ 10 nmol $l^{-1} d^{-1}$). On average, coastal upwelling waters were 11 times greater than open ocean northern temperate (NT) waters, eight times greater than gyre waters and four times greater than equatorial upwelling (EU) waters; suggesting that all upwelling waters upon reaching the surface ($\leqslant 20$ m), contain a microbial population that uses a relatively high amount of carbon (0.3–10 nmol $l^{-1} d^{-1}$), derived from methanol, to support their growth. In open ocean Atlantic regions, microbial uptake of methanol into biomass was significantly lower, ranging between 0.04–0.68 nmol $l^{-1} d^{-1}$. Microbes in the Mauritanian coastal upwelling used up to 57% of the total methanol for assimilation of the carbon into cells, compared with an average of 12% in the EU, and 1% in NT and gyre waters. Several methylotrophic bacterial species were identified from open ocean Atlantic waters using PCR amplification of *mxaF* encoding methanol dehydrogenase, the key enzyme in bacterial methanol oxidation. These included *Methylophaga* sp., *Burkholderiales* sp., *Methylococcaceae* sp., *Ancylobacter aquaticus*, *Paracoccus denitrificans*, *Methylophilus methylotrophus*, *Methylobacterium oryzae*, *Hyphomicrobium* sp. and *Methylosulfonomonas methylovora*. Statistically significant correlations for upwelling waters between methanol uptake into cells and both chlorophyll a concentrations and methanol oxidation rates suggest that remotely sensed chlorophyll a images, in these productive areas, could be used to derive total methanol biological loss rates, a useful tool for atmospheric and marine climatically active gas modellers, and air–sea exchange scientists.

Introduction

We have only just begun to understand methanol biogeochemistry and its significance in marine microbial carbon cycling in the oceans, and it represents one of the emerging scientific research areas. Recent analytical developments (for example, Beale *et al.*, 2011) coupled with proven published methods utilising ¹⁴C-labelled methanol (Dixon *et al.*, 2011a, b; 2012) have begun to uncover high seawater concentrations up to 429 nM (Beale *et al.*, 2011) coupled with biological turnover times as low as 1 day in the surface waters of the oligotrophic North Atlantic (Dixon *et al.*, 2011b). The occurrence

of methylotrophic bacteria (that is, bacteria that grow on methanol and other one carbon compounds) in the marine environment has been known for many years (for example, see Murrell *et al.*, 1992 and references therein), but there has been relatively little research on marine methylotrophs until recently. For example, using cultivation-independent methods such as DNA stable isotope probing, it was shown that methylotrophs and, in particular, *Methylophaga* species are prevalent in coastal waters (Neufeld *et al.*, 2007; 2008). More recently, Giovannoni and colleagues, through cultivation-based studies, have provided conclusive evidence that SAR 11 Alphaproteobacteria, which are some of the most abundant heterotrophs in the oceans, use one-carbon compounds like methanol for energy generation, and thus microbial oxidation of one carbon compounds could represent a significant *in situ* source of CO_2 in the upper ocean (Sun *et al.*, 2011). Using elegant genomics and environmental proteomics methods

they have also shown that Betaproteobacteria of the OM43 clade, which are commonly found in productive coastal ocean ecosystems, also metabolise methanol and other one carbon compounds (Giovannoni et al., 2008; Sowell et al., 2011; Halsey et al., 2012). All of this research indicates the potential for significant turnover of methanol in the marine environment, and thus it is important to understand how different ocean systems contribute to the cycling of methanol.

Upwelling regions of the global oceans inject inorganic nutrients to often impoverished waters, and are thus normally highly biologically productive regions, despite their limited geographical extent. The coastal Mauritanian upwelling area is one of two major coastal upwellings in the Atlantic Ocean, and globally one of the most biologically productive systems (Pauly and Christensen, 1995). However, this area remains relatively unexplored (Rees et al., 2011). In contrast, oligotrophic oceanic gyres, which are the oceans least productive waters, cover ~40% of the planet's surface and are thought to be expanding due to global warming (McClain et al., 2004; Polovina et al., 2008). The cyanobacterium Prochlorococcus and the SAR11 group of Alphaproteobacteria numerically dominate the oligotrophic gyres (Chisholm et al., 1988; Morris et al., 2002), while very small plastidic protists are the most numerous of the eukaryotes (Hartmann et al., 2012 and references therein). Thus, the aim of this study was to investigate the microbial demand for methanol as a carbon source for growth along a gradient of productivity from chlorophyll-rich coastal upwelling waters to oligotrophic oceanic gyres in the Atlantic Ocean.

Materials and methods

Sample collection
Seawater samples were collected from the Atlantic ocean (Figure 1) during two research cruises (a) SOLAS ICON (Surface Ocean Lower Atmosphere Study, UK 'The impact of coastal upwellings on the production of climatically active gases') aboard the RRS Discovery (D338, 15 April–27 May 2009) and (b) AMT19 (Atlantic Meridonal Transect cruise number 19) aboard RRS James Cook (JC039, 13 October–1 December 2009). Samples were collected with 20 l Niskin bottles deployed on a rosette
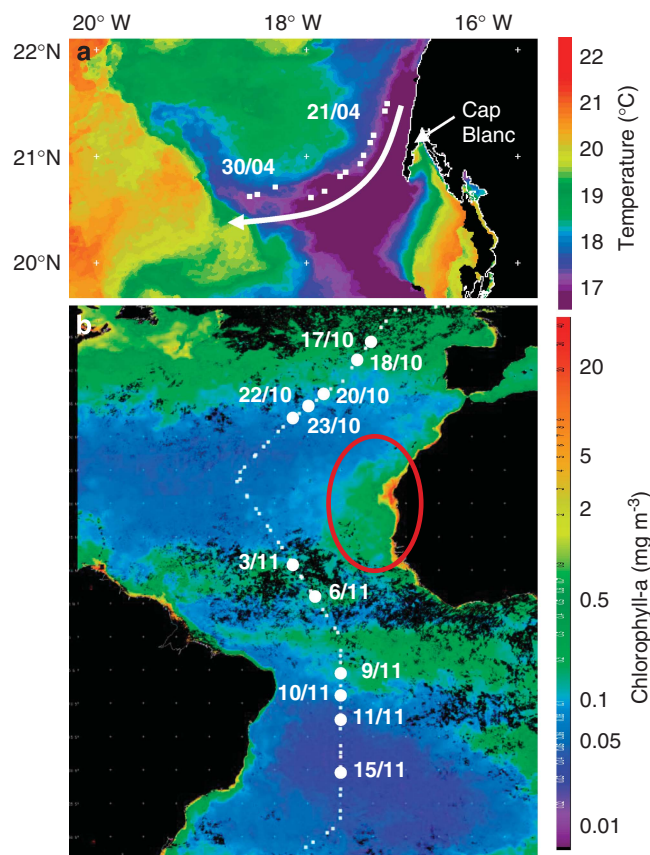
Figure 1 Study area showing (**a**) AVHRR image of sea surface temperature showing the cold water filament, which was sampled during 21–30 April 2009 where the arrow indicates the direction of filament flow away from the coast, and (**b**) remotely sensed MODIS—Aqua chlorophyll a composite image from November 2009. The red oval shows the high concentrations associated with the Mauritanian upwelling. White squares show station positions, while white circles and corresponding station numbers (sampling date) show location of methanol uptake experiments during AMT19. These images are courtesy of NEODAAS.

equipped with a Seabird conductivity, temperature and depth sensors. The seawater was collected directly into acid washed polycarbonate bottles that were in black plastic bags, and experiments were started within <1 h of sampling.

