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Seasonal variability in microbial methanol utilisation in coastal waters of the western English Channel.

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Abstract

Methanol is ubiquitous in seawater and the most abundant oxygenated volatile organic compound (OVOC) in the atmosphere where it influences oxidising capacity and ozone formation. Marine methylotrophic bacteria utilise methanol in seawater both as an energy and/or growth substrate. This work represents the first fully resolved seasonal study of marine microbial methanol uptake dynamics. Rates of microbial methanol dissimilation in coastal surface waters of the UK varied between 0.7 – 11.2 nmol l^{-1} h^{-1} and reached a maximum in February. Rates of microbial methanol assimilation varied between 0.04 – 2.64 x 10^{-2} nmol l^{-1} h^{-1} and reached a maximum in August. Temporal variability in microbial methanol uptake rates shows that methanol assimilation and dissimilation display opposing seasonal cycles, although overall <1% of methanol was assimilated. Correlative approaches with 16S rRNA pyrosequencing data suggested that bacteria of the SAR11 clade and *Rhodobacterales* could be significantly influencing rates of methanol dissimilation and assimilation, respectively, at station L4 in the western English Channel.

Introduction

Methanol is the single largest component of the total pool of oxygenated volatile organic compounds (OVOC) in the atmosphere (approximately 400 – 800 ppt in the Atlantic troposphere, Singh et al. 2001) and is ubiquitous in marine waters (e.g. Heikes et al. (2002),
5 Singh et al. (2003), Williams et al. (2004), Beale et al. (2013), Beale et al. (2015), Yang et al. (2014a). Methanol (and other OVOCs) substantially influences the oxidising capacity and ozone-forming potential of the atmosphere (Singh et al. 2001 and references therein), where it acts as a sink for hydroxyl radicals (Galbally & Kirstine 2002) producing formaldehyde (Millet et al. 2006) and carbon monoxide (Duncan & Logan 2008), among other products
10 and is thus a climate-relevant gas.

Knowledge of the sources and sinks of marine methanol is limited. A net air-to-sea flux was first demonstrated in Northern temperate waters by Beale et al. (2013). Recent direct flux measurements of methanol also demonstrated a consistent flux of methanol from the
15 atmosphere to surface waters along a meridional transect of the remote Atlantic Ocean (Yang et al. 2013) and the Greenland Sea (Yang et al. 2014b). Methanol is ubiquitous in atmospheric and marine environments where seawater concentrations have been reported ranging between <27 and 429 nM (Williams et al. 2004, Kameyama et al. 2010, Beale et al. 2013, Yang et al. 2013, Yang et al. 2014b, Beale et al. 2015). An annual study in the western
20 English Channel reported surface seawater methanol concentrations ranging between 16 and 78 nM over an annual cycle with no obvious seasonality (Beale et al. 2015). Recent analysis of methanol concentrations in rainwater of <6 nM to 9.3 μM suggest that wet deposition could also be a significant source of methanol to the surface ocean (Felix et al. 2014). Estimates of photochemical production of methanol in seawater from the Atlantic Ocean have

been found to be negligible, therefore surface seawater methanol concentrations are thought to be largely controlled by microbial metabolism (Dixon et al. 2013).

Most known aerobic methylotrophic bacteria can utilise methanol as a carbon and/or energy source usually through the use of methanol dehydrogenase (MDH) encoded by the gene *mxaF* (Chistoserdova et al. 2009, Chistoserdova 2011), which can be used as a functional gene marker (McDonald & Murrell 1997). These bacteria are widespread in both terrestrial and aquatic systems (Kolb 2009), but have not been studied as extensively in marine environments. For the purposes of this research, microbial methanol oxidation to derive energy is referred to as microbial methanol dissimilation (to CO_2) and the incorporation of methanol for growth (into cell material) is considered as microbial methanol assimilation. The concept of cells producing energy but not biomass from C_1 compounds has received relatively little attention. However, Sun et al. (2011) proposed a new term ‘methylovores’ to distinguish cells such as SAR11 that utilise methanol and other C_1 compounds only as a source of energy, from strict methylotrophs, which use C_1 compounds as sources of energy and carbon for growth.

Stable isotope probing (SIP) incubations using ^{13}C-labelled methanol have previously confirmed that surface waters at temperate coastal sampling site L4 (www.westernchannelobservatory.org.uk) harbour methylotrophs actively assimilating methanol into biomass (Neufeld et al. 2007, Neufeld et al. 2008). However, no accompanying uptake rate measurements were made and little is known about the seasonal variability in rates of microbial methanol utilisation at this site. A limited number of measurements of methanol dissimilation rates (to CO_2) in surface shelf waters have been made (Dixon et al. 2011a) and ranged between 2.1 and 8.4 nmol l^{-1} d^{-1}, although higher rates

of up to 146 nmol l^{-1} d^{-1} have been observed in more tropical remote Atlantic regions (Dixon et al. 2011b). Methanol assimilation rates have been previously shown to range between 0.04 and 10.00 nmol l^{-1} d^{-1} in Atlantic waters, with maximum rates associated with recently upwelled water (Dixon et al. 2013). There are no published rates of microbial methanol assimilation in temperate coastal waters. The average bacterial growth efficiency of methanol (BGE_M, percentage of methanol assimilated by microbes rather than dissimilated to CO_2) for Atlantic waters is 3%, but can reach up to 57% in recently upwelled coastal waters (Dixon et al. 2011b).

Bacteria belonging to the SAR11 clade (e.g. *Pelagibacter*), the most abundant heterotrophs in the ocean (Giovannoni et al. 2005), have also been shown to dissimilate C_1 compounds, including methanol, to CO_2 to derive reducing power and energy (Sun et al. 2011). This bacterial clade lacks methanol dehydrogenase, and cultivation experiments suggest that SAR11 cells do not assimilate carbon from methanol into biomass (Sun et al. 2011). The methanol dissimilation rates measured in these *in vitro* experiments were comparable to those measured *in situ* by microbial communities in the North East Atlantic (Dixon et al. 2011b). Thus methanol oxidation by SAR11 cells could represent a significant conduit by which dissolved organic carbon is recycled to CO_2 in the upper ocean.

