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ORIGINAL ARTICLE

Phylogenetic and functional gene analysis of the bacterial and archaeal communities associated with the surface microlayer of an estuary

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The surface microlayer (SML) is the thin biogenic film found at the surface of a water body. The SML is poorly understood but has been shown to be important in biogeochemical cycling and sea–air gas exchange. We sampled the SML of the Blyth estuary at two sites (salinities 21 and 31 psu) using 47 mm polycarbonate membranes. DNA was extracted from the SML and corresponding subsurface water (0.4 m depth) and microbial (bacteria and archaea) community analysis was performed using denaturing gradient gel electrophoresis of 16S rRNA gene PCR amplicons. The diversity of bacterial functional genes that encode enzyme subunits for methane monooxygenase (*pmoA* and *mmoX*) and carbon monoxide dehydrogenase (*coxL*) was assessed using PCR, clone library construction and restriction fragment length polymorphism (RFLP) analysis. Methanotroph genes were present only in low copy numbers and *pmoA* was detected only in subsurface samples. Diversity of *mmoX* genes was low and most of the clone sequences detected were similar to those of *mmoX* from *Methylomonas* spp. Interestingly, some sequences detected in the SML were different from those detected in the subsurface. RFLP analysis of *coxL* clone libraries indicated a high diversity of carbon monoxide (CO)-utilizing bacteria in the estuary. The habitats of the closely related *coxL* sequences suggest that CO-utilizing bacteria in the estuary are recruited from both marine and freshwater/terrestrial inputs. In contrast, methanotroph recruitment appears to occur solely from freshwater input into the estuary.

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Introduction

The sea surface microlayer (SML), the thin biogenic film on the surface of the ocean, provides the physical link between the sea surface and the lower atmosphere (Liss and Duce, 1997). The SML has been defined as the top 1–1000 μm (Liss and Duce, 1997) but is operationally defined by the depth sampled due to selection of sampling device used (Agogue *et al.*, 2004). In terms of its physical, chemical and biological properties, the SML is distinct from subsurface waters even a few centimetres below (Zhang *et al.*, 1998, 2003; Zhengbin *et al.*, 1998). The SML is a unique ecosystem, often referred to as the bacterioneuston. A recent study of the bacterial communities in the SML of the coastal North Sea showed greatly reduced diversity in the SML compared to subsurface waters (Franklin *et al.*, 2005).

The SML has the potential to modify the exchange of climatically active trace gases between ocean and atmosphere. Gases pass through the SML via slow molecular transport; hence there may be sufficient time for significant gas uptake and/or release by the bacterioneuston. Indeed, floating box experiments in the tropical Atlantic Ocean indicated methane (CH_4) and carbon monoxide (CO) processing by the SML (Conrad and Seiler, 1988) and laboratory mesocosm experiments showed that the presence of active CH_4-oxidizing bacteria in the SML significantly modified CH_4 fluxes, highlighting the potential significance of the bacterioneuston in air–sea gas exchange (Upstill-Goddard *et al.*, 2003).

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Active involvement of the bacterioneuston in the air–sea exchange of CH_4 and CO is potentially highly relevant because both strongly influence the Earth's climate and atmospheric chemistry. CH_4 accounts for ~15% of enhanced greenhouse forcing (IPCC, 2001) and participates in the formation of stratospheric H_2O and in photochemical reactions that regulate tropospheric concentrations of OH and O_3 (Crutzen, 1991). CO directly impacts global CH_4 via its competition for OH radicals, the primary tropospheric CH_4 sink (Crutzen, 1991; Monson and Holland, 2001).

The tropospheric CH_4 inventory is uncertain (Dlugokencky *et al.*, 1998, 2001). The marine (microbial) source of atmospheric CH_4 is believed to contribute about 2–3% of atmospheric CH_4 growth (Bange *et al.*, 1994; Lelieveld *et al.*, 1998). However, recent data from coastal waters, especially estuaries, suggest that this could be an underestimate (Upstill-Goddard *et al.*, 2000). Estuaries are also major contributors to the global marine source of tropospheric CO (SpringerYoung *et al.*, 1996) via the photolysis of dissolved organic matter (Zuo and Jones, 1995).

Methanotrophic *Bacteria* are ubiquitous in the biosphere and have been isolated from a range of ecosystems including soils (Whittenbury *et al.*, 1970), freshwater sediments (Auman *et al.*, 2000) and marine environments (Holmes *et al.*, 1995). Culture-independent studies of methanotroph diversity have relied upon probes for genes encoding subunits of particulate and soluble methane monooxygenase enzymes, *pmoA* and *mmoX*, respectively (Dumont and Murrell, 2005). PCR primers for *pmoA* and *mmoX* have been used to analyse methanotroph diversity in a range of habitats (Costello and Lidstrom, 1999; Horz *et al.*, 2002; Kolb *et al.*, 2003; Hutchens *et al.*, 2004), however, relatively few studies have considered estuarine environments (Sieburth *et al.*, 1993; McDonald *et al.*, 2005).

Microbial consumption may be the dominant marine CO sink (Xie *et al.*, 2005), yet very little is known about the diversity of bacteria involved in CO utilization (King, 2003; King and Weber, 2007). The *cox* genes encoding carbon monoxide dehydrogenase (CODH) are placed into two distinct groups; form I and form II (King, 2003). Studies of the functional diversity of CO-oxidizing *Bacteria* have relied on PCR probes for the carbon monoxide dehydrogenase gene (*coxL)* encoding the large subunit of the enzyme (King, 2003; Dunfield and King, 2004, 2005; Cleave, 2005).

For estuaries, which may dominate global air–sea exchange of both CH_4 (Upstill-Goddard *et al.*, 2000) and CO (Stubbins, 2001), an improved understanding of community structure both in the SML and subsurface is therefore critical. The main aims of this study were to compare microbial (*Bacteria* and *Archaea*) community structure in the SML with communities present in the subsurface of Blyth estuary, located on the North Sea coast of the United Kingdom, using a culture-independent molecular-based approach.