Microbial methanol uptake

Seawater samples (320 ml) were incubated with ~4.4 nM (final concentration) of ^{14}C labelled methanol ($^{14}CH_3OH$) for between 4.5–10 h in the dark at *in situ* temperature, to determine the microbial uptake of methanol into particulate cell biomass. For full details of experimental procedures please refer to Dixon *et al.* (2011a). Typical seawater methanol concentrations range between 27–212 nM (Beale, 2011, personal communication) and 100–429 nM (Williams *et al.*, 2004; Beale *et al.*, 2011; Dixon *et al.*, 2011a, b) in the Mauritanian upwelling and the wider Atlantic ocean respectively. Thus the radiotracer addition typically represents between 2–16% and 1–4% of *in situ* concentrations in upwelling and Atlantic waters, respectively. The data are reported as apparent rate constants, k (h^{-1}) calculated from a ratio of the sample ^{14}C counts (from the particles on the filter, $nCi\,ml^{-1}\,h^{-1}$) divided by total $^{14}CH_3OH$ added to the sample ($nCi\,ml^{-1}$). Evaluation of control samples suggests that $\leqslant 0.3\%$ is recovered on the filters. Methanol uptake rates in $nmol\,l^{-1}\,h^{-1}$ were calculated by multiplying the methanol counts obtained from the filter ($nCi\,ml^{-1}\,h^{-1}$) by the specific activity of ^{14}C methanol ($57.1\,mCi\,mmol^{-1}$).

Bacterial production

Heterotrophic bacterial production rates were determined using 1.7 ml seawater samples by measuring the incorporation of ^3H-leucine (20 nM final concentration) into bacterial protein synthesis following the method of Smith and Azam (1992). The relative error based on three replicates was on average 7.1%. Leucine incorporation rates were multiplied by carbon to leucine conversion factors. For the Mauritanian upwelling samples, an empirically derived conversion factor of $0.7\,kg\,C\,mol\,leu^{-1}$ from Alonso-Sáez *et al.* (2007) was used, which matches with upwelling location and sampling season. However, for an upper estimate, the theoretical conversion factor of $1.55\,kg\,C\,mol\,leu^{-1}$ derived from Simon and Azam (1989) and used notably by Hoppe *et al.* (2002) was applied. An average empirical conversion factor of $0.73\,kg\,C\,mol\,leu^{-1}$ (Morán *et al.*, 2002; 2004; Dixon, 2008; Calvo-Díaz and Morán 2009; Dixon *et al.*, 2011b) was also applied for all samples. For Atlantic transect samples, where surface chlorophyll a levels were relatively low (0.02–$0.68\,mg\,m^{-3}$, Table 1), a lower estimate of bacterial carbon production was estimated using a conversion factor of $0.1\,kg\,C\,mol\,leu^{-1}$ (Alonso-Sáez *et al.*, 2007; Dixon *et al.*, 2011b).

Primary production and Chlorophyll a concentrations

Primary production (PP) was estimated using the standard method of ^{14}C-bicarbonate incorporation (for example, Joint and Pomroy, 1993; Dixon, 2008), except that during the ICON upwelling cruise, samples were incubated for ~24 h (between 0600–0600), and for Atlantic Meridonal Transect (AMT) stations, samples were incubated from dawn to dusk. Chlorophyll a concentrations were determined by fluorometric analysis of acetone-extracted pigments (Holm-Hansen *et al.*, 1965).

Community composition

The numbers of bacterioplankton cells were determined by flow cytometry on SYBR Green I DNA-stained cells from 1.8 ml seawater samples fixed in paraformaldehyde (5%, final concentration). Numbers of *Prochlorococcus* sp. and *Synechococcus* sp. were analysed on unstained samples by flow cytometry. Water samples (250 ml) for plankton identification were collected pre-dawn and fixed in 2% acid Lugol's solution. Preserved samples were stored in cool, dark conditions until they were analysed in the laboratory by inverted settlement microscopy (Utermöhl, 1958). The plankton assemblage was characterised as detailed previously in Widdicombe *et al.* (2002).

Nutrient concentrations

Inorganic phosphate and nitrate concentrations were determined on discrete seawater samples using a 5-channel Technicon segmented flow autoanalyser for micromolar determinations (Woodward and Rees, 2001).

DNA extraction and bacterial identification

Seawater samples were only collected during AMT19 from the surface, 33% and 1% photosynthetically active radiation (PAR) equivalent depths and 200 m. Samples of 20 l were filtered through 0.2 μm Sterivex filters (Millipore, Watford, UK) using a peristaltic pump. DNA samples were incubated with 1.4 ml of RNAlater (Life Technologies, Paisley, UK) overnight at 6 °C. The RNAlater was removed before storage at −80 °C for the remainder of the cruise. Samples were transported back to the UK on dry ice and stored at −20 °C.

DNA was extracted from the Sterivex filters using the method previously described in Neufeld *et al.* (2007). Centrifugal filter devices (Amicon, Millipore) were used to clean the extracted DNA. PCR (35 cycles) amplification was carried out with functional gene primers for *mxaF* (encoding the large subunit of methanol dehydrogenase, found in Gram-negative methylotrophic bacteria)1003*f* and 1555*r* (McDonald and Murrell 1997; Neufeld *et al.*, 2007) with an annealing temperature of 55 °C. PCR products of the appropriate size (~550 bp) were

Table 1 Summary of parameters in the euphotic zone (down to 1% PAR depth)