OM43, a clade of *Betaproteobacteria* (Giovannoni et al. 2008), is commonly found in productive coastal waters. HTCC2181 was the first strain of the OM43 clade to be isolated and was shown to use C_1-compounds methanol and formaldehyde as both sources of carbon and energy (Giovannoni et al. 2008). This was the second methylotrophic isolate with the ability to grow on methanol yet lacking *mxaF* and *mxaI* (genes encoding the large and small subunits of methanol dehydrogenase respectively), instead possessing the *xoxF* gene

(Giovannoni et al. 2008). The *xoxF* gene is a homolog of the traditional *mxaF* gene and encodes an alternative pathway for methanol oxidation (Schmidt et al. 2010). Very recently, it has been used as a functional gene marker in cultivation-independent studies of methylotrophs in the environment (Taubert et al. 2015). Halsey et al. (2012) subsequently
5 showed that the growth of HTCC2181 is enhanced by a variety of C_1-substrates suggesting that HTCC2181 may be able to incorporate carbon from methanol whilst simultaneously using other C_1-compounds as energy sources.

The objective of this study was to examine the seasonal variability in rates of microbial
10 methanol utilisation, and investigate relationships between microbial methanol utilisation rates, environmental parameters and diversity of methylotrophs at a temperate coastal site. This study provides the first fully resolved seasonal evaluation of microbial methanol uptake in coastal waters.

15 Materials and methods

Sample collection

Coastal sampling station, L4, is located in the western English Channel (WEC, 50° 15.00 N, 4° 13.02 W, sampling depth ~5 m, Smyth et al. 2010) approximately 10 km from the coast of Plymouth, UK. This sampling station forms part of the Western Channel Observatory
20 (WCO, www.westernchannelobservatory.org.uk) and is a long-term time series station (Harris 2010). Sampling at L4 was carried out between April 2011 and April 2012 using a Conductivity Temperature Depth (CTD) rosette mounted with Niskin bottles to collect surface water (~5m) every two weeks. Seasons were defined as spring (March – May), summer (June – August), autumn (September – November) and winter (December –
25 February).

Microbial methanol utilisation

Rates of microbial methanol uptake were determined by adding ^{14}C-labelled methanol (American Radiolabelled Chemicals Inc, Saint Louis, MO, USA) to seawater as previously described in Dixon et al. (2011a). Incubations of 10 nM (final concentration) ^{14}C-labelled
5 methanol with surface seawater samples of 1 ml (methanol dissimilation to CO_2) and 320 ml (methanol assimilation into biomass) were used to measure microbial utilisation rates. *In situ* surface methanol concentrations during the sampling period ranged between 34 and 68 nM (Beale et al. 2015), thus our radioisotope additions represent between 15 and 29% of *in situ* concentrations. Incubations were conducted in triplicate at *in situ* temperatures and in the
10 dark, together with negative controls 'killed' with 5% w/v trichloroacetic acid, TCA, (final concentration), for dissimilation samples or 0.01% w/v mercuric chloride, $HgCl_2$ (final concentration) for assimilation samples (due to differences in sample volume). Incubation temperatures were matched to the *in situ* sea surface temperatures at Station L4, which ranged between 9 – 17°C throughout the sampling period.
15

Sample counts of ^{14}C-labelled methanol (mCi ml^{-1} h^{-1}) were generated either from the particles on the filter (assimilation method) or the precipitate containing the captured $^{14}CO_2$ as $Sr^{14}CO_3$ (dissimilation method). Sample counts of ^{14}C-labelled methanol (mCi ml^{-1} h^{-1}) were multiplied by the specific activity of ^{14}C-labelled methanol (5.71×10^{-4} mCi $nmol^{-1}$) and
20 by 1000 to calculate rates of microbial methanol dissimilation and assimilation (nmol l^{-1} h^{-1}).

The bacterial growth efficiency of methanol (BGE_M) was calculated as the rate of microbial methanol assimilation divided by the rate of total microbial methanol uptake, multiplied by 100 and was used as an indicator to quantify the fraction of carbon assimilated from methanol
25 directly into biomass (Dixon & Nightingale 2012).

Heterotrophic bacterial production

Rates of heterotrophic bacterial production were determined with 1.7 ml surface seawater samples using the incorporation of ^{3}H-leucine (final concentration of 25 nM, specific activity of 161 Ci mmol^{-1}, American Radiolabelled Chemicals Inc, Saint Louis, MO, USA) into bacterial protein, according to Smith & Azam (1992). Samples were incubated in triplicate at *in situ* temperature in the dark, and were corrected for abiotic sorption using control samples that were killed with trichloroacetic acid (5% final concentration).

A theoretical leucine-to-carbon conversion factor (1.55 kg C mol leu^{-1}, Smith & Azam 1989) was applied to rates of bacterial leucine incorporation to provide an upper estimate of bacterial production rates, as per an earlier study in the English Channel (Lamy et al. 2009). This theoretical conversion factor of 1.55 kg C mol leu^{-1} (Simon & Azam 1989) has also been used previously in a range of contrasting marine environments, from productive coastal waters to oligotrophic gyre regions (Lamy et al. 2009, Laghdass et al. 2012, Dixon et al. 2013).

Bacterial numbers, nutrients and chlorophyll a concentrations

Bacterial numbers, nutrient concentrations and concentrations of chlorophyll *a* were measured and data provided by the Western Channel Observatory (www.westernchannelobservatory.org.uk) using the following methodologies. Numbers of bacterial cells were determined using flow cytometry using SYBR Green I DNA-stained cells to determine high nucleic acid (HNA) and low nucleic acid (LNA) containing cells from 1.8 ml seawater samples fixed in paraformaldehyde (5% final concentration). *Synechococcus* sp. numbers were determined by flow cytometry on unstained samples based on their light

scattering and autofluorescence properties (Tarran et al. 2006). *In situ* chlorophyll *a* concentrations were determined through fluorometric analysis of acetone-extracted pigments (Tilstone et al. 2009). Nutrient analysis was conducted using methods described in Grasshoff (1976) and Zhang & Chi (2002) for nitrate and phosphate respectively.