Previous studies have focused on functional aspects of bacterioneuston communities such as resistance traits (Mudryk, 2002; Agogue *et al.*, 2005a) and enzyme profiles (Mudryk and Skorczewski, 2000). These studies, however, relied upon culture-dependent approaches and therefore are limited by associated culture biases. We also made a preliminary comparison of the metabolic potential in the SML (bacterioneuston) and subsurface (bacterioplankton) by analysis of diversity of bacterial functional genes that are involved in the oxidation of two globally significant trace gases CH_4 and CO.

Materials and methods

Sample site and sampling

Blyth River is a tidal estuary that feeds into the North Sea and has a small port, Blyth Harbour. Samples were collected in December 2005 using the sampling protocol as previously described (Franklin *et al.*, 2005) from two sites; site 1 (grid reference, 55°08′14.47″N: 1°31′09.91″N; salinity, 21.2 psu ± 3.1) and site 2 (grid reference, 55°07′16.09″N: 1°29′44.12″N; salinity, 31.4 psu ± 0.5). Forty-seven millimetre diameter polycarbonate membranes (Millipore, Watford, UK) were placed onto the surface of the water for 10 s using sterile forceps (sample depth ≈40 μm). A total of six membranes were taken within a radius of approximately 10 m. These samples were pooled and stored on dry ice. The subsurface was sampled by placing a presterilized 1 l bottle 0.4 m below the surface and removing the lid. The subsurface water (1 l) was filtered through a 0.22 μm Sterivex filter (Millipore) and the filter stored on dry ice.

DNA extraction

DNA extraction from the membranes was carried out following established methods (Somerville *et al.*, 1989; Murray *et al.*, 1998). Reagent volumes for DNA extraction from the Sterivex filters are in parentheses: 6 ml (1.6 ml) of SET lysis buffer (0.75 M sucrose, 40 mM EDTA, 50 mM Tris-HCl pH 9) was added to each of the pooled membranes before 675 μl (180 μl) of fresh lysozyme (9 mg lysozyme, 9 μl 1 M Tris-HCl pH 8, 890 μl sterile H_2O) was added to each tube before incubation for 30 min at 37 °C with constant rotation. After incubation, 750 μl (200 μl) of sodium dodecyl sulfate (10% w/v) and 206 μl (55 μl) fresh proteinase K (20 mg proteinase K, 50 μl 1 M Tris-HCl pH 8, 950 μl sterile H_2O) were added followed by incubation for 2 h at 55 °C with constant rotation. The lysate was then removed and placed into a fresh tube before an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) was added. The tube was then mixed gently and centrifuged for 10 min at 6500 *g* before being repeated. DNA was

Table 1 PCR primer sets used in this study for analysis of microbial community structure and bacterial functional gene diversity

Target group/enzyme	*Gene*	*Primers*		T*m* (°C)	*Reference*
Bacteria DGGE	16S rRNA	341F	518R	65–55	Muyzer *et al.* (1993)
Bacteria Clone Library	16S rRNA	27F	1492R	60	Lane (1991)
Archaea (PCR 1)	16S rRNA	109F	958R	45	DeLong (1992); Jurgens *et al.* (1997)
Archaea (PCR 2)	16S rRNA	$SA1F^{GC}$ & $SA2F^{GC}$	PARCH519R	53.5	Ovreas *et al.* (1997); Nicol *et al.* (2003)
Particulate methane monooxygenase	*pmoA*	189F	661R	55	Costello and Lidstrom (1999)
Soluble methane monooxygenase	*mmoX*	206F	886R	61	Hutchens *et al.* (2004)
Carbon monoxide dehydrogenase	*coxL*	CODH793F	CDOH2090R	58	Cleave (2005)

Abbreviation: DGGE, denaturing gradient gel electrophoresis.

precipitated with ammonium acetate and ethanol overnight at −20 °C.

PCR and denaturing gradient gel electrophoresis
Following DNA extraction, PCR amplification of 16S rRNA and functional genes was performed using primer sets listed in Table 1. For all PCRs, a total volume of 50 μl was used containing 20 μM dNTPs, 50 pmol of each primer, 0.75 μl (3.75 U) Taq DNA polymerase (Fermentas, Burlington, Ontario, Canada), 1.5 mM $MgCl_2$, 5 μl 10 × PCR buffer (Fermentas) and ≈30 ng DNA. A standard PCR programme consisted of initial denaturation at 94 °C for 5 min followed by 35 cycles of 95 °C for 1 min, annealing temperature (*T*m) varied according to primer set used (Table 1), 72 °C 1 min and a final elongation step at 72 °C for 10 min. For denaturing gradient gel electrophoresis (DGGE) with archaeal 16S rRNA genes, a nested PCR approach was used by first using primers 109F and 958R, followed by PCR with primers $SA1F^{GC}$/$SA2F^{GC}$ and PARCH519R (Table 1). DGGE was performed with a Dcode gel system (Biorad, Hercules, CA, USA) with minor modifications of the method of Cunliffe and Kertesz (2006). Gels were prepared with acrylamide/bisacrylamide in 1 × Tris-acetate-EDTA buffer with denaturant to yield a linear gradient gel containing 30–70% denaturant, with a 0% stacking gel. The gel was run in 1 × Tris-acetate-EDTA buffer at 60 °C for a total of 1008 volt hours (constant voltage 63 V, 16 h). Gels were stained with SYBR Green nucleic acid stain (Invitrogen, Paisley, UK) before the image was captured on an FLA-5000 imaging system (Fuji Film). Bacterial and archaeal DGGE profiles were compared using GelCompareII (Applied Maths, Sint-Martens-Latem, Belgium). A similarity coefficient was calculated using a curve-based Pearson correlation before constructing an UPGMA dendrogram.