	Methanol uptake (d^{-1})	Chl a ($mg\,m^{-3}$)	PP ($mgC\,m^{-3}\,h^{-1}$)	BN ($\times 10^6$ cells ml^{-1})	BP[a] ($pM\,Leu\,d^{-1}$)	Nitrate ($\mu mol\,l^{-1}$)	Phosphate ($\mu mol\,l^{-1}$)	Syns ($\times 10^3$ cells ml^{-1})	Pros ($\times 10^5$ cells ml^{-1})	Peuks[b] ($\times 10^3$ cells ml^{-1})	Nano[c] ($\times 10^3$ cells ml^{-1})
Coastal upwelling											
Av ± 1s.d.	0.033 ± 0.020	2.8 ± 2.0	114 ± 112[d]	1.2 ± 0.3	1465 ± 495	6.6 ± 1.5	0.47 ± 0.10	0.35 ± 0.20	ND	0.54 ± 1.1	1.1 ± 0.7
Range	0.004–0.117	0.5–6.5	5–338[d]	0.5–1.8	190–2279	4.5–13.3	0.35–0.81	0.12–1.3	ND	0.03–5.3	0.2–2.4
n	43	43	43	43	43	42	42	43		43	43
Northern Temperate											
Av ± 1s.d.	0.003 ± 0.001	0.18 ± 0.15	4.7 ± 2.7	NA	203 ± 112	0.09 ± 0.005	0.06 ± 0.03	20.2 ± 19.2	1.6 ± 0.8	5.1 ± 3.8	0.62 ± 0.44
Range	0.001–0.004	0.02–0.35	1.4–8.3		34–341	0.08–0.09	0.03–0.13	2.3–41.9	0.02–2.2	1.1–11.3	0.18–1.3
n	8	8	8		8	8	8	8	8	8	8
Northern Gyre											
Av ± 1s.d.	0.004 ± 0.003	0.15 ± 0.16	2.7 ± 1.4	NA	344 ± 369	0.28 ± 0.69[e]	0.06 ± 0.05[e]	2.4 ± 2.0	1.3 ± 0.4	1.6 ± 1.7	0.22 ± 0.07
Range	0.001–0.010	0.06–0.59	1.2–5.3		124–1488	0.01–2.5[e]	0.04–0.21[e]	0.1–5.6	0.5–1.8	0.8–6.5	0.05–0.36
n	12	12	12		12	12	12	12	12	12	12
Equatorial upwelling											
Av ± 1s.d.	0.008 ± 0.004	0.27 ± 0.18	10.5 ± 6.0	NA	391 ± 175	0.08 ± 0.07	0.02 ± 0.03	30.9 ± 24.1	2.5 ± 1.1	3.4 ± 4.1	0.9 ± 1.4
Range	0.003–0.015	0.13–0.68	1.2–19.8		135–635	0.02–0.20	0.01–0.08	1.0–81	1.1–3.6	1.5–13.3	0.3–4.3
n	8	8	8		8	8	8	8	8	8	8
Southern Gyre											
Av ± 1s.d.	0.004 ± 0.002	0.13 ± 0.12	3.2 ± 2.2	NA	525 ± 757	0.18 ± 0.41[f]	0.15 ± 0.07[f]	2.0 ± 1.2	2.2 ± 1.1	2.5 ± 2.9	0.28 ± 0.16
Range	0.001–0.007	0.03–0.45	0.7–7.9		51–3217	0.02–1.6[f]	0.06–0.29[f]	0.01–3.7	0.7–3.8	0.6–11.5	0.16–0.77
n	16	16	16		16	16	16	16	16	16	16

Abbreviations: BN, bacterial numbers; ND, not detected; NA, not available; PP, primary productivity; Pros, Prochlorococcus sp; Syns, Synechococcus sp.
[a]Bacterial leucine incorporation.
[b]Picophytoplankton (<2 µm).
[c]Nanophytoplankton (~2–12 µm).
[d]For coastal Mauritanian upwelling primary productivity was carried out over 24 h period hence units of mgC m^{-3} d^{-1}, whereas for all other regions incubations were from dawn to dusk and are shown in mgC m^{-3} h^{-1}.
[e]For one depth profile on 20 October 2009 the 1% PAR equivalent depth of 57 m was within the start of the nitricline, where concentration of nitrate was 2.5 µmol l^{-1} and phosphate was 0.21 µmol l^{-1}.
[f]The 1% PAR equivalent depth was within the beginning of the nitricline.
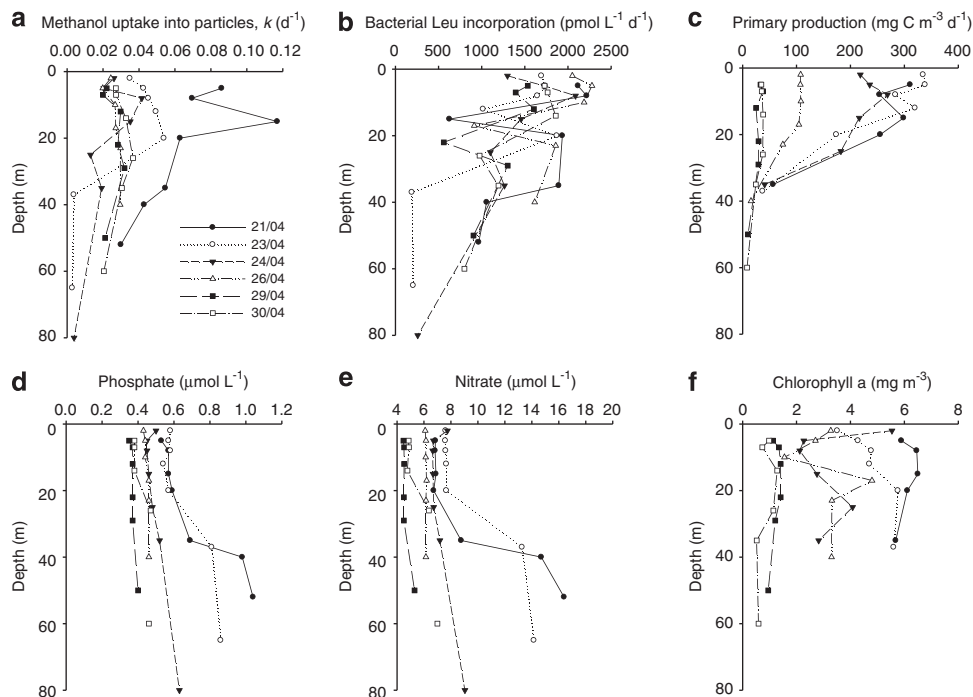
Figure 2 Depth profiles (97–1% PAR equivalent depth) of biogeochemical parameters (**a**) methanol uptake into particles, (**b**) bacterial leucine incorporation, (**c**) rates of primary production and concentrations of (**d**) phosphate, (**e**) nitrate and (**f**) chlorophyll a determined while tracking an upwelling filament of water originating off Cap Blanc in Mauritanian waters.

purified from agarose gels using the QIAquick gel extraction kit (Qiagen, Crawley, UK) and cloned into JM109 high efficiency competent cells using the pGEM-T easy vector system (Promega, Southampton, UK). Colonies were selected for colony PCR using M13 primers, PCR products were cleaned using a QIAquick PCR purification kit (Qiagen) and DNA sequenced (using M13f as a sequencing primer). Nucleotide sequences retrieved were trimmed in Chromas Lite (2.01) and BLAST (Basic Local Alignment Search Tool, NCBI) was used to search for similar sequences within the nucleotide collection (nr/nt) database using the Blastn algorithm.

