Substrate-specific clades of active marine methylotrophs associated with a phytoplankton bloom in a temperate coastal environment

Diversity of active marine methylotrophs

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ABSTRACT

Marine microorganisms that consume one-carbon (C_1) compounds are poorly described, despite their impact on global climate via an influence on aquatic and atmospheric chemistry. This study investigated marine bacterial communities involved in the metabolism of one-carbon compounds. These communities were of relevance to surface seawater and atmospheric chemistry in the context of a phytoplankton bloom that was dominated by phytoplankton known to produce dimethylsulfoniopropionate. In addition to using 16S rRNA gene fingerprinting and clone libraries to characterize samples taken from a bloom transect in July 2006, seawater samples from the phytoplankton bloom were incubated with ^{13}C-labelled methanol, monomethylamine, dimethylamine, methyl bromide, and dimethylsulfide to identify microbial populations involved in turnover of C_1 compounds using DNA stable isotope probing (DNA-SIP). The ^{13}C-DNA samples from a single time point were characterized and compared using denaturing gradient gel electrophoresis (DGGE), fingerprint cluster analysis and 16S rRNA gene clone library analysis. Bacterial community DGGE fingerprints from ^{13}C-labelled DNA were distinct from those obtained with the DNA of the non-labelled community DNA and suggested some overlap in substrate utilization between active methylotroph populations growing on different C_1 substrates. Active methylotrophs were affiliated with *Methylophaga* spp. and several clades of undescribed *Gammaproteobacteria* that utilized methanol, methylamines (both monomethylamine and dimethylamine) and dimethylsulfide. Ribosomal RNA gene sequences corresponding to populations assimilating ^{13}C-labelled methyl bromide and other substrates were associated with *Alphaproteobacteria* (e.g. *Rhodobacteraceae* family), *Cytophaga-Flexibacter-Bacteroidetes*, and unknown taxa. This study expands the known diversity of marine

methylotrophs in surface seawater and provides a comprehensive dataset for focussed cultivation and metagenomic analyses in the future.

INTRODUCTION

Methylotrophic bacteria represent an important functional guild, contributing to the metabolism and assimilation of one-carbon (C_1) compounds. As the carbon sources that they depend on in the marine environment are present at low concentrations, characterizing marine methylotrophs has involved the use of enrichment and cultivation approaches with a variety of C_1 substrates. The C_1 substrates of relevance to the marine environment include methane, methanol, methylated amines, methyl halides and methylated sulfur compounds. Methane is supersaturated in surface seawater and several studies have isolated methanotrophs from the marine environment (14, 16, 27, 46). Methyl halides are produced by a number of phytoplankton species (e.g. 41) and these ozone-depleting compounds have been used to isolate methylotrophic *Alphaproteobacteria* that belonged to the *Roseobacter* clade (42, 44). Methanol represents a marine C_1 substrate derived from phytoplankton (13) and the atmosphere (7), which may be actively metabolised by marine methylotrophs (21). Methanol has been estimated at between 100 nM (47) and 300 nM (10) and has been directly measured in one study, ranging between 50-250 nM in several tropical Atlantic samples (53). Enrichment and isolation studies using methanol as a sole carbon source have generated molecular fingerprint phylotypes and characterised isolates of *Methylophaga* spp. (*Gammaproteobacteria*). *Methylophaga* spp. have also been isolated using dimethylsulfide (DMS; 8, 43) and can grow on monomethylamine (e.g. 23), both of which occur at nM concentrations in surface seawater (11, 24). Together, these cultivation-based approaches have revealed the presence of organisms capable of C_1 cycling in the marine environment. Their

involvement in methylotrophic metabolism *in situ* can be experimentally addressed using stable isotope probing (39).

DNA stable-isotope probing recently identified *Methylophaga*-like organisms as active methylotrophs that assimilated methanol and methylamine in surface waters of the English Channel (35). This study also demonstrated that 16S rRNA gene sequences representing clades of uncultivated *Gammaproteobacteria* were also retrieved from the heavy DNA for each of these compounds that clustered close to *Methylophaga*. A SIP experiment with methanol substrate dilution to concentrations anticipated to reflect those *in situ* (33) confirmed the involvement of *Methylophaga* spp. in methanol consumption and retrieved functional genes involved in methanol metabolism from these active methylotrophs using metagenomic libraries.

The goal of the current study was to extend our previous observations that were made during non-bloom conditions, by studying methylotrophic populations in the context of a phytoplankton bloom dominated by *Emiliania huxleyi* and *Karenia mikimotoi* (formerly *Gyrodinium aureolum*). Both coccolithophores (e.g. *Emiliania*) and small dinoflagellates (e.g. *Karenia*) are associated with dimethylsulfoniopropionate (DMSP) production (22, 28) and phytoplankton blooms are known to produce relevant C_1 compounds or their precursors, including methanol (13), methylated sulfur compounds (24), and methyl halides (41). As with our previous marine SIP studies (33, 35), seawater samples were incubated with methanol and methylamine, and in this investigation, SIP incubations were also carried out with ^{13}C-labelled methane, dimethylamine, methyl bromide and DMS in order to identify microbial populations that are actively involved in the cycling of these C_1 compounds during phytoplankton blooms *in situ*.

MATERIALS AND METHODS

Bloom sampling. A transect across a phytoplankton bloom dominated by *E. huxleyi* and *K. mikimotoi* (D. Schroeder, personal communication) was sampled in the English Channel bordering the south coast of the UK. Surface seawater was taken from inside the bloom (49.3222 N, 5.1446 W to 49.5105 N, 5.1217 W), at the edge of the bloom (49.5472 N, 4.3966 W to 49.5523 N, 4.4045 W), and outside the bloom area (50.1158 N, 4.1998 W to 50.1053 N 4.2062 W). The distances between the beginning and end of sampling differed for the three sampling stations and were 1.3 km, 0.8 km, and 20 km for the outside, edge, and inside of bloom, respectively. All samples were taken between 10.00 h and 22.00 h on July 26, 2007. Water samples were returned to the laboratory and aliquots were taken for filtration (for DNA extraction) and to establish SIP incubations on July 27, 2006. Upon arrival, multiple aliquots of approximately one-litre were filtered through 0.2-µm Sterivex filters (Durapore, Millipore) and frozen at -80°C until processed for nucleic acid extraction.