Two consecutive rounds of PCR were used for *pmoA* and *mmoX* genes. One microliter of PCR product from the first round was used as template DNA for a second round of PCR (2 × 35 cycles). PCR products were analysed by running 5 μl of PCR product on a 1.2% (w/v) agarose gel containing ethidium bromide.

Bacterial 16S rRNA gene and functional gene clone library construction and coverage estimation
PCR amplification products from bacterial 16S rRNA gene (PCR primer set 27F and 1492R), bacterial functional genes *mmoX* and *coxL* from site 2 were cloned using the TOPO TA cloning kit (Invitrogen) as per the manufacturer's instructions. Clone libraries were created from pooled replicate PCRs to avoid bias in library construction. Clones (24 clones per library) were screened using restriction fragment length polymorphism (RFLP) analysis of inserts digested with restriction endonucleases *Eco*RI/*Rsa*I. DNA fragments were resolved by electrophoresis through a 2% (w/v) agarose gel before each clone was assigned to an operational taxonomic unit (OTU). Evaluation of *mmoX* and *coxL* gene clone libraries was performed by repeated calculation of a nonparametric richness estimator, S_{Chao1}, along an increasing number of inserts sampled for RFLP. S_{Chao1} is calculated as:

$$S_{\mathrm{Chao1}} = S_{\mathrm{obs}} + \frac{F_1^2}{2(F_2+1)} - \frac{F_1 F_2}{2(F_2+1)^2}$$

where S_{obs} is the number of functional gene OTUs observed in the library, F_1 and F_2 are the numbers of OTUs occurring once or twice. This calculation creates an estimate of the probable total number of OTUs present in a given sample and therefore source community (Lee and Chao, 1994).When the estimator becomes asymptotic, the library is considered large enough to cover all OTUs present. Sequences (500–600 bp) of selected clones for 16S rRNA genes, *pmoA*, *mmoX* and *coxL* were obtained using the University of Warwick Central Molecular Biology Services Laboratory. Sequences from the NCBI database that were closely related to clone sequences and excised DGGE sequences in this study were identified by ARB (Ludwig *et al.*, 1998) and Basic Local Alignment Search Tool (Altschul *et al.*, 1990). Partial sequences of 16S rRNA were imported into an ARB database (Ludwig *et al.*, 1998) and pre-aligned against the closest relative and alignments were manually checked for consistency and valid secondary structure. The dendrogram was derived using maximum likelihood (Axml in ARB) based on alignment columns corresponding to *Escherichia coli* positions 166–625. Partial gene

sequences encoding the α subunit of soluble *mmoX* and the large subunit of *coxL* were imported into ARB databases of known orthologues, translated *in silico* and the protein sequences were aligned using CLUSTALX. Phylogenetic dendrograms of MmoX and CoxL were constructed using distance methods in ARB and the PAM Dayhoff matrix. For MmoX dendrogram calculation, the sequence of *Pseudomonas butanovora* soluble butane monooxygenase α subunit was used as a filter, the input mask corresponded to the amino-acid positions 93–236 of MmoX of *Methylococcus capsulatus* (Bath). For CoxL, the sequence of *Oligotropha carboxydohydrogena* CoxL (positions 295–459) was used as filter. Scale bars in dendrograms indicate an estimated 10% evolutionary divergence. Bootstrapping analyses (100 replicates) were carried out using programs from the PHYLIP package (Felsenstein, 1993), SEQBOOT, PROTDIST (for MmoX and CoxL) or DNADIST (for 16S rRNA sequences), NEIGHBOR and CONSENSE. Only values higher than 75% are shown in dendrograms. Values of 75–95% are indicated as a white circle, values above 95% are shown as a solid circle on the corresponding node.

The gene sequences retrieved in this study are available under the following accession numbers: bacterial 16S rRNA gene clone sequences, DGGE band sequences and archaeal 16S rRNA gene DGGE band sequences EU340035 to EU340075; *pmoA* gene sequences EU419955 to EU419957; *mmoX* gene sequences EU371925 to EU371932; and *coxL* sequences EU503130 to EU503138 in GenBank.

Results

Microbial community structure and diversity

In this study, we made a comparison of the microbial communities in the SML and subsurface at two sites along a small tidal estuary using DGGE. DNA was extracted and community composition determined by analysing bacterial and archaeal 16S rRNA gene PCR amplicons (Table 1). DGGE profiles revealed that microbial community composition remained relatively similar (similarity 88%) in subsurface waters at both sample sites (Figures 1a and b), even though salinity increased from 21 to 31 psu (a salinity of 1≡1 g sea salt in 1 kg of water) between the two sites. Many of the dominant 16S rRNA gene amplicons, present as intense bands in subsurface DGGE profiles, were also present in DGGE profiles of 16S rRNA genes from the SML. Here we infer that 16S rRNA gene amplicons, which are dominant DGGE bands, are also dominant taxa in the sample taken and therefore represent abundant taxa in the community. However, several amplicons detected in the DGGE profiles from the SML were not present in subsurface profiles, indicating SML-specific microbial populations (Figure 1a).

A total of four dominant bacterial 16S rRNA gene amplicons present only in the SML at site 2 were excised and sequenced (Figure 1a). The partial 16S rRNA gene sequences obtained revealed that these genes were similar to the sequences of 16S rRNA from known *Betaproteobacteria*, *Gammaproteobacteria* and *Bacteriodetes* retrieved from a range of marine and estuarine habitats (Table 2). A dominant amplicon present in the SML (BAC-DGGE-6) was also dominant in the subsurface and was closely affiliated (98% sequence identity) to known *Glaciecola* spp. (*Gammaproteobacteria, Alteromonadales*) (Tolli, 2003).