Results

Mauritanian upwelling filament
The upwelling filament was tracked using SF_6 from its upwelling in surface waters close to the Mauritanian coast for 10 days (21–30 April 2009), over a distance of over 200 km. The top 200 m of this filament was 50–80% dominated by North Atlantic Central waters (Rees *et al.*, 2011). Microbial uptake of methanol into the particulate phase was initially $0.086 \pm 0.008\,d^{-1}$ in the most recently upwelled surface waters (Figure 2a), with maximum rates determined in subsurface waters (15 m) of $0.117 \pm 0.002\,d^{-1}$. Rates thereafter generally decreased in the euphotic zone until the 26 April, when methanol uptake rates stabilised to an average of $0.026 \pm 0.007\,d^{-1}$ (Figure 2a). The overall range in methanol

uptake rate constants found in Mauritanian upwelling waters was between $0.004–0.117\,d^{-1}$ (Table 1), which equates to $0.2–9.8\,nmol\,l^{-1}\,d^{-1}$ (calculated using the specific activity of added ^{14}C methanol). If the methanol uptake rate constants are multiplied by the range in methanol concentrations measured in the upwelling filament, methanol uptake varies between $0.1–24.8\,nmol\,l^{-1}\,d^{-1}$. Depth profiles consistently showed subsurface maxima between 8–20 m, with rates generally decreasing at depths >20 m. On average, during the filament, 91–97% of the microbial assimilation of methanol carbon was associated with the 0.2–2.0 μm size fraction.

Surface bacterial leucine incorporation rates did not show any progressive changes with filament age. Overall leucine uptake rates varied between $190–2279\,pmol\,Leu\,l^{-1}\,d^{-1}$ (Table 1) and typically displayed subsurface minima between 12–22 m (Figure 2b). Surface rates decreased from 2116 to $1299\,pmol\,Leu\,l^{-1}\,d^{-1}$ in the initial 3 days of filament progression, but peaked on day 4 at $2279\,pmol\,Leu\,l^{-1}\,d^{-1}$ before gradually decreasing until the end of the experiment (Figure 2b). Rates of PP were exceptionally high during the first 3 days of the filament ranging between $218–338\,mg\,C\,m^{-3}\,d^{-1}$ in surface waters, before reducing to 107 and to $35\,mg\,C\,m^{-3}\,d^{-1}$ 5 and 9 days later (Figure 2c). Rates of PP typically decreased significantly below 20 m ($\sim 7\%$ PAR). On average 90% of the PP in the top 20 m was in large cells >2 μm during first 4–5 days of bloom before decreasing slightly to 77%. Phytoplankton abundance was largely dominated

(51–82%) by flagellates and diatoms (13–22%), although their contribution decreased slightly in the last 2 days of sampling the filament. The diatoms were numerically dominated (on average 52% ranging between 31–78%) by small *Chaetoceros* species. However, in terms of carbon biomass, diatoms comprised 74–80% of the total phytoplankton carbon for most of the filament, except during the final 2 days, when their dominance dropped to 51–61%. Chlorophyll a concentrations were maximum at 6.5 mg m^{-3} in the most recently upwelled waters, and decreased to a minimum of 0.5 mg m^{-3} 10 days later (Figure 2f and Table 1), with depth profiles often showing subsurface maxima (Figure 2f). Concentrations of the inorganic nutrients nitrate and phosphate ranged between 6.1–7.7 µM and 0.43–0.58 µM, respectively, in surface waters (down to 20 m) during the first 5 to 6 days of the filament, before reducing to between 4.5–4.9 µM and 0.35–0.38 µM in the last 2 days (Figures 2d and e, Table 1). The N:P ratio was on average 15 for the most part of the filament development, but dropped to 13 in the last 2 days. Typically the nitricline was between 20–40 m, below which maximum concentrations of nitrate and phosphate were 16.4 and 1.0 µM, respectively. *Prochlorococcus* cells were notably absent from the Mauritanian upwelling waters, while *Synechococcus* numbers ranged between 0.12–1.3 × 10^3 cells ml^{-1} (Table 1). Pico-plankton and nano-phytoplankton abundance ranged between 0.03–5.3 and 0.2–2.4 × 10^3 cells ml^{-1} (Table 1).

Methanol uptake rates (calculated using the specific activity of ^{14}C methanol) were integrated to the 1% PAR light equivalent depth and were initially 2.6 ng C m^{-2} d^{-1} before decreasing to an average of 1.1 ± 0.2 ng C m^{-2} d^{-1} on all subsequent sampling days (Table 2). Surface chlorophyll a was also the highest initially at 6.2 mg m^{-3}, but decreased to an average of 3.5 and 1.2 mg m^{-3} between 2–6 and 7–9 days later, respectively. Integrated methanol carbon rates were compared with calculated bacterial carbon production and suggest that in the most recently upwelled waters between 3–6% of the carbon assimilated by heterotrophic bacteria could be derived from methanol. This percentage reduced to between 1–4% as the upwelled filament aged (Table 2).

Microbial methanol uptake in regions of the Atlantic Ocean
We have used the MODIS–Aqua surface chlorophyll a composite image from November 2009 (Figure 1b) to broadly split the Atlantic Ocean into four regions: Northern temperate (NT, Chl ⩾ 0.15 mg m^{-3}), Northern Gyre (NG, Chl < 0.15 mg m^{-3}), Equatorial upwelling (EU, Chl ⩾ 0.15 mg m^{-3}), Southern Gyre (SG, Chl < 0.15 mg m^{-3}), using a similar approach taken by Hill *et al.* (2011). Microbial uptake of

Table 2 Summary of % contribution of microbial methanol uptake to bacterial carbon production

Cruise/date	Zone[a]	Surface Chl a (mg m^{-3})	Methanol uptake Σ1%[b] (ng C m^{-2} d^{-1})	Bacterial leucine incorporation (pmol Leu L^{-1} d^{-1})	Bacterial carbon production[c] Σ1% (ng C m^{-2} d^{-1}) s-CF Min-CF Max-CF			Methanol % contribution to Bacterial carbon production
Mauritania filament								
21 April 09	U	6.2	2.6	1760 ± 578 (626–2217)	44.4	42.5	94.2	3–6
23 April 09	U	3.9	1.3	1358 ± 645 (190–1870)	35.0	33.6	74.3	2–4
24 April 09	U	3.9	0.93	1488 ± 361 (1101–2087)	36.2	34.8	77.0	1–3
26 April 09	U	3.0	0.97	1817 ± 503 (913–2279)	51.3	49.2	109	1–2
27 April 09	U	3.2		1442 ± 472[d]				
28 April 09	U	1.1		1228 ± 612[d]				
29 April 09	U	1.2	1.2	1217 ± 405	42.8	41.0	90.9	1–3
30 April 09	U	0.86	1.6	1504 ± 393	55.8	53.5	119	1–3
Atlantic transect								
17 October 09	NT	0.31	0.19	289 ± 74 (182–341)	13.3	1.82	28.2	1–10
18 October 09	NT	0.16	0.09	117 ± 63 (34–183)	4.01	0.55	8.53	1–9
20 October 09	NG	0.06	0.17	261 ± 42 (207–295)	10.4	1.44	22.3	1–17
22 October 09	NG	0.08	0.19	159 ± 30 (124–197)	7.65	1.05	16.3	1–19
23 October 09	NG	0.07	0.27	611 ± 589 (216–1488)	59.6	8.17	127	0.2–3
03 November 09	EqU	0.14	0.18	487 ± 137 (307–635)	14.4	1.96	30.5	1–9
06 November 09	EqU	0.14	0.27	294 ± 168 (135–445)	17.3	2.37	36.8	1–13
09 November 09	SG	0.08	0.19	249 ± 28 (220–283)	17.0	2.33	36.1	1–10
10 November 09	SG	0.10	0.24	1188 ± 1364 (324–3217)	111	15.1	235	0.1–2
11 November 09	SG	0.04	0.19	536 ± 319 (256–995)	57.3	7.85	122	0.2–2
15 November 09	SG	0.03	0.07	128 ± 68 (21–187)	13.4	1.84	28.4	0.3–4