Incubation with ^{13}C labelled substrates. Samples taken from the edge of the bloom were chosen to set up SIP incubations with several ^{13}C-labelled C_1 substrates. Seawater sample aliquots of 750 ml were added to 1-liter serum bottles with the addition of 0.1% (750 µl) marine ammonium mineral salts medium (MAMS; modified from 12) and substrate. A total of 75 µmol of ^{13}C-labelled substrate was added to bottles for methanol, monomethylamine, dimethylamine, methyl bromide, and methane (final concentration of 100 µM assuming complete dissolution). For dimethylsulfide, 187.5 µmol of substrate were added to make up a final concentration of 250 µM. All serum bottles were crimp-sealed with butyl rubber bungs to prevent loss of volatile substrates. All ^{13}C-labelled compounds were 99% or greater purity and obtained from Cambridge Isotope Laboratories (Hook Hampshire, UK) except methylated amines (Sigma, Gillingham,

UK). Dimethylsulfide ($^{13}C_2$-labelled) was prepared by a method adapted from that for labelled dimethylsulfoxide synthesis (5). Sodium sulfide nonahydrate (6.5 g) was dissolved in 6.5 mL of sterile deionised water in a glass test-tube and cooled to 0°C in an ice-water bath with vigorous stirring. Subsequently, 5 g of ^{13}C-methyl iodide (Cambridge Isotope Laboratories LTD, Andover, MA) was added dropwise over a period of 30 min prior to incubating the reaction mixture at 0°C for 5 h with stirring. Five mL of each 2 M sodium hydroxide solution and 1 M sodium thiosulfate solution were added and the reaction vessel was then connected to a receiving tube held at -170°C in liquid nitrogen and the reaction mixture allowed to warm to 40°C in a water bath. The $^{13}C_2$-dimethylsulfide was distilled from the reaction mixture for 90 minutes and then re-distilled into a sterile receiving vessel for 1 hour. Sterile deionised water was added to the receiving vessel to dissolve the $^{13}C_2$-dimethylsulfide and the resulting solution transferred, with washings, to a sterile 1-l serum vial which was then sealed with a butyl rubber bung. The concentration and purity of the $^{13}C_2$-dimethylsulfide solution were assessed by gas chromatography with a flame ionization detector. A total of 250 ml of a 7 mM solution of pure $^{13}C_2$-dimethylsulfide was obtained.

For all substrates, parallel incubations were set up as ^{12}C-unlabelled controls and ^{13}C and ^{12}C-substrate incubations were harvested at a single time point. With the exception of monomethylamine and dimethylamine, substrate utilization was monitored by gas chromatography, using a flame ionization detector. Measurement of DMS and methyl bromide concentrations in sterile seawater controls confirmed that the degradation observed in SIP incubations was due to a biological processes and not due to chemical decomposition. The concentrations of the methylamines were assumed to mirror those of methanol; recovery of ^{13}C-DNA from methylamine and dimethylamine incubations confirmed that methylated amines

had been assimilated. Following substrate depletion, SIP incubations were filtered through 0.22-µm Sterivex filters and frozen at -80°C until processed for nucleic acid extraction.

DNA extraction, SIP gradient centrifugation and fractionation. Total nucleic acids were extracted directly from Sterivex filters according to a previously described protocol (35). Briefly, lysozyme, proteinase K and sodium dodecyl sulfate (SDS) were used to lyse cells and lysates were transferred to 15-ml phase lock tubes (Qiagen, West Sussex, UK) for phenol-chloroform and chloroform extractions. Purified DNA was quantified on a 1% (w/v) agarose gel. Aliquots (1-5 µg) of DNA extracts from each of the SIP incubations were added to cesium chloride (CsCl) solution (average density of ~1.725 g ml^{-1}) and transferred to an ultracentrifuge gradient tube for centrifugation and fractionation as previously described (36). Briefly, tubes were added to a Vti 65.2 rotor (Beckman Coulter, Fullerton, CA) and centrifuged at 44,100 rpm (177,000 g_{av}) for 40 h at 20°C. Gradients were fractionated from bottom (fraction 1; highest density) to top (fraction 12; lowest density) into 425-µl fractions. DNA was purified from CsCl and quantitatively recovered by precipitation with glycogen (20 µg) and polyethylene glycol (30% PEG 6000 and 1.6 M NaCl). Purified DNA was suspended in 30 µl of sterile LoTE buffer (3 mM Tris at pH 8, 0.2 mM EDTA) and 5-µl aliquots were run on a 1% (w/v) agarose gel for quantification and to identify the distribution of labelled ^{13}C-DNA relative to background unlabelled ^{12}C-DNA (Figure S1 in the supplemental material). These data indicated that the ^{13}C-labelling of DNA was very high for methanol, monomethylamine, and dimethylsulfide incubations; most of the DNA for these ^{13}C-incubated samples eluted in heavy fractions (fractions 7 and 8; ~1.725-1.730 g ml^{-1}). The detection of ^{13}C-labelled DNA confirmed that the substrate was incorporated into microbial biomass. For dimethylamine and methyl bromide incubations, the extent of DNA labelling was

less pronounced. For methyl bromide incubations, there was almost no difference between the smears of DNA across gradients associated with ^{12}C and ^{13}C methyl bromide SIP incubations (Figure S1 in the supplemental material).