5

DNA extraction and bacterial identification

Triplicate DNA samples were collected by filtration of six litres of surface water through 0.22 μm Sterivex polyethersulfone filters (Millipore, Watford, UK) using a peristaltic pump. Filters were stored immediately at -80°C. A modified phenol:chloroform:isoamyl alcohol
10 extraction method, as previously described in Neufeld et al. (2007), was used to extract DNA from Sterivex filters. PCR amplification (32 cycles) was conducted on extracted DNA using 16S rRNA gene primers 341F (Muyzer et al. 1993) and 907R (Muyzer et al. 1998) with an annealing temperature of 55°C. PCR products were purified from agarose gels using the QIAquick gel extraction kit (Qiagen, Crawley, UK) before being sent to Molecular Research
15 LP (MR DNA, www.mrdnalab.com) for 454 pyrosequencing using the GS-flx platform.

16S rRNA gene sequences were depleted of barcodes and primers, and then sequences less than 200 bp, with ambiguous bases or with homopolymer runs exceeding 6 bp were removed. Sequences were denoised and chimeras removed. After the removal of singleton sequences,
20 operational taxonomic units (OTUs) were defined at 97% 16S rRNA gene identity using Quantitative Insights Into Microbial Ecology (QIIME, http://qiime.org, Caporasa et al. 2010). OTUs were assigned taxonomically using BLASTn (Basic Local Alignment Search Tool, NCBI) against the Silva database (www.arb-silva.de). Sequences were randomly re-sampled to the lowest number of sequences per sample (816 sequences per DNA sample) to
25 standardise the sequencing effort.

Data analysis

Spearman's Rank Correlation Coefficients were calculated to investigate possible relationships between environmental parameters. Microbial methanol utilisation rates were compared to phylogenetic data of groups known to metabolise methanol (including SAR11 and *Rhodobacterales*) using partial Mantels' tests (Mantel & Valand 1970) of Bray-Curtis matrices.

Results

Temporal variability of microbial methanol utilisation

Microbial rates of methanol oxidation to CO_2 at station L4 (referered to as 'dissimilation', Figure 1) ranged between 0.7 and 11.2 nmol l^{-1} h^{-1} and were higher during autumn and winter (average 8.2 ± 2.2 nmol l^{-1} h^{-1}, n = 8) compared to spring and summer (average 3.7 ± 2.3 nmol l^{-1} h^{-1}, n = 14). Rates of microbial methanol assimilation (Figure 1) varied between 0.04 and 2.64 x 10^{-2} nmol l^{-1} h^{-1} in surface waters from April 2011 to April 2012. These assimilation rates were up to 1,000-fold lower than rates of methanol dissimilation, with an overall average rate of 0.67 ± 0.68 x 10^{-2} nmol l^{-1} h^{-1} (n = 30). Methanol assimilation rates were on average higher between May and September (average of 1.10 ± 0.08 x 10^{-2} nmol l^{-1} h^{-1}, n = 13) and lower between October and April (0.30 ± 0.20 x 10^{-2} nmol l^{-1} h^{-1}, n = 14). Maxima of 2.64 x 10^{-2} nmol l^{-1} h^{-1} and 2.23 x 10^{-2} nmol l^{-1} h^{-1} were observed in August and September, respectively.

The rates of microbial methanol assimilation and dissimilation at station L4 exhibited contrasting seasonal trends; rates of methanol assimilation reached a maximum during summer months, whilst rates of methanol dissimilation (to CO_2) were highest during autumn

and winter (Figure 1). Methanol assimilation rates correlated significantly with bacterial production rates ($r = 0.420$, $n = 17$, $P < 0.05$), numbers of heterotrophic bacteria ($r = 0.762$, $n = 17$, $P < 0.01$) and in particular the abundance of high nucleic acid containing bacteria ($r = 0.755$, $n = 17$, $P < 0.01$). In contrast, methanol dissimilation rates displayed a significant
5 negative correlation with rates of bacterial productivity ($r = -0.708$, $n = 17$, $P < 0.01$).

Bacterial growth efficiency for methanol (BGE_M)

The BGE_M varied from <0.01 to 0.94% (Figure 2a) and generally showed a pattern of higher BGE_M during spring and summer compared to winter months. The annual pattern in BGE_M
10 correlated with the numbers of heterotrophic bacterial (July 2011 to April 2012, $r = 0.529$, $n = 17$, $P < 0.05$, Figure 3a) and rates of bacterial productivity ($r = 0.511$, $n = 17$, $P < 0.05$, Figure 2a). The proportion of methanol assimilated into biomass remained less than 1% throughout the year, which is in agreement with previously reported BGE_M from the NE Atlantic (west of the Iberian Peninsula, Dixon & Nightingale 2012) and oligotrophic regions
15 (Dixon et al. 2011b), but is much lower than previous measurements (12-57%) for productive coastal upwelled and shelf waters (Dixon et al. 2011a, b, Dixon et al. 2013). The BGE_M values reported in this study are also similar to results by Sun et al. (2011) who reported less than 6% of ^{14}C-methanol assimilated by bacterioplankton populations in seawater incubations from the western Sargasso Sea. Cultivation studies have demonstrated negligible methanol
20 assimilation in the SAR11 strain HTCC1062 (Sun et al. 2011), whilst the OM43 strain HTCC2181 dissimilated 3.5 times more methanol than that assimilated in cultivated cells (Halsey et al. 2012).

Bacterial numbers and productivity

Total numbers of heterotrophic bacteria (Figure 3a) varied between 2.0 – 15.8 x 10^5 cells ml^{-1} with maxima in May, July and September. The seasonal pattern and numbers of bacteria found in this study agreed well with those found at L4 in previous years (2.0 to 15.0 x 10^5 cells ml^{-1}, data from 1998 to 2001 and 2003 to 2004, Mary et al. 2006). Heterotrophic
5 bacterial numbers were subdivided into high nucleic acid (HNA) bacteria and low nucleic acid (LNA) bacteria, which ranged between 1.3 and 12.9 x 10^5, and 0.3 –and 5.7 x 10^5 cells ml^{-1}, respectively (Figure 3a). Bacterial communities were dominated throughout the year by HNA bacteria (43 to 85%), which accounted for 81% of total numbers of bacteria during the July peak. Numbers of LNA bacteria were highest from July to September.