[a]U, Mauritanian upwelling; NT, north east temperate Atlantic; NG, North Atlantic gyre; EqU, Equatoral upwelling; SG, southern gyre.
[b]Missing data due to incomplete depth profile, for Atlantic transect dark incubation data shown to compare with upwelling filament.
[c]Integrated (to 1% PAR) bacterial production has been calculated three ways: Max-CF using the theoretical leucine to carbon conversion factor of 1.55 kg C mol leu^{-1} (Simon and Azam, 1989; Hoppe *et al.*, 2002), which is also the maximum conversion factor found, Min-CF is the lowest empirical conversion factor found in the literature matched to season and place where possible, 0.7 kg C mol leu^{-1} has been applied to Mauritanian upwelling waters and 0.1 kg C mol leu^{-1} has been applied to low chlorophyll a Atlantic transect samples (Alonso-Sáez *et al.*, 2007) s-CF using a conversion factor of 0.73 kg C mol leu^{-1} applied to all the samples (Morán *et al.*, 2002, 2004; Dixon, 2008; Calvo-Díaz and Morán, 2009; Dixon *et al.*, 2011b).
[d]Only two depths sampled in the top 1–97% PAR depths therefore only average ± 1 s.d. shown.

methanol into the particulate phase during the meridonal transect of the Atlantic Ocean was overall relatively low ($0.001–0.015 \, d^{-1}$) compared with the highly productive Mauritanian upwelling waters (Table 1 and Figure 3a). This range is equivalent to between $0.04–0.68 \, nmol \, l^{-1} \, d^{-1}$ (using the specific activity of added ^{14}C methanol). Samples taken in NT waters showed the lowest microbial methanol uptake rates ($0.001–0.004 \, d^{-1}$), with an average of $0.003 \pm 0.001 \, d^{-1}$, which is ~ 10 times lower than Mauritanian upwelling waters (Table 1). The highest rates (during AMT19) were found in EU waters ($0.003–0.015 \, d^{-1}$) followed by NG ($0.001–0.010 \, d^{-1}$) and SG ($0.001–0.007 \, d^{-1}$). Sub surface peaks in the microbial uptake of methanol into the cells were found in waters receiving $\sim 33\%$ PAR light (equivalent to 10–25 m). Below which methanol uptake rates generally decreased with depth (Figure 3a). On average, during the Atlantic Ocean transect, 82–95% of the microbial uptake of methanol was associated with the 0.2–2.0 µm size fraction.

The average bacterial leucine incorporation rates found in NT, NG, EU and SG waters were between 3–7 times lower than incorporation rates in Mauritanian upwelling waters. Overall average rates of bacterial leucine uptake were lowest in NT waters, where they varied between $34–341 \, pM \, Leu \, d^{-1}$, with an average of $203 \, pM \, Leu \, d^{-1}$ (Table 1). Average values were highest in SG waters, but depth profiles reveal two hotspots of anomalously high activity (3217 and $995 \, pM \, Leu \, d^{-1}$) associated with two profiles (out of four) at depths equivalent to 14% PAR (45–55 m, Figure 3b). If these are excluded from

calculations, then average bacterial leucine rates decrease to $299 \pm 179 \, pM \, Leu \, d^{-1}$. Similarly, in NG waters, one depth profile (out of three) also showed a large hotspot of bacterial activity at the 14% PAR light level ($1488 \, pM \, Leu \, d^{-1}$ at 45 m), and if this is excluded from calculations, then the average bacterial leucine incorporation rates decreases from 344 ± 369 to $240 \pm 85 \, pM \, Leu \, d^{-1}$, which are similar to rates found in SG depth profiles. If these outliers are ignored, the EU waters show the highest average bacterial leucine uptake rates of $391 \pm 175 \, pM \, Leu \, d^{-1}$. However, for each hotspot in bacterial leucine incorporation shown in gyre water from between 45–55 m, three replicate samples were obtained with good precision (1–5%), so although the data seem anomalous when compared with the whole data set, it is difficult to justify their complete removal as they could represent hot spots in activity associated with detrital material (Azam *et al.*, 1994).

Rates of PP were characteristically low in gyre waters (Figure 3c), with an average of $2.7 \pm 1.4 \, mg \, C \, m^{-3} \, h^{-1}$ and $3.2 \pm 2.2 \, mg \, C \, m^{-3} \, h^{-1}$ in NG and SG depth profiles, respectively. PP was on average slightly higher in NT profiles ($4.7 \pm 2.7 \, mg \, C \, m^{-3} \, h^{-1}$), but not surprisingly showed the highest rates in EU waters ($10.5 \pm 6.0 \, mg \, C \, m^{-3} \, h^{-1}$). In NT, NG and EU waters, on average (for each depth profile) between 67–75% of the PP was associated with the 0.2–2 µm fraction. However for SG profiles, this decreased to 58%.