Denaturing gradient gel electrophoresis (DGGE). For each fractionated gradient, two fractions were selected for analysis of the 'heavy' (^{13}C) DNA (fractions 7 and 8; ~1.725-1.730 g ml^{-1}), and one fraction was selected for the characterization of 'light' (^{12}C) DNA (either fraction 11 or 12; ~1.710-1.705 g ml^{-1}). One-µl aliquots of gradient fractions were used as template for PCR to obtain 16S rRNA gene fragments suitable for DGGE analysis. Each 50-µl reaction mix consisted of 25 pmol each of primers 341f-GC and 534r (31), 1 X $(NH_4)_2SO_4$ buffer (Fermentas, York, UK), 1.5 mM MgCl, 33.6 µg non-acetylated bovine serum albumin (Sigma, Gillingham, UK), 40 nmol dNTPs, 1.25 U *Taq* polymerase (Fermentas). The reaction tubes were loaded directly into the block at 95°C (simplified hot start), followed by an initial denaturation at 95°C for 5 min and 30 cycles of 94°C for 1min (denature), 55°C for 1 min (anneal), and 72°C for 1 min (extension). A final extension at 72°C for 7 minutes was followed by a holding step at 10°C. Five-µl aliquots were quantified on a 1% (w/v) agarose gel.

For DGGE, 5-µl aliquots (100-300 ng) were run on a 10% polyacrylamide gel with a 30-70% denaturing gradient (100% denaturant is 7.0 M urea and 40% deionized formamide) according to the D-Code System instructions (Bio-Rad, Hercules, CA, USA). Gels were run overnight (14 h) at 85 V, then stained for 1 h in SYBR Green I (Invitrogen, Paisley, UK). Gel images were captured with a FLA-5000 imaging system (Fujifilm, Tokyo, Japan). Bands selected for sequence analyses were sampled from the gel by means of sterile pipette tips and amplified from the gel using the PCR conditions described above for DGGE. Sequencing was

done with the 341f primer and the BigDye terminator version 3.1 kit (Applied Biosystems, Foster City, USA) and the sequencing products were run on an ABI PRISM 3130 x l Genetic Analyser (Applied Biosystems) by the Molecular Biology Service, University of Warwick. DGGE band sequences were approximately 150 bases in length.

For determining the relatedness of the DGGE fingerprints, gels were imported into Gelcompar II (Applied Maths, Sint-Martens-Latem, Belgium) and normalized to ladder bands and additional internal standard bands. A UPGMA dendrogram was generated by performing a Pearson correlation on background-subtracted densitometric curves which takes band intensities into account. The output of the clustering analysis was independent of the input order of DGGE fingerprints.

16S rRNA gene libraries. Clone libraries of bacterial 16S rRNA genes were generated from the original seawater samples (outside bloom, edge of bloom, inside of bloom; 36 clones sequenced from each) and for the heavy DNA associated with the five substrates (methanol, monomethylamine, dimethylamine, methyl bromide, dimethylsulfide) that yielded ^{13}C-labelled DNA (24 clones sequenced from each). The PCR to amplify the 16S rRNA gene used primers 27f and 1492r (25) and the same amplification reaction as for DGGE except with an extension time of 1.5 min. Products were cloned into the TOPO-TA vector according to the manufacturer's protocol (Invitrogen). Screening was done as described previously (37) and cloned 16S rRNA gene inserts were sequenced at the Edinburgh node of the NERC Molecular Genetics Facility using the 27f primer. The program Pintail (3) was used to identify suspected chimeras and identified one heavy-band sequence which was likely chimeric in origin and several water library sequences that were likely chimeric, these sequences were excluded from further analyses. For

seawater samples, classification of 16S rRNA gene sequences was done using the RDP-II classifier (52) after manually verifying base calls. For 16S rRNA gene libraries constructed using ^{13}C-DNA from SIP experiments, manually verified 16S rRNA gene sequences were compared to Genbank (6) to retrieve three closest matches for each library sequence. Sequences were aligned within Arb (29) and an alignment was exported to MEGA4 (48). Evolutionary distances were computed using the Maximum Composite Likelihood method (49) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 466 nucleotide positions in the final dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (9). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. In the absence of cultivated methylotrophic organisms that fell within the groups of 16S rRNA genes derived from SIP experiments, clades were defined based on the consistent association with particular substrates but a specific cut-off value was not used.

Genbank sequence deposition. All sequences were deposited in Genbank for the marine samples taken from the edge (EU399242-EU399272), inside (EU399273-EU399306) and outside (EU399307-EU399340) of the phytoplankton bloom. The 16S rRNA gene clone library sequences from heavy DNA were deposited with the following accession numbers for SIP incubations with dimethylamine (EU399341-EU399364), dimethylsulfide (EU399365-EU399386), methyl bromide (EU399387-EU399407), monomethylamine (EU399408-EU399428) and methanol (EU399429-EU399451). DGGE band sequences from heavy DNA

were deposited with the following accession numbers for SIP incubations with methanol (EU399452-EU399457), monomethylamine (EU399458-EU399464), dimethylamine (EU399465-EU399469), dimethylsulfide (EU399470-EU399474) and methyl bromide (EU399475-EU399477).

RESULTS

Phytoplankton bloom microbial community analysis. This study was conducted on samples from an extensive mixed phytoplankton bloom with a predominance of both *Emiliania huxleyi* and *Karenia mikimotoi* (D. Schroeder, personal communication). Based on remotely sensed observations from the day prior to sampling, three sampling stations within the western English Channel were selected to represent areas of varying chlorophyll concentrations (Figure 1A), indicating regions internal to the bloom ('inside'), on the edge of the bloom ('edge') and external to the bloom ('outside'). Prior to assessing the methylotrophs in the bloom (edge sample), we assessed the background bacterial community composition of the three water samples using 16S rRNA gene fingerprinting (Figure 1B) and clone libraries (Figure 1C). The DGGE profiles indicate that the bacterial communities of these three water samples were represented by unique predominant band phylotypes, although several bands were shared between the three samples (Figure 1B). Almost all sequences collected from the 16S rRNA gene clone libraries were most similar to Genbank sequences derived from other marine surface water samples, reflecting a composition similar to previous studies (data not shown). All libraries were dominated by *Alphaproteobacteria* and Cyanobacteria, although *Bacteroidetes* were also prevalent in the 'Inside' and 'Edge' libraries (Figure 1C). Overall, the communities shared similar division-level

composition but also indicated that local sample heterogeneity existed across this relatively short bloom transect.