10

Measured rates of ^{3}H-leucine incorporation at L4 varied between 2.6 and 137 pmol l^{-1} h^{-1} and were highest in July and lowest during January/February i.e. winter months (Figure 2a). Calculated bacterial production rates ranged from 4.1 to 212 ng C l^{-1} h^{-1} which are typical of this temperate coastal environment (Lamy et al. 2009). The highest rates of bacterial
15 production were found between June and July (maximum 212 ng C l^{-1} h^{-1}). Rates subsequently decreased from July to September and remained low thoughout autumn and winter months (with an average of 18.1 ± 18.4 ng C l^{-1} h^{-1}), with the exception of a relatively small maximum towards the end of September (70.3 ng C l^{-1} h^{-1}). This coincided with a secondary autumnal phytoplankton bloom (chlorophyll *a* concentration of 1.95 mg m^{-3}).
20 Bacterial production rates in April 2012 were similar to those of April 2011, with an average of 34.4 ± 8.0 ng C l^{-1} h^{-1}.

Bacterial community composition

The bacterioplankton community of L4 surface waters during the sampling period was dominated by *Flavobacteria* and *Proteobacteria* (including SAR11 clade*, Rhodobacterales*
25 and a relatively minor contribution from *Alteromonadales*) which together comprised 49 to

94% of the 16S rRNA gene sequences per sample (Figure 3b). *Flavobacteriales* represented 6 to 69% of the 16S rRNA gene sequences, dominating the bacterial community from April to August 2011. The SAR11 clade (*Alphaproteobacteria*, Giovannoni et al. 2005) was the second most numerically abundant bacterial order (1 to 46% relative abundance of 16S rRNA gene sequences), becoming more prevalent in the bacterial community from October to March, reaching a maximum in February.

Spearman's rank correlation coefficient was used to determine possible relationships between methanol utilisation and phylogenetic data of the microbial community. Significant correlations were identified between methanol dissimilation and numbers of *Proteobacteria* (r = 0.513, n = 23, P < 0.02), as well as methanol assimilation and numbers of *Verrucomicrobia* (r = 0.461, n = 23, P < 0.02), however from this 16S rRNA sequence data no known methylotrophic species could be identified within *Verrucomicrobia*. Rates of methanol dissimilation were further correlated with numbers of *Alphaproteobacteria* (r = 0.599, n = 23, P < 0.01) with no significant correlation found between *Betaproteobacteria* or *Gammaproteobacteria*. Therefore, bacteria known to utilise methanol were identified and selected from the bacterial community sequenced for further analysis. Sequence numbers of bacterial clade SAR11 significantly correlated with methanol dissimilation rates (r = 0.714, n = 23, P < 0.01, Figure 4a) during this seasonal study (April 2011 to April 2012). *Rhodobacterales* (*Alphaproteobacteria*) varied between 4 and 26% of 16S rRNA gene sequences, following an opposing seasonal trend to SAR11 (Figure 4b) and contributed >15% to the total population sequenced from April to September. Numbers of *Rhodobacterales* 16S rRNA gene sequences were found to have a statistically significant positive correlation with microbial methanol assimilation rates between June 2011 and March 2012 (r = 0.731, n = 16, P < 0.01).

Synechococcus (*Cyanobacteria*) varied from 0 and 36% of the 16S rRNA gene sequences and were present in all but one sample between June and March (0.6 to 8.5 x 10^4 cells ml^{-1}, flow cytometry data Figure 2e, maximum of 36% during October) and almost absent April to June
5 (<0.1 to 0.3 x 10^4 cells ml^{-1}, Figure 2e). No statistically significant relationship was found between rates of methanol utilisation and numbers of picoeukaryotes or nanoeukaryotes (Figure 2f). *Acidimicrobiales* (*Acidimicrobia*) accounted for 0 to 18% of the 16S rRNA gene sequences of the bacterial community throughout the year, with sequence numbers higher during winter months. Unclassified bacteria varied between 1 and 16% of the 16S rRNA
10 gene sequences per sample throughout the study: the fraction of these sequences was also higher during winter months.

The number of operational taxonomic units (OTU) retrieved at Station L4 varied between 113 and 341 throughout the time-series, increasing from the minimum number of OTUs in
15 July 2011 to the maximum in February 2012. The number of bacterial OTUs per sample statistically correlated with corresponding total (dissimilation plus assimilation) methanol utilisation rates ($r = 0.606$, $n = 16$, $P < 0.05$).

Discussion

20 The patterns of methanol utilisation by microbes exhibited opposite seasonal trends, with microbial methanol dissimilation rates being higher during winter and assimilation rates being higher during summer months. Comparison of microbial methanol dissimilation rates from June 2011 (1.3 ± 0.5 nmol l^{-1} h^{-1}, this study) indicated that they were up to five-fold higher than rates from June 2006 (0.2 – 0.4 nmol l^{-1} h^{-1}, Dixon et al. 2011a). This suggests
25 that methanol dissimilation could exhibit inter-annual variability at this dynamic coastal

station, although a longer time series conducted over multiple years would be needed to confirm this.

Halsey et al. (2012) made measurements of methanol utilisation in natural coastal waters (Newport Harbour), estimating methanol dissimilation rates to be 0.40 µmol C per 10^{12} cells h^{-1} (equating to 3.2 nmol l^{-1} h^{-1} using the bacterial count of concentrated seawater, 8 x 10^{6} cells ml^{-1}) and methanol assimilation rates to be 0.35 µmol C per 10^{12} cells h^{-1} (equating to 2.8 nmol l^{-1} h^{-1} using the bacterial count of concentrated seawater, 8 x 10^{6} cells ml^{-1}). The rate of methanol disimilation reported by Halsey et al. (2012) is within the range measured during this study. However, the rate of methanol assimilation is considerably higher than reported from station L4, which could be a result of differences in the abundance of OM43 strain HTCC2181 between sampling sites. This strain was isolated from Newport Harbour and produced similar rates of methanol assimilation in culture to the natural seawater incubation (Halsey et al. 2012). Other marine microbes have been shown to assimilate methanol. The low abundance or activity of other methylotrophic bacteria within the microbial community at station L4 could also result in the lower methanol assimilation rates observed. Only a single 16S rRNA sequence was identified as HTCC2181 within our data from station L4. It should be noted that filtering for bacterial community composition using 0.22 µm could have resulted in the loss of SAR11 and/or OM43 cells, which could help to explain the relatively low numbers of these groups in our data.