Concentrations of nitrate and phosphate in NT, SG, EU and SG profiles were also very low compared with Mauritanian upwelling coastal
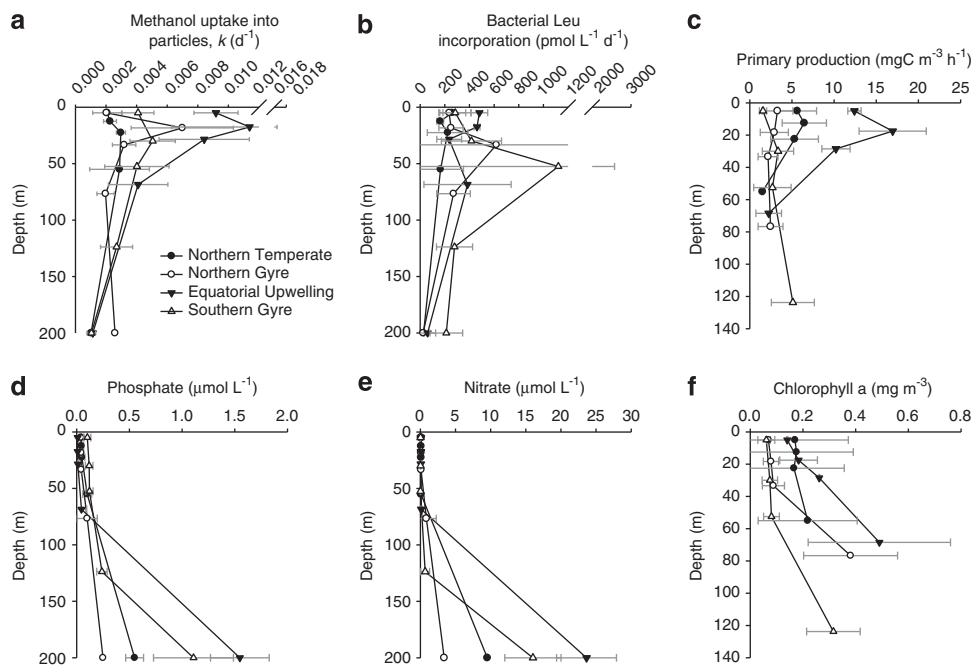
Figure 3 Depth profiles (97–1% PAR equivalent) of biogeochemical parameters (**a**) methanol uptake into particles, (**b**) bacterial leucine incorporation, (**c**) rates of primary production and concentrations of (**d**) phosphate, (**e**) nitrate and (**f**) chlorophyll a determined in different provinces of the Atlantic Ocean. The profiles represent the average found in each province where for NT $n = 2$, NG $n = 3$, EU $n = 2$, SG $n = 4$. For each individual profile the number of replicate at each depth were $\geqslant 3$.

waters (Table 1 and Figures 3d and e). Nitrate concentrations in NT profiles were consistently low, ranging between 0.08–0.09 μM. The ranges shown for gyre waters are slightly extended because on occasion the 1% PAR equivalent depth was within the start of the nitricline (Table 1). Phosphate concentrations were on depth average 0.06 ± 0.03 μM, 0.06 ± 0.05 μM and 0.02 ± 0.03 μM in NT, NG and EU profiles waters, respectively, but showed a relatively higher average concentration of 0.15 ± 0.07 μM in SG waters. The resulting N:P ratios were very low and ranged between 0.9–3.3, 2–2.5, 2–13.2 and 0.2–5.8 for NT, NG, EU and SG depth profiles, respectively. For SG profiles, the exceptionally low N:P values of 0.2–0.4 were due to relatively more P compared with other regions, while in three out of four profiles, N was relatively enriched at the 1% PAR depth (95–125 m) due to incursion within the nitricline resulting in N:P ratios of between 2.5–5.8.

Numbers of *Synechococcus* were characteristically low in the gyres (2.4 ± 2.0 cells ml^{-1} and $2.0 \pm 1.2 \times 10^3$ cells ml^{-1} for NG and SG, respectively), but were on average, two orders of magnitude higher in NT and EU waters (20.2 ± 19.2 cells ml^{-1} and $30.9 \pm 24.1 \times 10^3$ cells ml^{-1} for NT and EU, respectively) compared with Mauritanian upwelling waters (Table 1). Although NT and EU profiles had relatively high variability due largely to a rapid decrease in numbers below 14% PAR equivalent depth, when light starts to become limiting. *Prochlorococcus* cells favoured EU and SG waters showing average numbers of $2.5 \pm 1.1 \times 10^5$ cells ml^{-1} and $2.2 \pm 1.1 \times 10^5$ cells ml^{-1}, respectively. They showed lower averages of $1.6 \pm 0.8 \times 10^5$ cells ml^{-1} and $1.3 \pm 0.4 \times 10^5$ cells ml^{-1} in NT and NG waters, respectively. Pico-plankton numbers throughout the Atlantic open ocean regions were on average higher than in coastal upwelling waters and varied between $1.6–5.1 \times 10^3$ cells ml^{-1} across the regions, while nano-phytoplankton abundance was on average lower in the open ocean compared with coastal upwelling ranging between $0.22–0.9 \times 10^3$ cells ml^{-1} (Table 1).

Integrated methanol uptake rates in open ocean waters were lower than their coastal upwelling counterparts, ranging between 0.09–0.19 ng C m^{-2} d^{-1}, 0.17–0.27 ng C m^{-2} d^{-1}, 0.18–0.27 ng C m^{-2} d^{-1} and 0.07–0.24 ng C m^{-2} d^{-1} for NT, NG, EU and SG regions, respectively. However, because integrated bacterial carbon production is generally lower in the open ocean compared with the chlorophyll a-rich eutrophic coastal upwelling area (except when integrated rates have been elevated due to the measured 'hot spots' of bacterial leucine incorporation as discussed above), the contribution that methanol carbon could make to bacterial carbon incorporation can be as high as 10%, 19%, 13% and 10% in NT, NG, EU and SG regions, respectively, although the ranges shown in Table 2 suggest that methanol contribution to bacterial carbon

incorporation is relatively less important in the SG compared with the other Atlantic regions.

Preliminary characterisation of methylotrophic bacteria in regions of the Atlantic Ocean

Using cultivation-independent molecular biological methods, based on retrieval of *mxaF*, the gene encoding the large subunit of methanol dehydrogenase, a key enzyme in methanol metabolism in Gram-negative methylotrophs, the presence of several methylotrophic bacteria was noted in open ocean water samples of the Atlantic Ocean (Table 3). Of the 14 *mxaF* sequence types (operational taxonomic units) retrieved by PCR from DNA from these samples, seven of them had no significant taxonomic affiliation and were assigned as *mxaF* clones from uncultivated methylotrophic bacteria. However, it was obvious from sequence analysis that they were bona fide *mxaF* sequences (82–87% identity at the amino acid level to *mxaF* from *Methylophaga spp.* over 145 amino acyl residues). The *mxaF* phylotype 33–7_A02 was found in all samples (NT, NG, EU and SG) and was identified as *mxaF* from *Methylophaga* (Neufeld *et al.*, 2007; 2008). A total of 89 clones were screened (19 from NT, 20 from NG, 26 from EgU, 19 from SG and 5 from ST Atlantic region).

While some *mxaF* clones, identified as originating from members of the Burkholderiales (RZ18–153), Methylococcaceae (ET-SHO) and also *Methylophaga* sp. (DMS021), were present in DNA samples from several open ocean Atlantic regions, other *mxaF* sequences, such as those identified as belonging to the methylotrophs *Ancylobacter aquaticus*, *Paracoccus denitrificans*, *Methylophilus methylotrophus*, *Methylobacterium oryzae*, *Hyphomicrobium* sp. and *Methylosulfonomonas methylovora* (Table 3 and references therein), were only identified in DNA from seawater samples in specific regions and under particular light.