DNA-SIP incubations. Enrichment incubations with six C_1 substrates were established on the day following sampling (day 0) with substrate concentrations of 100 µM (250 µM for DMS). Substrate had been depleted by day 3 in methanol incubations and these were filtered for DNA extraction as were those containing methylamines. Approximately 110 µM of ^{12}C and ^{13}C-DMS were consumed by the fourth day (data not shown) and these incubations were subsequently sacrificed for DNA extraction. Methyl bromide incubations (^{12}C and ^{13}C) had consumed >90% of the 100 µM of substrate originally present by day 18 and were filtered for DNA extraction. Changes in headspace concentrations of methane (100 µM total in bottle; ~0.63% in headspace) for seawater incubations with methane were unchanged for several months (data not shown) and these incubations were not analyzed further.

16S rRNA gene fingerprinting of DNA from SIP experiments. Denaturing gradient gel electrophoresis (DGGE) was used to profile the bacterial communities associated with 'heavy' and 'light' fractions for both ^{12}C-control samples and ^{13}C-incubated samples. The ^{12}C incubated samples showed no profile difference between the 'heavy' and 'light' fractions (data not shown), whereas unique fingerprints were evident for all C_1-substrate incubations (Figure 2). As expected, the fingerprints for the light fractions of all tubes clustered together. The 'background' bacterial communities in each SIP incubation were more similar to one another than to the 'heavy' ^{13}C-fraction fingerprints of the same incubation. However, for the methyl bromide SIP incubation, the fingerprint of the 'heavy' DNA was less clearly unique from the light DNA than

for the other substrate incubations, reflecting that only a small amount of DNA was labelled and was just detectable above background ^{12}C-DNA. All other DGGE fingerprints from heavy fractions (fractions 7 and 8) of ^{13}C-substrate incubations clustered in distinct clades apart from the 'light' DNA, with monomethylamine and methanol fingerprints clustering closely, with some similarity to the dimethylamine fingerprints. Dimethylsulfide ^{13}C-DNA fingerprints were distinct from all other patterns in this study, reflecting a unique composition of active methylotrophs enriched in these SIP incubations. Individual bands from fingerprints representing 'heavy' DNA from $^{13}C_1$ incubations were selected for PCR reamplification and sequencing. These sequences were used to assign band sequences to specific SIP-related 16S rRNA gene clades derived from this study and from a previous SIP study that was carried out during non-bloom conditions in the English Channel (35). The results indicate that *Methylophaga* spp. were associated with methyl bromide, methanol and methylamine SIP incubations, whereas additional clades were affiliated with dimethylamine and dimethylsulfide, likely contributing to their more distinct fingerprint profiles (Figure 2).

16S rRNA gene clone libraries of ^{13}C-DNA. As the diversity of active methylotrophs was anticipated to be relatively low, 24 clones were sequenced from each library associated with SIP incubations with each of the five substrates analyzed in this study. The results of the sequencing confirmed relative low diversity of methylotrophs within each SIP incubation, but across the different substrates applied indicated a broad diversity of active marine methylotrophs in this study.

Methanol-assimilating phylotypes. Phylogenetic analysis demonstrated that sequences associated with SIP incubations with methanol clustered in the *Methylophaga* spp. clade of the *Gammaproteobacteria* together with sequences from a previous SIP incubation (35) and with several characterised *Methylophaga* isolates (Figure 3, Figure S2 in the supplemental material). In addition, one cloned 16S rRNA gene sequence obtained from the methanol SIP was a member of a clade of unknown phylogenetic affiliation, which also contained one DMS and DMA-SIP derived cloned 16S rRNA gene respectively. The methanol clade identified in a previous methanol SIP experiment (Figure 3; Figure S2 in the supplemental material) has closest affiliation to *Gammaproteobacteria* sequences in Genbank that were retrieved from multiple marine Arctic surface sediments or detected on the surface of submerged artificial substrates incubated in marine water near China. This clade however, was not detected in the current study.

Phylotypes assimilating methylated amines. As with the methanol SIP incubation, a previously characterised clade of sequences associated with a monomethylamine SIP incubation (35) was also represented by sequences from the monomethylamine SIP from the current study, and also from the dimethylamine SIP incubation. In particular, 22 of the 24 sequences generated from the dimethylamine SIP incubation and most of the corresponding DGGE band sequences (Figure 2) fell within this clade (Figure 3, Figure S3 in the supplemental material). This clade also contained several sequences isolated from Arctic sediment (Li et al, unpublished), a mangrove ecosystem (Liao et al. 2007, Microb Ecol. 54(3):497-507), and a deep-sea coral (Penn et al unpublished) and a strain isolated from the Yellow Sea (Kim and Cho, unpublished, Genbank accession EF468718). Additional 16S rRNA gene sequences obtained from the

monomethylamine SIP incubation belonged to the *Methylophaga* clade many species of which can grow on methylated amines.

Phylotypes assimilating DMS. Almost all 16S rRNA gene sequences derived from the dimethylsulfide SIP 'heavy' DNA were nearly identical and formed an additional clade with low relative diversity (Figure 3; Figure S4 in the supplemental material). The DMS clade was most closely related to the methanol SIP clade associated with the *Gammaproteobacteria* and identified in a previous study (35) and shared close similarity (96%) with sequences retrieved from clone libraries associated with DMS-enriched seawater samples from the Sargasso Sea (51) and was approximately 91% similar to the *Methylophaga* sp. clade, based on the percent similarity between sequences DMS_584_22 and *Methylophaga marina* (accession number X95459) over 722 bases. Another sequence from the 'heavy' DNA of the DMS SIP was affiliated with a clade of unknown phylogeny.