Restriction fragment length polymorphism analysis of bacterial 16S rRNA gene clone libraries (50 clones per library) from site 2 for both subsurface

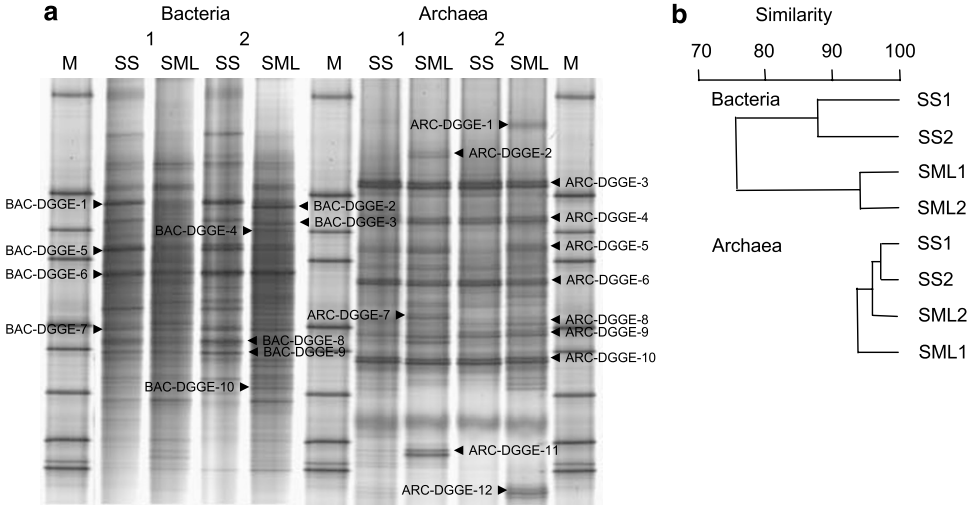

Figure 1 (**a**) Denaturing gradient gel electrophoresis (DGGE) profiles of archaeal and bacterial 16S rRNA gene PCR amplicons showing microbial community structure in the surface microlayer (SML) and subsurface (SS) of two sites (site 1, salinity 21 and site 2, salinity 31) along the Blyth estuary. M is a DGGE marker created from a bacterial 16S rRNA clone library. The highlighted DGGE bands are those which were excised and sequenced (Table 1). (**b**) Dendrogram showing similarity of DGGE profiles.

Table 2 Phylogenetic affiliations of bacterial (BAC) and archaeal (ARC) 16S rRNA gene sequences obtained from Blyth River estuary surface microlayer and subsurface DNA samples after DGGE (Figure 1)

	DGGE band	*Closest BLAST match (accession no.)*	*Identity (%)*	*Habitat*	*Taxon*	*Reference*
Surface microlayer	BAC-DGGE-2	Uncultured clone (AY053477)	99	Marine	*Betaproteobacteria*	Lanoil *et al.* (2001)
	BAC-DGGE-3	*Pseudomonas* sp. BWDY-44 (DQ314546)	98	Estuary	*Gammaproteobacteria*	Unpublished
	BAC-DGGE-4	Uncultured DGGE band (AJ583816)	96	Marine	*Bacteroidetes*	Unpublished
	BAC-DGGE-10	Uncultured clone (AY062127)	91	Wastewater	*Betaproteobacteria*	Zilles *et al.* (2002)
Ubiquitous phylotypes	BAC-DGGE-1	Uncultured clone (AY830016)	98	Marine	*Bacteroidetes*	Unpublished
	BAC-DGGE-5	DGGE band (*Alteromonas* sp.) (AF466898)	100	Marine	*Gammaproteobacteria*	Winter *et al.* (2001)
	BAC-DGGE-6	*Glaciecola* sp. WHOI JT-6b (AY349463)	98	Marine	*Gammaproteobacteria*	Tolli (2003)
	BAC-DGGE-7	*Ruegeria* sp. SC15 (DQ001317)	92	Marine	*Alphaproteobacteria*	Anand *et al.* (2006)
	BAC-DGGE-8	Uncultured clone (DQ450187)	95	Marine	*Alphaproteobacteria*	Piccini *et al.* (2006)
	BAC-DGGE-9	Uncultured clone (AY828401)	91	Marine	*Alphaproteobacteria*	Unpublished
Surface microlayer	ARC-DGGE-2	Uncultured clone (DQ424907)	98	Marine	*Crenarchaeota*	Unpublished
	ARC-DGGE-11	Uncultured clone (DQ129987)	93	Hypersaline Lake	Not known	Jiang *et al.* (2006)
	ARC-DGGE-7	Uncultured clone (DQ190066)	96	Freshwater aquifer	Not known	Unpublished
	ARC-DGGE-1	Uncultured clone (AY627472)	97	Marine	Not known	Unpublished
	ARC-DGGE-12	Uncultured clone (AY396004)	96	Estuarine sediment	Not known	Roling *et al.* (2004)
	ARC-DGGE-5	Uncultured clone (DQ190066)	96	Freshwater aquifer	Not known	Unpublished
Ubiquitous phylotypes	ARC-DGGE-3	Uncultured clone (AF180687)	98	Estuary	*Crenarchaeota*	Crump and Baross (2000)
	ARC-DGGE-4	Uncultured clone (AF355964)	97	Marine	*Crenarchaeota*	Huber *et al.* (2002)
	ARC-DGGE-6	Uncultured clone (U78195)	98	Marine	*Crenarchaeota*	Massana *et al.* (1997)
	ARC-DGGE-8	Uncultured clone (AF355942)	92	Marine	Not known	Huber *et al.* (2002)
	ARC-DGGE-9	Uncultured clone (AF223131)	90	Marine	*Euryarchaeota*	Massana *et al.* (2000)
	ARC-DGGE-10	Uncultured clone (AY627472)	96	Marine	Not known	Unpublished

Abbreviations: BLAST, Basic Local Alignment Search Tool; DGGE, denaturing gradient gel electrophoresis.