Microbial methanol assimilation rates are comparable with values measured in the remote Atlantic Ocean (0.17 to 2.83 x 10^{-2} nmol l^{-1} h^{-1}, Dixon et al. 2013), with the highest rates of methanol carbon assimilation observed during increased bacterial productivity and dissolved organic carbon concentrations (Spring and Summer, Figure 2a, b). This could reflect an

increased dominance of obligate methylotrophs which may be using a synergistic metabolic approach of utilising methanol for growth whilst simultaneously using other C_1-compounds (part of the DOC pool) strictly as a source of energy, as previously suggested for OM43 strain HTCC2181 (Halsey et al. 2012). This metabolic strategy could also help explain why
5 rates of microbial methanol assimilation, reported from highly productive upwelled waters of coastal Mauritania (0.42 nmol l^{-1} h^{-1}, Dixon et al. 2013), where elevated DOC substrates were available, were approximately 16 times higher than rates measured at station L4. Obligate methylotrophs may only use C_1 compounds as both carbon and energy sources, whereas facultative methylotrophs may, under certain conditions, use multi-carbon substrates for
10 growth whilst utilising C_1 compounds for energy production (Chistoserdova 2011).

Methanol was predominately used as a source of energy (dissimilation), with less than 1% assimilated into biomass throughout the year at station L4. This is similar to previously measured BGE_M in northern temperate and remote gyre waters of 1% (Dixon et al. 2013), but
15 considerably lower than previous estimates from upwelling areas (12 to 57%, Dixon et al. 2013). This may be a result of the biogeochemical differences between these regions, for example differences in the availability of organic carbon substrates which is likely to impact the success of methylotrophic bacteria. The adoption of a synergistic approach could provide an advantage to those microbes, such as members of the HTCC2181 strain, over other
20 obligate methylotrophs when methanol is less abundant.

Significant negative correlations between microbial methanol dissimilation and both bacterial productivity and heterotrophic bacterial numbers show that during times of lower bacterial productivity (typically winter months) and lower DOC substrate availablity (Figure 2a, b), an
25 increase in rates of microbial methanol dissimlation occurs. This could reflect a shift in the

methylotrophic community composition, from obligate to facultative methylotrophs where increased dominance of facultative methylotrophs during the winter months could result in increased methanol dissimilation rates if multi-carbon substrates are being used for growth.

A significant Spearman's rank correlation found between the number of OTUs per sample and total methanol utilisation (dissimilation and assimilation) rates suggests that temporal changes in the bacterial population assemblage may be linked to variations in methanol utilisation. This link could be due to the presence of methylotrophic microbial groups. Previous work has shown that bacterial species diversity is highest during winter in the western English Channel (Gilbert et al. 2012), and that activities of specific bacterial groups can vary throughout the year, particularly in summer when inorganic nutrients can be more limiting (Alonso-Sáez & Gasol 2007). Methylotrophic bacteria were tentatively quantified from the bacterial community sequence data using 16S rRNA gene sequences from known methylotrophic species but were found to be numerically rare (e.g. *Methylophaga thiooxidans* DMS010, Boden et al. 2010, Neufeld et al. 2007, Schäfer 2007). The successful design of new primer sets targeting *xoxF* (Taubert et al. 2015) an alternative methanol dehydrogenase like XoxF (Giovannoni et al. 2008, Wilson et al. 2008) will enable future studies to investigate the prevalence and role of *xoxF* at station L4.

A correlative approach, using 16S rRNA pyrosequencing data and rate measurements of microbial methanol utilisation, suggests that members of the SAR11 and *Rhodobacterales* clades may play important roles in methanol cycling in the coastal marine environment. The strong significant correlation between SAR11 clade 16S rRNA gene sequences and methanol dissimilation rates observed during this seasonal study (Figure 4a) strongly links this type of organism to the dissimilation of methanol. Although this group of bacteria lack *mxaF* in their

genomes (Giovannoni et al. 2008, Sun et al. 2011), SAR11 cells have been shown to possess an iron-containing alcohol dehydrogenase (Fe-ADH) which Sun et al. (2011) propose may be capable of oxidising methanol and other short chain alcohols.

5 Although no statistically significant correlation between methanol assimilation and numbers of *Rhodobacterales* 16S rRNA gene sequences was found during the complete sampling period (April 2011 to April 2012), these measurements do appear to follow a similar overall seasonal trend. When the analysis is confined to using values from June 2011 to March 2012, a statistically significant correlation is found, suggesting that *Rhodobacterales* may be
10 contributing to rates of microbial methanol assimilation into cell biomass, at least in some circumstances (Wilson et al. 2008, Chen 2012). The trend is not as clear during the period from April to June 2011 which could reflect the uncharacteristic environmental conditions experienced at Station L4 in early 2011 (Spring 2011 was exceptionally warm and dry, with increased light levels), although it is plausible that from April to June 2011 other
15 methylotrophic bacteria are dominating methanol assimilation. Members of the *Rhodobacterales* have been shown to metabolise methanol: for example, *Rhodobacter sphaeroides* possesses a *xoxF* gene (Wilson et al. 2008) which has also been identified in 40 genomes of the marine *Roseobacter* clade (Chen 2012). Newly designed primers targeting *xoxF* have demonstrated the widespread presence of this gene in coastal waters, including
20 station L4 where some of the most abundant *xoxF* sequences detected were related to *xoxF* of members of the *Rhodobactaceae* family (Taubert et al. 2015). This supports the hypothesis that the *Rhodobacterales* order are significantly contributing to methanol assimiliation in the western English Channel. However, further experimental work using RNA sequencing and environmental proteomics over a seasonal cycle is required to confirm if SAR11 and

Rhodobacterales cells at L4 are actively expressing methanol utilisation genes and their cognate proteins.