DISCUSSION

The study site was chosen based on the mixed *Emiliania* and *Karenia* bloom that occurred in the English Channel in July of 2006. The growth of phytoplankton in oceanic surface water has been associated with the direct or indirect production of methanol (13), methylamines (reviewed in 32), methyl halides (2, 4, 30, 41), methylated sulfur compounds (19, 20, 26) and methane, through decomposition (15, 38). In sampling from the edge of the bloom for SIP analysis (Figure 1), the objective was to retrieve sequences of methylotrophs relevant to bloom C_1 substrate production. Although the sample chosen was relevant to C_1 metabolism, it is

340 important to note that the substrate concentrations (100 µM) were far higher than those normally present in marine surface water samples. This was done because for a previous bloom in Bergen, Norway, the application of C_1 substrates at low µM concentrations did not result in the detection of ^{13}C-labelled DNA, possibly due to relatively high bacterial biomass associated with the bloom (Murrell *et al*., unpublished). In this study, the objective was to identify phylotypes associated
345 with the use of labelled C_1 substrates and the use of elevated substrate concentrations may have biased the results obtained. Typically, SIP experiments require substrate concentrations that exceed those found naturally and the data may have to be interpreted with caution (34). Nonetheless, a comparison of near *in situ* substrate concentrations (1 µM) with a marine methanol SIP incubation detected the same *Methylophaga* spp. phylotypes as detected in the
350 present study (33). As a result, for C_1 substrates in the marine environment, the results may be consistent despite the range of substrate concentrations used. In all SIP incubations thus far, the incubation times were extended to days and an addition of nutrients may have also selected for a fast growing species of methylotrophs. However, the uncultivated methylotrophs detected here are consistently present, which suggests that they do play an active role in C_1 metabolism in
355 coastal marine environments.

This study represents a comprehensive survey of active methylotrophs in a marine surface water sample during a bloom of phytoplankton associated with production of DMSP. The methylotrophs detected in this survey are consistent with the results of our pilot study with only methanol and monomethylamine under non-bloom conditions obtained a year prior to the
360 current sampling event (35); however, use of a wider range of C_1 substrates allowed the identification of a larger diversity of methylotrophs than found previously, including populations assimilating dimethylamine, DMS and methyl bromide. DMS SIP clones obtained were most

closely related to clones obtained from DMS enrichments from Pensacola and the Sargasso Sea by Vila-Costa and colleagues (51), suggesting that the latter had similar metabolic activities and indeed represented DMS degrading populations. Those sequences were classified as "uncultivated *Methylophaga*"; however, given the relatively low similarity of the 16S rRNA gene sequences of these cloned 16S rRNA gene sequences to those of *Methylophaga* isolates (around 92%) and their distinct clustering supported by bootstrap analysis (see Fig. S2 and S4), it is also possible that these represent DMS-degrading populations belonging to a different genus. Conversely, none of the DMS SIP clones were closely related to previously isolated DMS-degrading *Methylophaga* isolates (43), which belonged to the *Methylophaga* clade detected on methanol, monomethylamine and methyl bromide, strongly suggesting that populations closely related to the isolated strains may have a preference for other C_1 substrates and/or are outcompeted by those represented by the DMS clade under the specific incubation conditions. The methyl bromide SIP sequences suggest that methyl bromide may be used by members of the *Methylophaga* genus and an organism with a 16S rRNA gene sequence most similar to *Phaeobacter gallaeciensis* (formerly *Roseobacter*) within the *Rhodobacteraceae*. The notion that the *Phaeobacter*-related population degraded methyl halides would be supported by previous cultivation based identification of marine methyl halide degrading organisms which were closely related (42, 44, 45); however, screening of several *Methylophaga* isolates has failed to show their ability to degrade methyl halides (Schäfer, unpublished). The observation of *Methylophaga*-like sequences in the ^{13}C-methyl bromide incubation could therefore be due to the slow hydrolytic conversion of methyl bromide to methanol (1) and subsequent utilisation of the resulting methanol by these organisms. If *Methylophaga* populations in the methyl bromide incubations became labelled with ^{13}C due to uptake of methanol produced by conversion of methyl halides to

methanol, this would further underline their ability to take up methanol at ambient concentrations and support using SIP incubations with elevated substrate concentrations to investigate substrate responsive populations in seawater. Together, these data suggest that marine waters harbour a diverse suite of active methylotrophs that, apart from *Methylophaga* spp. have been unnoticed by previous cultivation studies (8, 17, 23, 42-44) and are almost completely without representation in marine clone libraries. The sequences represented here represent important targets for directed cultivation and focussed activity-based studies of marine methylotrophy.

Given the focus of past marine metagenomic studies on abundant community members, it is perhaps not surprising that few genes (phylogenetic or 'functional') have reflected the predominance of methylotrophic bacteria. Although formaldehyde oxidation genes were identified in the Sargasso Sea metagenomic libraries (50), genes for methane, methylamine, and methanol oxidation were not detected (18). Furthermore, the only presumed methylotroph 16S rRNA gene sequences identified in a marine metagenomic library was from *Methylophilus* spp. and these sequences occurred at ~0.4% of the total 16S rRNA gene dataset from the global ocean survey (40). The contribution of *Methylophilus* to marine C_1 cycling remains unclear and *Methylophilus* spp. have not been detected in ^{13}C DNA from the incubations carried out in this study. One possibility is that *Methylophilus* spp. represent *K*-selected organisms that are adapted to concentrations of carbon and nutrients that are lower than those used in this study. Cultivation-based approaches (17), enrichment cultures (43, 51) and SIP incubations (current study; 33, 35) have all demonstrated that *Methylophaga* spp. and related *Gammaproteobacteria* from multiple disparate marine samples (including estuary sediment; unpublished data) are present in the seawater samples and rapidly respond to the presence of C_1 substrates. It is possible that these organisms may represent low-abundance and *r*-selected bacteria that are capable of opportunistic

growth in the presence of relatively high concentrations of growth substrates during phytoplankton blooms, for example.