(bacterioplankton) and SML (bacterioneuston) samples produced 35 and 31 OTUs respectively (data not shown). Some OTUs were present in both the SML and subsurface, indicating, as with DGGE experiments, that many bacteria are found in both the SML and the subsurface samples. However, most OTUs in both libraries were unique to that library, indicating that SML-specific or subsurface-specific bacterial populations were sampled from that environment. The bacterial 16S rRNA sequences from the clone libraries were dispersed over five classes of the *Proteobacteria* and the *Bacteriodetes* (Figure 2).

As with the bacterial community DGGE profiling, profiles generated from archaeal partial 16S rRNA gene amplicons were relatively similar (similarity $\geqslant 93\%$) between sampling sites, suggesting that the subsurface (archaeaplankton) community structure changed little along the stretch of estuary sampled (~ 2 km). Dominant subsurface amplicons were also dominant in the SML, yet novel amplicons were also detected in the SML (Figure 1). The habitats, where

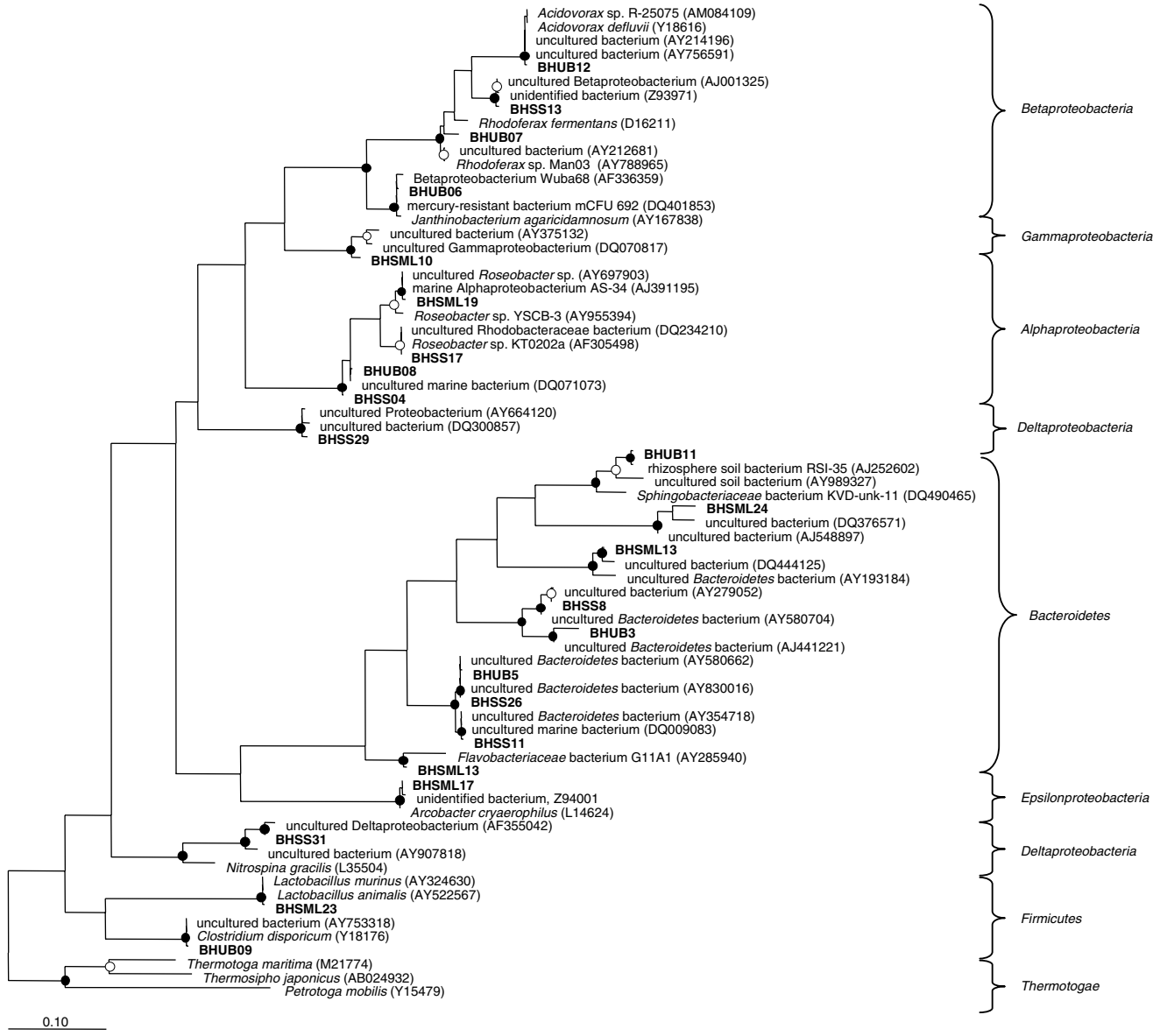

Figure 2 Phylogenetic analysis of bacterial 16S rRNA gene sequences obtained from the surface microlayer and subsurface waters (40 cm depth) of Blyth estuary and sequences available from public nucleotide databases. The tree was derived using a maximum likelihood analysis of 16S rRNA gene positions 166–626 (*Escherichia coli*) and the scale bar represents 10% sequence divergence. The clone designations are as follows: UB, clones found in both the surface microlayer (SML) and the subsurface libraries; SML, found only in the SML library; and SS, found only in subsurface library. Filled circles (●) at branching points indicate that bootstrap values obtained by neighbour-joining analysis that were >95%; open circles (○) indicate bootstrap values between 75% and 95%.

closely affiliated archaeal 16S rRNA gene sequences were found, ranged from hypersaline to freshwater environments (Table 2). Many of the dominant archaeal 16S rRNA gene DGGE bands found in the subsurface and SML, assumed here as dominant populations *in situ*, were affiliated with known marine *Crenarchaeota*, with only one DGGE band being affiliated with known marine *Euryarchaeota*. One of the dominant archaeal populations detected in the Blyth River estuary (ARC-DGGE-3) was closely affiliated (98% sequence similarity) to a clone detected in the Columbia River estuary, USA (Crump and Baross, 2000).