Discussion

We measured the highest ever microbial methanol uptake rates (k) of 0.117 ± 0.002 d^{-1} in the most recently upwelled coastal waters, which approximately equates to 10 nmol l^{-1} d^{-1}. However, this microbial incorporation of methanol carbon into cells reduced and stabilised to an average of 2 nmol l^{-1} d^{-1} in the euphotic zone within 5 days of following the upwelled waters. This maximum rate is >100-fold higher than that reported for North Atlantic oligotrophic waters (Dixon *et al.*, 2011b), and about nine times higher than the average found in surface waters of the NE Atlantic (55.2° N 12.7 °W) where chlorophyll a concentrations ranged between 0.7–1.0 mg m^{-3} (Dixon *et al.*, 2011a). On average coastal upwelling waters were 11 times greater than NT waters, eight times greater than gyre

Table 3 Occurrence of *mxaF* genes in DNA samples from different Atlantic Ocean areas

Phylogenetic affiliation of mxaF clones	Atlantic area					Light level (% PAR)				Genbank accession number	Reference
	NT	NG	EqU	SG	ST	97%	33%	1%	200 m (dark)		
Ancylobacter aquaticus	*					*				AB455976	Suzuki *et al.*, 2009
Uncultured bacterium 33–7_A02	*	*	*	*		*			*	EU001854	Neufeld *et al.*, 2007
Burkholderiales bacterium RZ18–153	*			*	*	*		*	*	EU548067	Kalyuzhnaya *et al.*, 2008
Methylococcaceae bacterium ET-SHO	*	*	*	*		*	*		*	AB453966	Nakamura *et al.* unpublished.
Uncultured clone L4_E01	*			*				*	*	EU001809	Neufeld *et al.*, 2007
Methylophilus methylotrophus	*								*	EU194905	Madhaiyan *et al.*, 2009
Paracoccus denitrificans	*								*	U34346	Ras *et al.*, 1991
Uncultured clone C04		*		*				*	*	JQ254993	Irvine *et al.*, 2012
Uncultured *Methylophaga* sp.		*	*						*	EU031899	Neufeld *et al.*, 2007
Uncultured clone C7		*					*	*	*	JN665049	Antony *et al.*, 2012
Methylophaga sp. DMS021		*	*	*					*	EU001861	Neufeld *et al.*, 2007
Uncultured clone 24–7_F06		*	*						*	EU001823	Neufeld *et al.*, 2007
Methylobacterium oryzae NBHP3		*					*			HQ221359	Meena *et al.*, 2012
Hyphomicrobium sp. B294				*		*				Y08081	Fesefeldt and Gliesche, 1997
Methylosulfonomonas methylovora		*							*	U70525	McDonald and Murrell, 1997
Uncultured bacterium clone C23				*		*				EF212333	Chen *et al.*, 2007

Abbreviations: NT, north east temperate Atlantic; NG, North Atlantic gyre; EqU, Equatoral upwelling; SG, southern gyre; ST, Southern temperate.
*indicates presence of *mxaF* genes.

waters and four times greater than EU waters in this study. These results suggest that coastal and EU waters upon reaching the surface ($\leqslant 20$ m), contain a microbial population that use a relatively high amount of carbon (0.3–10 nmol l^{-1} d^{-1}), derived from methanol, to support their growth. Preliminary characterisation of methylotrophic bacteria in the equatorial region of the Atlantic Ocean reveal a number of uncultivated methylotrophic bacteria, Methylococcaceae (ET-SHO), *Methylophaga* sp. (DMS021) and *Methylosulfonomonas methylovora,* the latter is a marine methanesulfonate degrading methylotroph that also uses methanol for growth (Holmes *et al.*, 1997). When the use of methanol for incorporation into cell biomass was compared with the total amount of methanol used (for energy and uptake into cellular structures), then analysis of coastal upwelling samples revealed up to 57% of the total methanol was used to assimilate carbon into cells (average of 32% over all depths and days), which compares with an average of 12% in the EU, 1% in NT and NG regions, and 8% in the SG. This agrees with previously published results, which also suggest that $\leqslant 4$% of methanol is used for assimilation in open ocean gyre waters (Dixon *et al.*, 2011b), but between 12–50% for more seasonally eutrophic shelf and coastal areas of the North-East Atlantic (Dixon *et al.*, 2011a, b).

Dissolved organic carbon production by phytoplankton is believed to be the primary organic carbon source for marine bacteria (Dixon *et al.*, 2011b).

However there is current debate over whether apparent mismatches can, or do occur, that is, where bacterial carbon demand exceeds that apparently available from phytoplankton production (for example, del Giorgio and Duarte, 2002; Duarte *et al.*, 2001; Carlson *et al.*, 2007; López-Sandoval *et al.*, 2011). Dixon *et al.* (2011b) have previously suggested that for oligotrophic regions, methanol could be a significant, perhaps non-phytoplankton derived, organic carbon and energy source for bacteria. These current results further suggest that, even in highly productive, nutrient rich coastal upwelling regions, methanol could also be a significant carbon source for microbial anabolism (and energy), which is perhaps counter intuitive in such phytoplankton-rich waters. However, what remains uncertain is where the methanol originates, that is, whether the methanol is produced *in situ*, perhaps from photochemical degradation of dissolved organic carbon compounds, ultimately derived from phytoplankton or from air-sea transfer.

Relationships between the microbial assimilation of methanol carbon and other biogeochemical variables (including bacterial production, PP, cellular abundance and inorganic nutrient concentrations) have been explored in order to try and understand what controls, or determines the uptake of methanol into particulate cellular material. Statistically significant relationships are summarised in Table 4. We did not find a significant relationship between methanol uptake and bacterial leucine incorporation

Table 4 Summary of statistically significant relationships between methanol uptake and biogeochemical variables

Methanol uptake	Primary production	Chlorophyll a	Synechococcus sp.	Prochlorococcus sp.	Nanophytoplankton
Mauritanian upwelling (D338)	0.611**	0.610**	NS	NA	0.381*
Open Ocean (AMT19)	0.676**	NS	0.518**	0.611**	NS

Abbreviation: NS, not significant; $*P \geqslant 0.05$; $**P \leqslant 0.001$; NA, not applicable because the upwelling did not have any *Prochlorococcus* cells.