Previous studies of the microbial community at L4 have shown that both SAR11 and
5 *Rhodobacterales* represent the most abundant 16S rRNA genes (Gilbert et al. 2012), with SAR11 peaking during winter (at 46% relative abundance) and representing on average 13% of the bacterial community (Mary et al. 2006). A metaproteomic study of a coastal upwelling system found 36% of proteins detected to be best matched to proteins of SAR11 and 17% to proteins from strains in the *Roseobacter* clade (Sowell et al. 2010). Therefore, the extent to
10 which SAR11 and *Rhodobacterales* dominate the bacterial community composition could significantly influence the temporal variability observed in methanol dissimilation and assimilation rates at station L4. Strains of *Flavobacterium* have also been shown to grow on methanol (Moosvi et al. 2005, Boden et al. 2008), or to contain *mxaF* (Madhaiyan et al. 2010). However, numbers of *Flavobacterium* 16S rRNA gene sequences did not correlate
15 with the seasonal trends for microbial methanol utilisation.

Overall, these results suggest that at Station L4 methylotrophic bacteria with alternative methanol oxidation enzymes other than MxaFI may be largely responsible for methanol dissimilation. Stable Isotope Probing experiments using ^{13}C-methanol have provided
20 valuable insights into active methanol-assimilating microbes (Neufeld et al. 2007, Grob et al. 2015). However, this methodology does not aid the identification of microbes that dissimilate methanol because it relies on the incorporation of ^{13}C into biomass. With new emerging methods, such as DNA Stable Isotope Probing combined with metagenomics and metaproteomics (Grob et al. 2015) and new primer sets for *xoxF* (Taubert et al. 2015) to
25 complement the traditional *mxaF* approach, new tools are now available to examine the

diversity of methylotrophs using a combined approach of functional gene probes alongside process measurements. The use of RNA sequencing and environmental proteomics would also enable the identification of active enzymes involved in C_1 metabolism in the marine environment. A combined approach that included both the identification and quantification of the activity of methanol dissimilating bacteria in the marine environment is required to understand the controls on microbial methanol utilisation in coastal waters and identify key microbial players in the marine cycling of methanol.

Conclusions

This research provides the first fully resolved seasonal study of microbial methanol uptake dynamics combined with molecular characterization of the bacterial community. We show that microbial methanol dissimilation (higher throughout winter) and microbial methanol assimilation (higher during summer) exhibit opposing seasonal patterns. Overall <1% of the total methanol utilised was assimilated into cell carbon. Temporal trends in the utilisation of methanol suggest that the composition of the methanol-utilising community changes throughout a yearly cycle, with methanol being used as an alternative source of energy and/or carbon during times of increased competition for other preferred sources of carbon. Statistically significant correlations implicate SAR11 and *Rhodobacterales* in the dissimilation and assimilation of methanol at Station L4, respectively. Further research should use a combined approach of RNA sequencing, environmental proteomics and functional gene probes (*mxaF* and *xoxF*), together with process measurements, to explore active methanol utilisation and identify key players in the marine cycling of methanol.

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Conflict of Interest Statement

The Authors declare no conflict of interest with this manuscript.

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Titles and legends to figures

Figure 1. Temporal variability in microbial rates of methanol uptake: dissimilation (white circles) and assimilation (black circles) form April 2011 to April 2012 in surface waters at
5 station L4. Shaded areas show the seasons. Error bars denote ± 1 SD of three replicates.

Figure 2. Temporal variability between April 2011 and April 2012 at station L4 in (a) bacterial growth efficiency for methanol (BGE_M, black circles), rates of bacterial production (white squares), (b) numbers of heterotrophic bacteria, divided into high nucleic acid (HNA,
10 black circles) and low nucleic acid (LNA, white circles), and dissolved organic carbon (DOC) concentrations (white squares). Error bars denote ±1 SD of three replicates. Monthly averages of (c) nitrate (black diamonds) and phosphate (white diamonds) concentrations, (d) sea surface temperatures (black diamonds) and chlorophyll *a* concentrations (white diamonds). Error bars represent ±1 SD of monthly values. Numbers of (e) *Synechococcus*,
15 (f) picoeukaryotes (black diamonds) and nanoeukaryotes (white diamonds). Error bars denote ±1 SD of three replicates.

Figure 3. Bacterial community composition (identified using 16S rRNA gene sequencing) at station L4 at the order level. Analysis is based on a rarefied sample of 816 sequences per
20 sample. Bacterial orders individually contributing to less than 5% of the total sample sequences were pooled together into 'Others (<5%)' for clarity.

Figure 4. Comparison between the temporal variability of microbial methanol (a) dissimilation (white circles) with the sequence abundance of the SAR 11 clade (white bars)
25 and (b) assimilation (black circles) with the sequence abundance of *Rhodobacterales* (shaded bars), as identified from 16S rRNA gene fragments at L4. Analysis is based on a rarefied sample of 816 sequences per sample. Error bars denote ± 1 SD of three replicates.

Figure 1.