Functional gene diversity of trace gas-utilizing bacteria

Bacteria have a significant involvement in the biogeochemical cycling of gases, including CH_4 and CO. PCR primers targeting genes encoding subunits of *pmoA*, *mmoX* and *coxL* (Table 1) were used with template DNA extracted from the SML and subsurface of the two sampling sites on the Blyth estuary.

A PCR product for *pmoA* was only obtained with DNA from the subsurface at sampling site 2 after two rounds of PCR (total 70 cycles). PCR products for *mmoX* genes were detected in the SML of site 1 and in both the subsurface and SML at site 2. The need

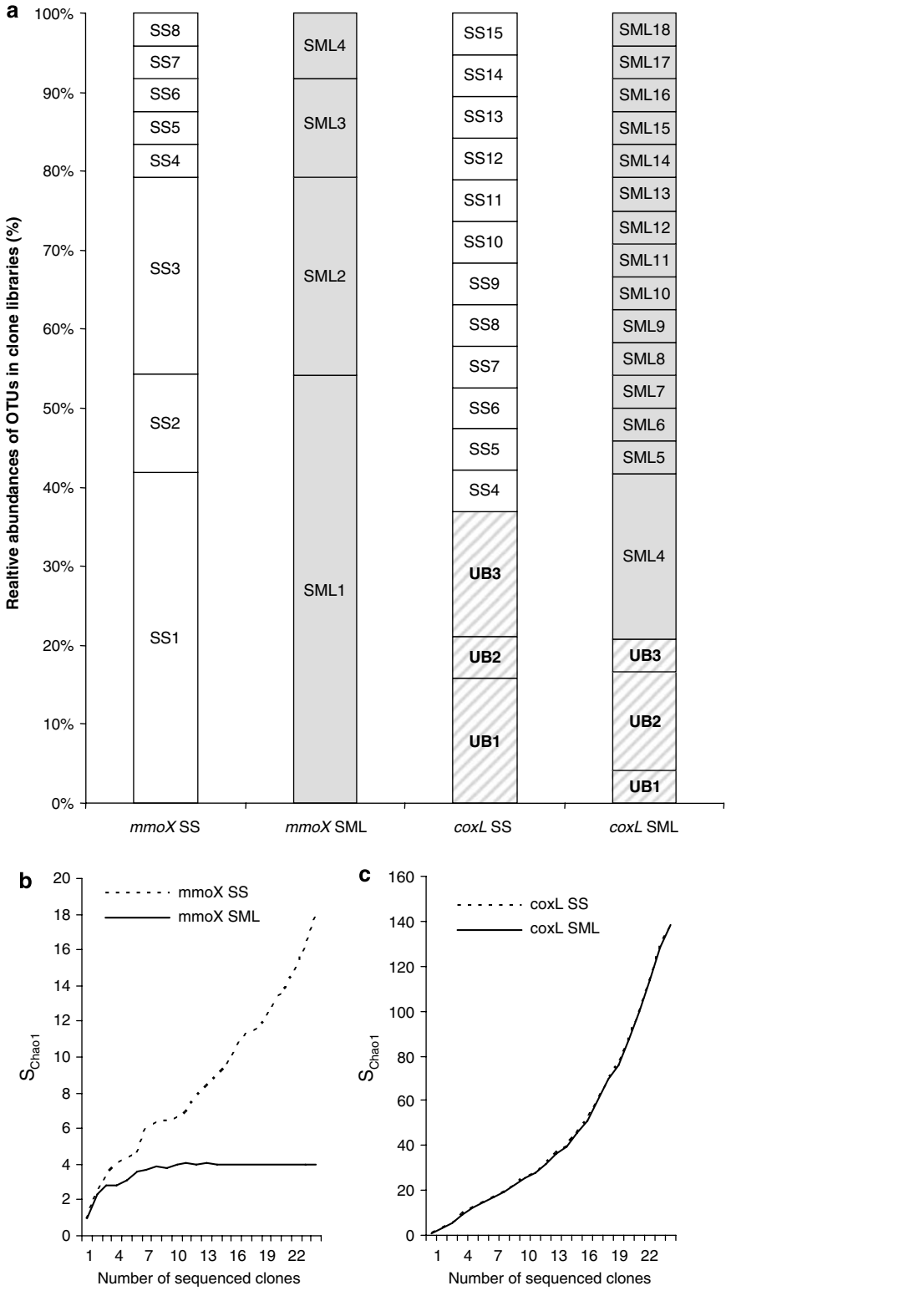

Figure 3 (**a**) Relative abundances of operational taxonomic units (OTUs) created from restriction fragment length polymorphism (RFLP) banding patterns of bacterial soluble methane monooxygenase (*mmoX*) and carbon monoxide dehydrogenase (*coxL*) subunit-encoding genes. Clone libraries created from DNA extracted from the subsurface (SS, white) and surface microlayer (SML, grey) at sampling site 2 on the Blyth estuary. Hatched segments show ubiquitous OTUs (UB) that were present in both SML and subsurface DNA samples. Selected clones from the RFLP analysis were sequenced and these sequences were compared to gene sequences from extant Bacteria (Figures 4 and 5). (**b** and **c**) Rarefaction curves for *mmoX* and *coxL* libraries calculated using the nonparametric richness estimator, S_{Chao1}.

for two rounds of PCR to detect *pmoA* suggests that there were low numbers of methanotrophs present in the Blyth SML. Conversely, *coxL* genes were detected in all samples after one round of PCR (35 cycles), indicating the ubiquity and higher abundance of CO utilizers in the Blyth River estuary.