This study represents a comprehensive cultivation-independent survey of active marine methylotrophs and demonstrates that previously unrecognized bacterial groups are present in seawater, which are capable of responding to the presence of added C_1 substrates. The presence of numerous clades of presumed substrate-specific methylotrophs presents a challenge to microbiologists to focus cultivation and quantitative molecular approaches to better understand the metabolism and distribution dynamics of these organisms with potentially enormous biogeochemical significance.

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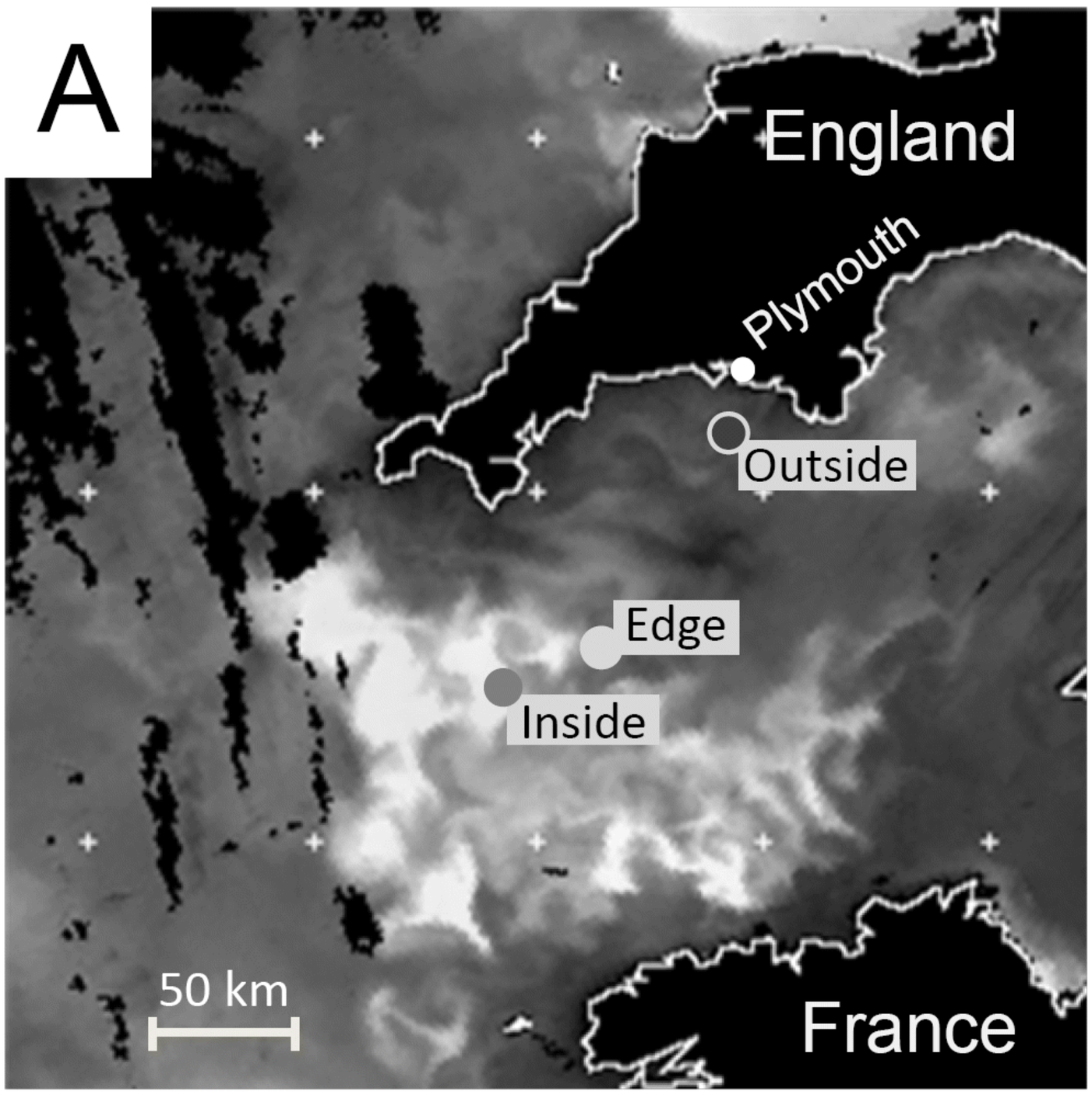
Figure 1. Physical location and bacterial composition of samples. A chlorophyll-*a* satellite image (A) from July 24, 2006 shows regions of high chlorophyll associated with a mixed bloom dominated by *Emiliania huxleyi* and *Karenia*. (B) Bacterial DGGE fingerprints of samples taken from the locations indicated in (A). (C) Frequency of 16S rRNA gene clones belonging to major phylogenetic groups across the different gene libraries analysed.

Figure 2. DGGE fingerprint comparison of 'light' and 'heavy' DNA associated with DNA SIP incubations with different carbon sources (MMA, monomethylamine; DMA, dimethylamine; MOH, methanol; MBr, methyl bromide; DMS, dimethylsulfide). The dendrogram scale bar refers to percent similarity of Pearson correlations between fingerprint densitometric curves. Shading of the triangle pointers indicates the phylogenetic affiliations of sequenced bands, most of which were associated with clades in Figure 3. Numbers to the bottom right of fingerprints correspond to sequenced bands submitted to Genbank. For example, the open triangle for fraction 7 of the ^{13}C-methylbromide SIP (MBr_7) will be labelled MBr_7_3 for the Genbank submission. Several bands were not associated with clades but were affiliated with sequences in Figure 3: MBr_7_3 is identical to MBr_587_7, DMA_7_6 is closest to MBr_587_24, DMS_7_2 is identical to DMS_584_3.