for this data, in contrast to previous experiments, which were from more limited geographical and biogeochemical areas (Dixon *et al.*, 2011a; 2012). Methanol assimilation into the particulate phase for both the coastal upwelling and open ocean Atlantic samples (NT, NG, EU and SG combined) showed positive statistically significant relationships with rates of primary productivity ($r = 0.611$, $n = 43$, $P \leqslant 0.001$ and $r = 0.676$, $n = 44$, $P \leqslant 0.001$, respectively). For open ocean samples, when carbon fixation is dominated by unicellular cyanobacteria such as *Synechococcu*s and *Prochlorococcus* sp. (and small plastidic protists, Hartmann *et al.*, 2012), statistically significant relationships are also shown between their abundance and the uptake of methanol into cellular biomass ($r = 0.518$ and 0.611, respectively, for $n = 50$, $P \leqslant 0.001$), in agreement with previously published results from diel experiments in the subtropical region of the NG, and in waters west of the Iberian peninsula located within the NT region (Dixon *et al.,* 2011b; 2012). However, these relationships were not shown for more shelf waters of the north-east Atlantic (Dixon *et al.,* 2011a). For the Mauritanian coastal upwelling waters where PP was dominated by cells $> 2\,\mu m$, a statistically significant relationship, albeit weaker, was shown between methanol uptake and the numbers of nano-phytoplankton ($2–20\,\mu m$, $r = 0.381$ $n = 43$, $P \geqslant 0.05$), rather than cyanobacterial cells. The link between the uptake of methanol into microbial cells and rates of carbon fixation by photosynthesis is unlikely to be direct in the coastal upwelling because 91–97% of the methanol uptake was in the $0.2–2\,\mu m$ particle fraction, whereas on average 90% of the PP, was associated with particles $> 2\,\mu m$ (nanoplankton). Thus, we suggest that mixotrophy of large plastidic protists, that is, preying on methylotrophic bacteria could have been occurring. Mixotrophy in natural populations of nano-plankton has been previously documented in other coastal upwelling regions, for example, North West Iberian upwelling system (Crespo *et al.*, 2011; Vargas *et al.*, 2011; Hartmann *et al.*, 2012 and references therein) and in both temperate and oligotrophic regions of the Atlantic ocean (Zubkov and Tarran, 2008; Hartmann *et al.*, 2012). Alternatively, the correlation found in Mauritanian upwelling waters between microbial methanol assimilation and primary productivity could be an indirect effect, of possibly a distinct group of methylotrophs associated with upwelling waters that uniquely use up to 57% of the methanol for
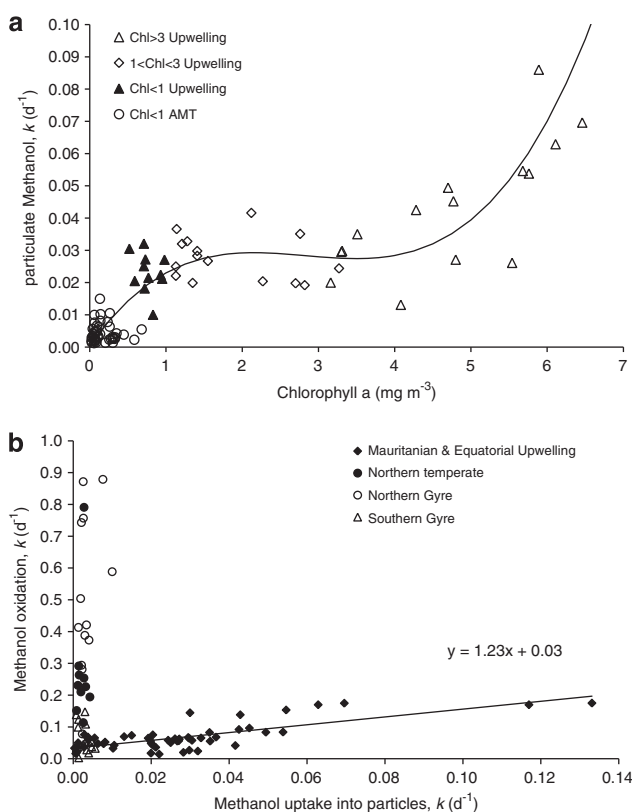
Figure 4 Microbial uptake of methanol into cellular particles (**a**) as a function of chlorophyll a concentration for all data, which is best described by the third order polynomial $y = 0.0015x^3 - 0.0127x^2 + 0.034x + 0.004$ ($r = 0.915$, $n = 86$, $P < 0.001$) and (**b**) relationship with methanol oxidation.

cellular incorporation. This percentage is much higher than previously reported in oligotrophic and temperate coastal waters ($\leqslant 4\%$, Dixon *et al.,* 2011a, b; 2012), but more similar to that predicted in Murrell and McDonald (2000).

Because the two primary productivity data sets cannot be combined (different incubation lengths), microbial methanol uptake as a function of chlorophyll a concentrations for all the data is shown in Figure 4a. The relationship is best described by a third-order polynomial ($r = 0.915$, $n = 86$, $P < 0.001$), although no statistically significant linear relationship between methanol uptake and chlorophyll a is apparent for the open ocean data only (Table 4). However, these results do suggest that for upwelling waters, the rate of methanol incorporated in cellular biomass could be estimated from chlorophyll a concentrations. The relationship between methanol

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oxidation (used as an energy source) and uptake into particles is shown in Figure 4b. The upwelling data from the Mauritanian coast and Equator have been combined, and show a statistically significant linear relationship ($r = 0.755$, $n = 54$, $P \leqslant 0.001$). No relationship was found in NT or gyre waters where methanol oxidation rates tend to be much higher, and $\geqslant 97\%$ of methanol is used by microbes for obtaining energy (Dixon et al., 2011b). The relationships found for upwelling waters suggest that remotely sensed chlorophyll a images of the sea surface could be used to predict the total amount of methanol loss due to biological activity, which could potentially be a very useful tool when trying to determine global methanol budgets (for example, Jacob et al., 2005).

As a first attempt to assess the robustness of the relationships described in Figure 4, we have estimated the concentration of chlorophyll a from remotely sensed MODIS Aqua images (to coincide for when we have in situ methanol oxidation rates for the upwelling D338 cruise we have used a composite image from 21 to 27 April 2009 of the Mauritanian upwelling area, and for the EU we have used a composite AMT19 image from October 2009). For the Mauritanian upwelling we estimate chlorophyll a concentrations between 2–4 mg m^{-3} from the image. This equates to between 0.028–0.029 d^{-1} for methanol uptake into particles and consequently between 0.065–0.066 d^{-1} for methanol oxidation (using the relationships in Figures 4a and b, respectively). We have five surface in situ measurements over the image time giving an average ^{14}C-derived methanol oxidation of 0.088 ± 0.054 d^{-1} ($n = 5$). For the EU area we estimate chlorophyll a from the image to be between 0.3–0.7 mg m^{-3}, which equates to between 0.009–0.018 d^{-1} particulate methanol uptake, and between 0.042–0.053 d^{-1} for methanol oxidation. This compares with in situ oxidation measurements using ^{14}C-labelled methanol to between 0.033–0.052 d^{-1} ($n = 2$).

This research offers the first ever microbial methanol assimilation rates from highly productive coastal upwelling waters, which sharply contrasts with oligotrophic oceanic gyre water. Maximum microbial methanol carbon incorporation rates up to 10 nmol l^{-1} d^{-1} have been shown, which suggest that up to 57% of the total methanol is assimilated into carbon biomass. In comparison, microbes in oligotrophic gyre waters use $\geqslant 97\%$ methanol as an energy source (Dixon et al., 2011b). The statistically significant correlations for upwelling water between methanol uptake into cells and both chlorophyll a and methanol oxidation rates suggest that remotely sensed chlorophyll a images could be used in these productive areas to derive total methanol biological loss rates, a potentially useful tool for atmospheric and marine climatically active gas modellers and air–sea exchange scientists. Although we have identified some methylotrophic bacterial species

from the open Atlantic Ocean, further research should identify which microbial species are utilising methanol, in addition to further development and testing of methanol production and loss processes parameterisations.

Acknowledgements

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