We used the nonparametric richness estimator, S_{Chao1}, to determine coverage of both the *mmoX* and *coxL* gene clone libraries (Figures 3b and c). These data suggest that the total diversity of *mmoX* genes present in the SML is considerably less compared to the subsurface water as the rarefaction curve calculated for the SML is asymptotic. Diversity was higher in the subsurface due to the presence of several OTUs with only single representatives in the clone libraries. Rarefaction curves calculated for the *coxL* gene clone libraries indicates a very high diversity of this gene in both samples compared to the *mmoX* gene clone libraries. This is because in both libraries most OTUs were represented by single RFLPs.

mmoX clone libraries were made using PCR products from site 2 subsurface and SML samples. Results from RFLP analysis were markedly different for *mmoX* genes in the subsurface compared to the SML. All of the *mmoX* OTUs were specific for each environment (Figures 3 and 4), indicating that different soluble methane monooxygenase (sMMO)-containing methanotrophic communities were present in the SML compared to the corresponding subsurface samples. The *mmoX* sequence identification indicated the dominance of *Methylomonas*/*Methylococcus*-related methanotrophs in the samples taken, from both the subsurface water and SML (Figures 3 and 4).

The diversity of *pmoA* gene sequences retrieved from the subsurface water at site 2 was very low with only three OTUs being present. The clone library RFLP analysis revealed one major OTU (88% of clone library) with closest sequence similarity to a known *Methylobacter pmoA* gene sequence (AAF08210) (Costello and Lidstrom, 1999). The two remaining low-abundance OTUs, both with a relative abundance of 6%, were similar to *pmoA* clones from uncultivated bacteria collected from a river plain aquifer (AAZ06139) (Erwin *et al.*, 2005) and a sand water filter (ABC59829) (Stoecker *et al.*, 2006).

As with the bacterial 16S rRNA gene clone libraries described earlier, *coxL* clone libraries contained OTUs that were present in both the subsurface and SML DNA samples (Figure 3). The number of *coxL* gene OTUs was high relative to those of *mmoX* and *pmoA* genes and a large number of single representative OTUs were detected in the clone libraries that were detected only in either the SML or the subsurface DNA. The dominant *coxL* gene OTUs and some of the low-abundance *coxL* gene OTUs were sequenced and analysed. The *coxL* gene sequences from the SML and subsurface clustered into four main groups: *Silicibacter pomeroyi* type *coxL* form I gene sequences, *Bradyrhizobium* spp. type *coxL* form II gene sequences, *Burkholderia* spp. type *coxL* form II gene sequences and *Roseobacter* group type *coxL* form II gene sequences (Figure 5). As with the *mmoX* gene

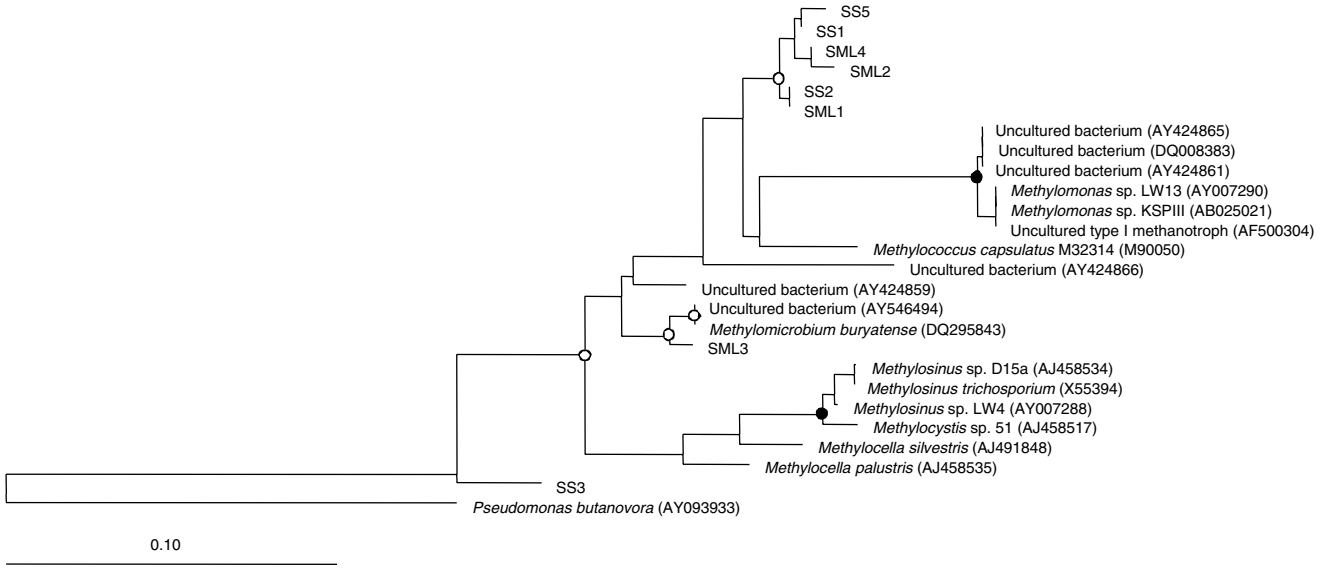

Figure 4 Phylogenetic analysis of the derived amino-acid sequences of *mmoX* genes that encode the active-site subunit of soluble methane monooxygenase, from the Blyth estuary surface microlayer and subsurface water. The dendrogram was derived as described in Materials and methods and rooted with BmoX (soluble butane monooxygenase) from *Pseudomonas butanovora* (Sluis *et al.*, 2002). The scale bar represents 10% sequence divergence. The relative abundances of Blyth estuary clones are given in Figure 3. Filled circles (●) at branching points indicate that bootstrap values obtained by neighbour-joining analysis that were >95%; open circles (○) indicate bootstrap values between 75% and 95%.