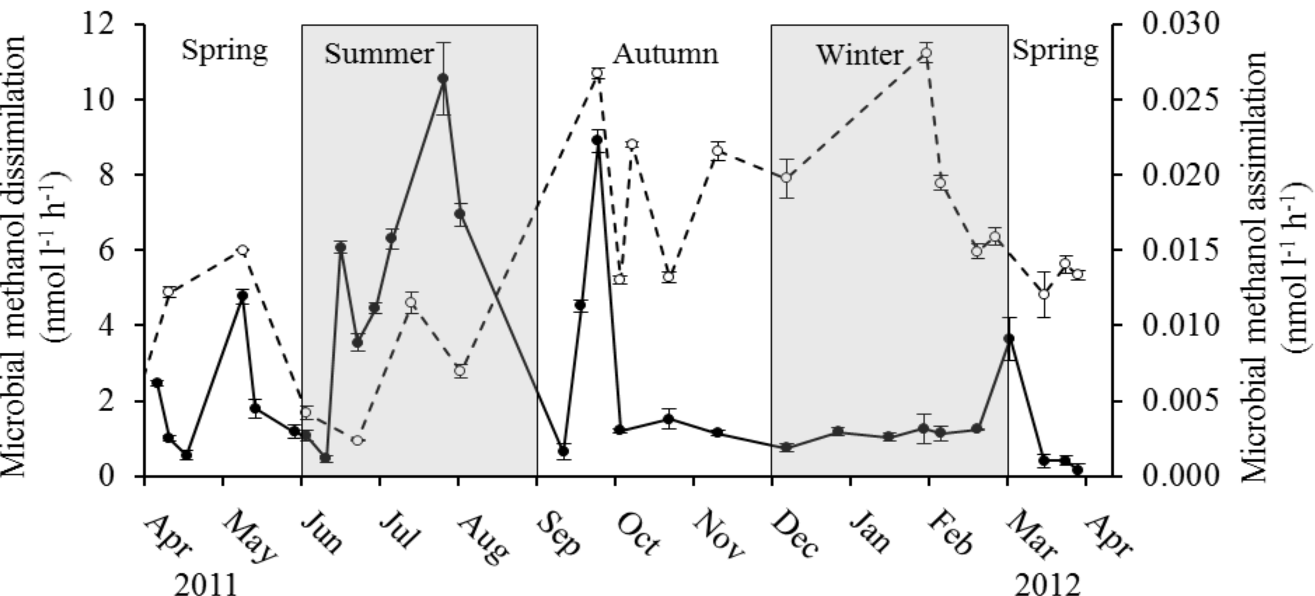

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Figure 2.

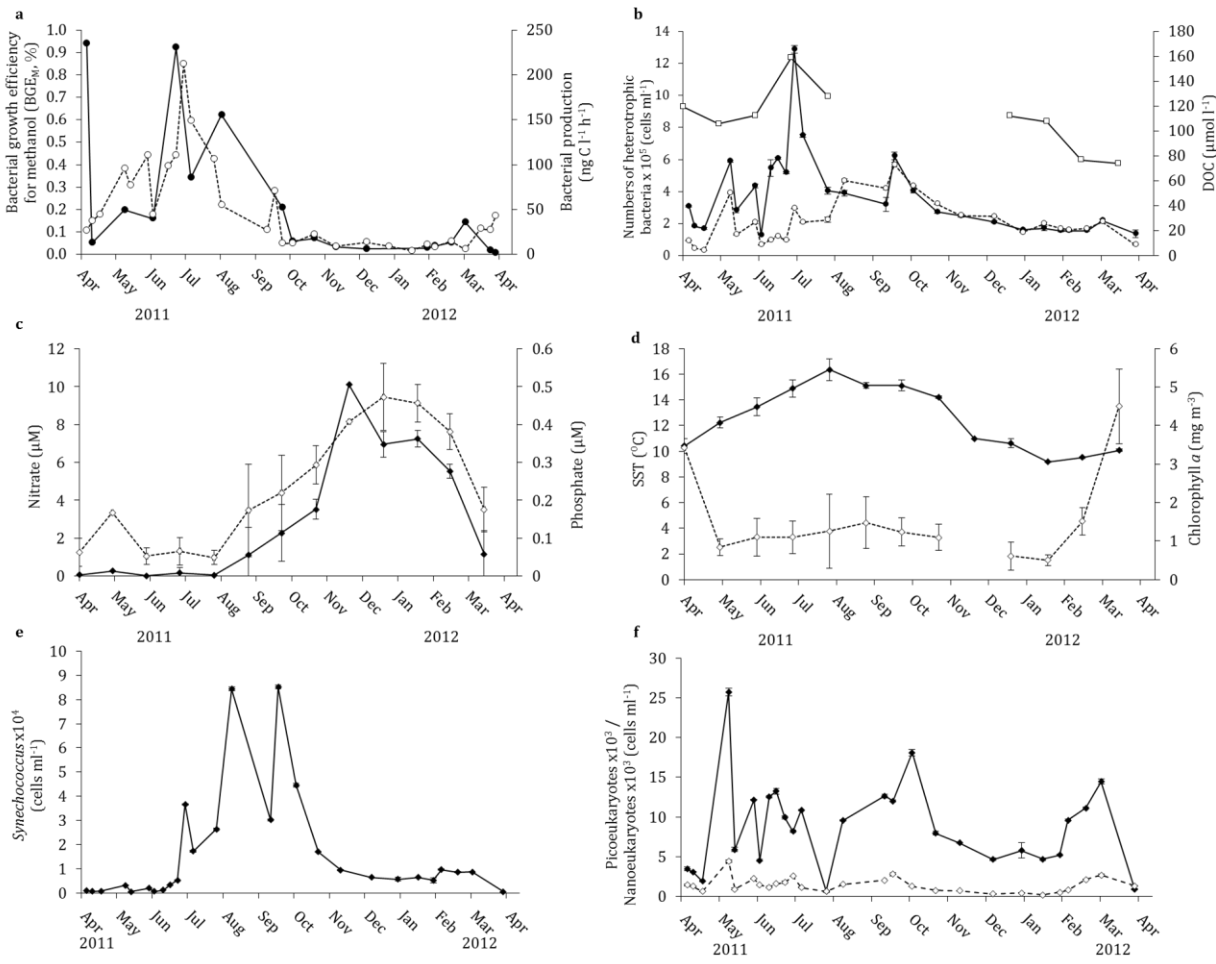

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Figure 3.