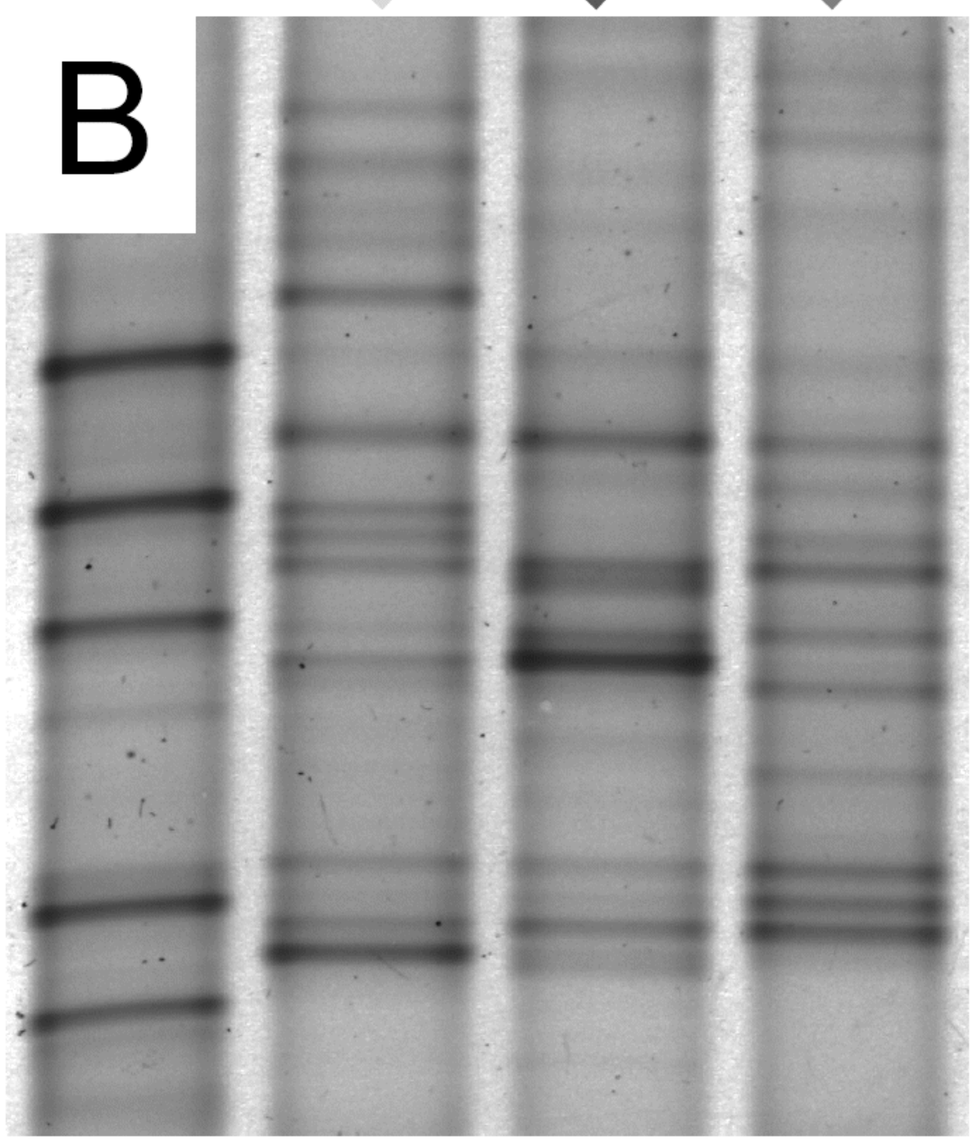
Figure 3. Phylogenetic affiliations of 16S rRNA gene sequences obtained by $^{13}C_1$-SIP incubations with methanol (MOH), monomethylamine (MMA), dimethylamine (DMA), methyl bromide (MBr) and dimethylsulfide (DMS) SIP incubations. Selected Genbank sequences from uncultivated clones and reference strains are included for comparison. Bootstrap values are included for all branch points on this neighbour-joining tree. Genbank accession numbers are

included within parentheses. The scale bar (within tree) represents 5% sequence divergence. The collapsed clades are expanded in Figures S2, S3 and S4 found in the supplemental material. The division-level affiliation of sequences indicated in the boxes along the right-hand side. CFB is *Cytophaga–Flavobacterium–Bacteroides*.

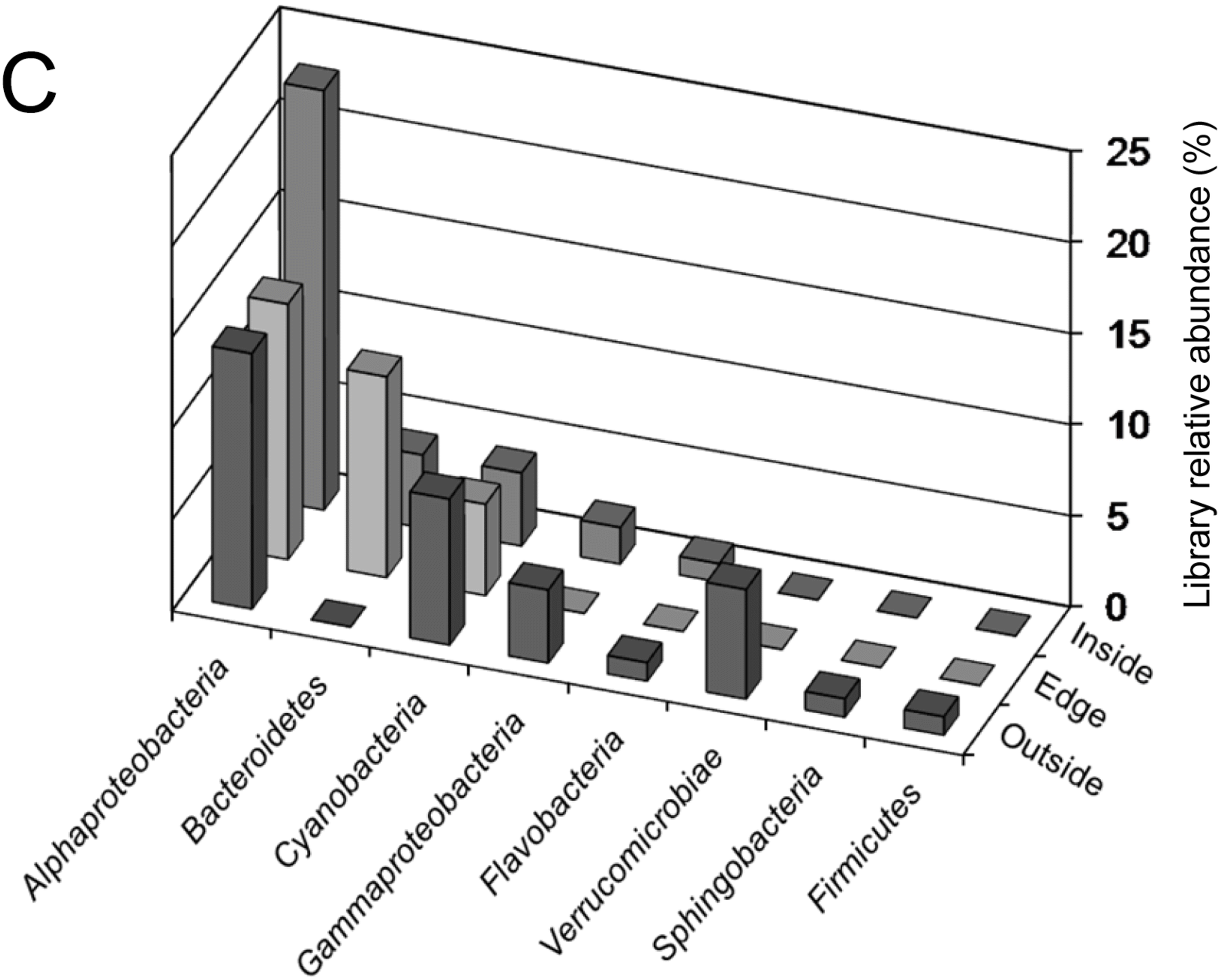
A


England
Plymouth
Outside
Edge
Inside
50 km
France

B


ladder
edge
outside
inside

C


25
20
15
10
5
0
Library relative abundance (%)
Inside
Edge
Outside
Alphaproteobacteria
Bacteroidetes
Cyanobacteria
Gammaproteobacteria
Flavobacteria
Verrucomicrobiae
Sphingobacteria
Firmicutes

50

100

DMA_11

MOH_11

DMS_12

MMA_11

MBr_11

‘Light’ DNA

MBr_8

MBr_7

1,2,3

MMA_7

1,2,3,4

MMA_8

1,2,3

MOH_7

1

MOH_8

1,2,3,4,5,6

DMA_8

DMA_7

1,2,3,4,5,6

DMS_8

1

DMS_7

1,2,3,4

‘Heavy’ DNA

* Failed reaction

Methylamines cluster

Δ Other clades

▲ *Methylophaga* spp. cluster

▲ Dimethylsulfide cluster

Methylophaga

Methyl bromide SIP: 15/20
Monomethylamine SIP: 6/20
Methanol SIP: 22/23
Neufeld *et al*. (2007) methanol SIP: 14/24
Neufeld *et al*. (2007) methylamine SIP: 2/19
Methylophaga isolates: 23

Methanol

Neufeld *et al*. (2007) methanol SIP: 10/24

DMS

Dimethylsulfide SIP: 21/22

Uncultured clone from seawater enriched with DMS (AM238571)
Uncultured clone from seawater enriched with DMS (AM238592)
Uncultured clone from seawater enriched with DMS (AM238610)
Uncultured clone from seawater enriched with DMS (AM238607)

Methylamines

Monomethylamine SIP: 12/20
Dimethylamine SIP: 22/24
Neufeld *et al*. (2007) methylamine SIP: 17/19

Cycloclasticus spirillensus (AY026915)
Pseudomonas sp. SI85-2B (AF326382)
Microbulbifer hydrolyticus (AJ608704)
Marinobacter lutaoensis (AF288157)
Microbulbifer hydrolyticus (AJ608704)
MMA SIP heavy band sequence (MMA_579_23)
Glaciecola mesophila (AJ548479)
Glaciecola sp. DMS049 (DQ660931)
Uncultured marine bacterium (DQ009205)
MBr SIP heavy band sequence (MBr_587_11)
MMA SIP heavy band sequence (MMA_579_1)
Uncultured bacterium clone (DQ372848)
Ruegeria atlantica (D88526)
Sulfitobacter mediterraneus (Y17387)
Leisingera methylohalidivorans strain MB2 (AY005463)
Phaeobacter daeponensis strain H-S3-33 (EU363304)
MBr SIP heavy band sequence (MBr_587_7)
MBr SIP heavy band sequence (MBr_587_4)
MBr SIP heavy band sequence (MBr_587_10)
MBr SIP heavy band sequence (MBr_587_24)
Uncultured Flavobacteriaceae bacterium clone (DQ810324)
Uncultured bacterium (AB199581)
MOH SIP heavy band sequence (MOH_577_20)
DMS SIP heavy band sequence (DMS_584_3)
DMA SIP heavy band sequence (DMA_581_24)
DMA SIP heavy band sequence (DMA_581_13)
Uncultured marine bacterium clone PB2.29 (DQ071098)

0.05

Gammaproteobacteria
Alphaproteobacteria
CFB
Unknown