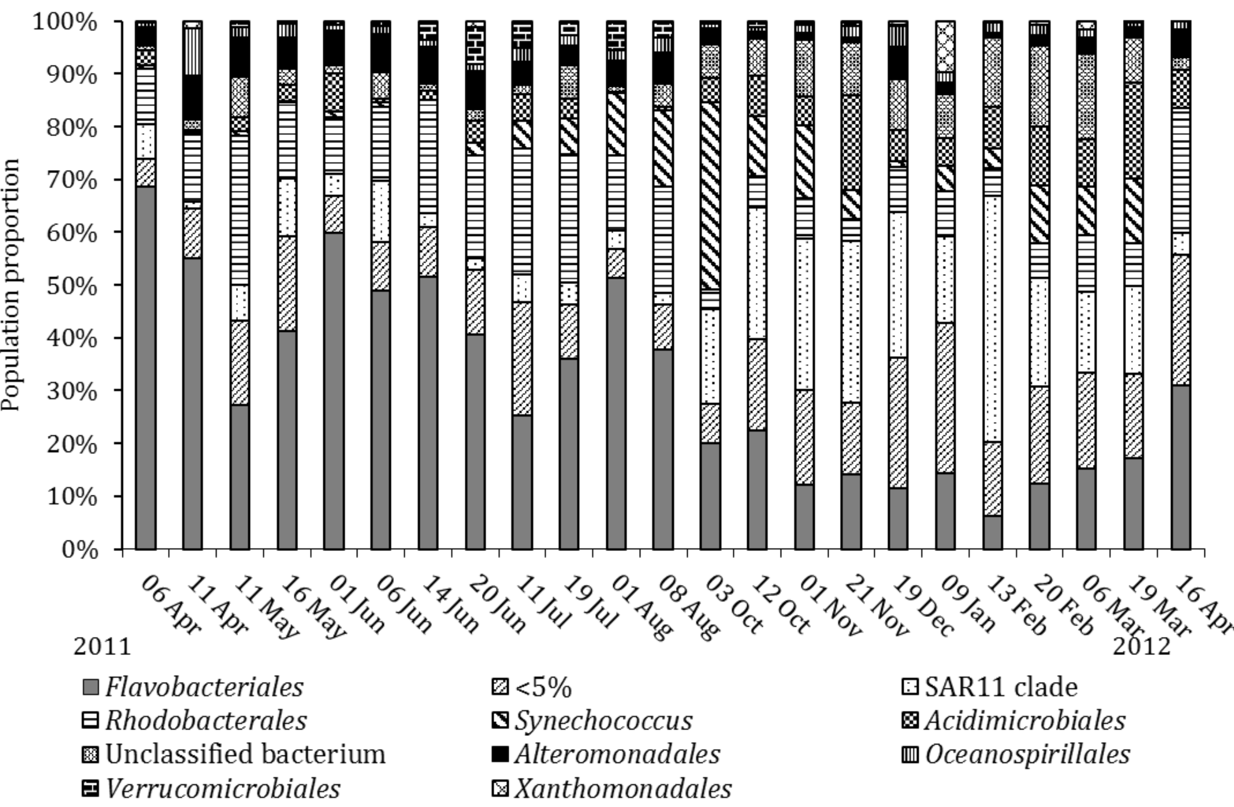

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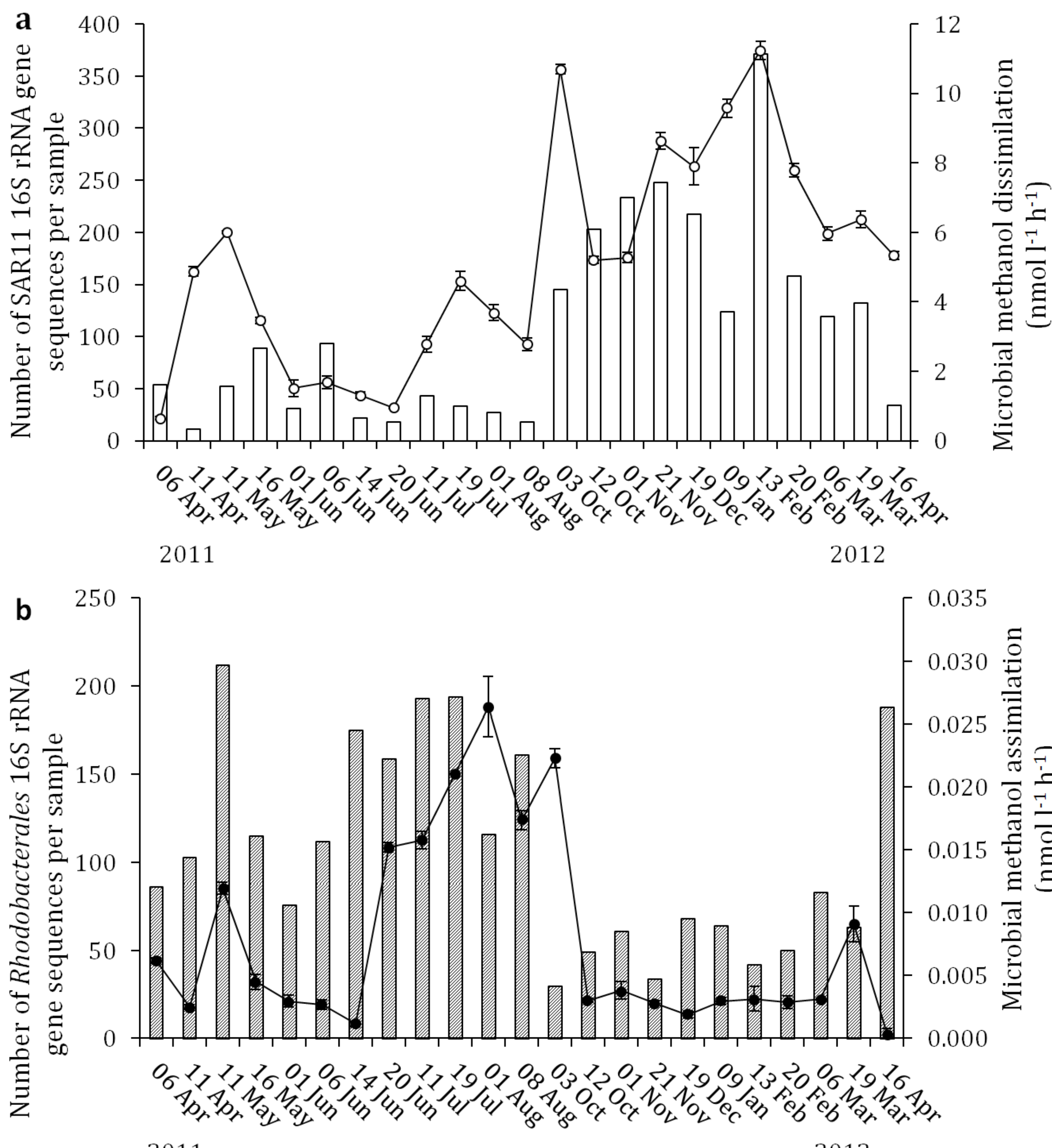
Figure 4.



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