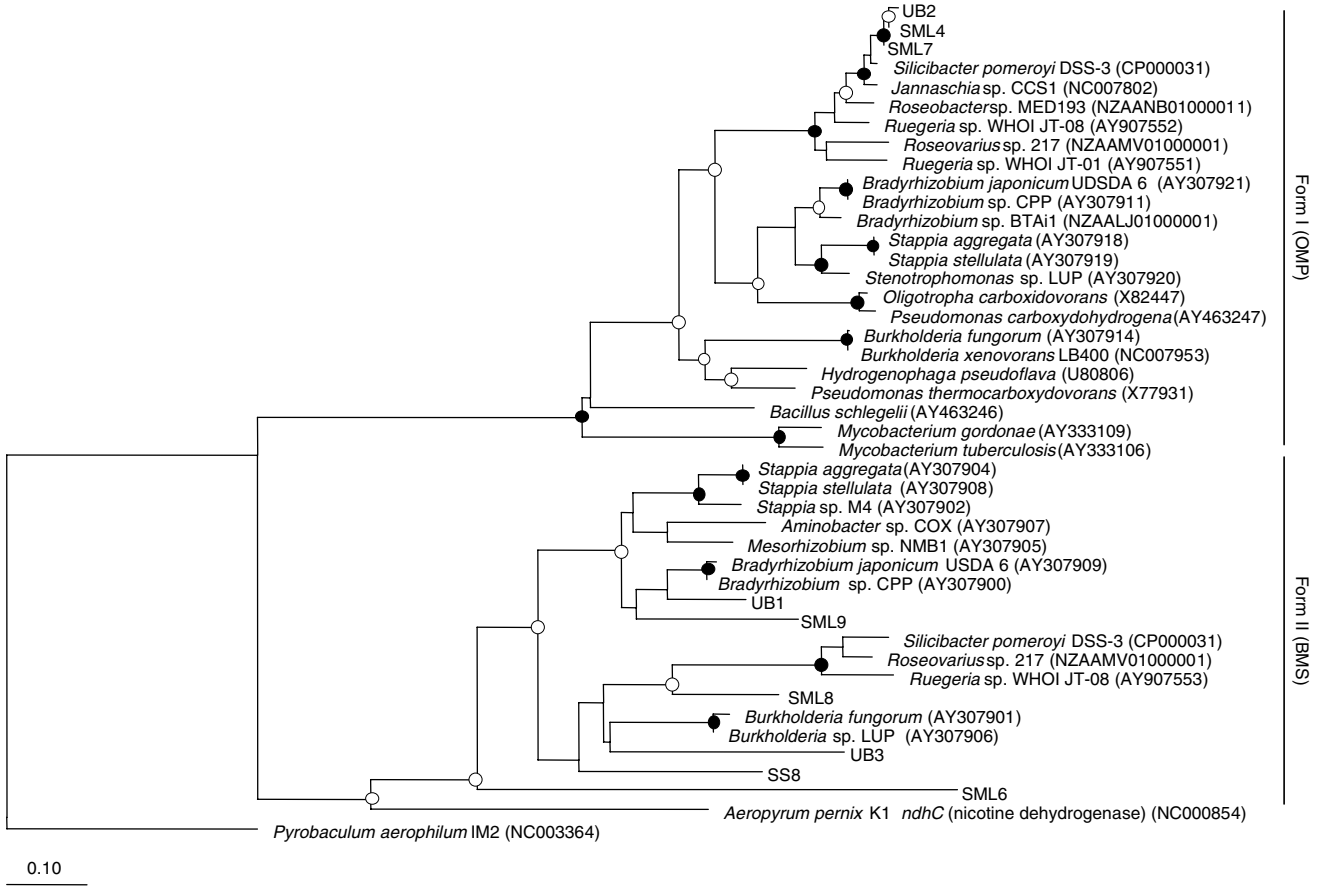

Figure 5 Phylogenetic analysis of the derived amino-acid sequences of *coxL* genes that encode a subunit of carbon monoxide dehydrogenase from the Blyth estuary surface microlayer and subsurface water. The dendrogram was derived as described in Materials and methods and was rooted with a carbon monoxide dehydrogenase from *Pyrobaculum aerophilum* (NC003364). The scale bar represents 10% sequence divergence. *coxL* genes are divided into two forms: form I (OMP represents derived *coxL* sequences from *Oligotropha*, *Mycobacterium*, *Pseudomonas*) and form II (BMS represents derived *coxL* sequences from *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*) (King, 2003). The relative abundances of Blyth estuary clones are given in Figure 3. Filled circles (●) at branching points indicate that bootstrap values obtained by neighbour-joining analysis that were >95%; open circles (○) indicate bootstrap values between 75% and 95%.

sequences, *coxL* gene sequences found in the SML were closely related to *coxL* gene sequences found in the corresponding subsurface samples. In contrast to the methanotrophic *Bacteria* discussed above, the Blyth estuary has a mix of both marine and freshwater/terrestrial sequences.

Discussion

Microbial community structure and diversity

To gain insight into microbial community structure in the SML and to compare this with subsurface water, we profiled bacterial and for the first time archaeal communities using DGGE. Further analysis of bacterial diversity was made by analysis of 16S rRNA gene clone libraries to make direct comparisons with previous work on the bacterioneuston of marine coastal waters (Franklin *et al*., 2005).

Estuaries are characterized by steep gradients in salinity and hence ionic strength, pH, temperature and chemical composition. Each of these variables potentially impacts on microbial community structure. In the Blyth River estuary, the difference in salinity between the two sample sites had no apparent effect on microbial community structure in the subsurface water.

Bacteria and *Archaea* DGGE band sequences and bacterial 16S rRNA gene clone library sequences were closely associated to other sequences collected from a range of non-estuarine habitats, indicating that the microbial community present in the Blyth estuary is predominantly allochthonous (Table 2). This has also been reported for other estuarine systems; archaeal populations detected in the Columbia River system, for example, were also largely allochthonous, being associated with both terrestrial and marine sequences (Crump and Baross, 2000).

The broad range of sampling devices available to study the SML collect different SML thicknesses and therefore influence the data collected (Agogue

et al., 2004). These include glass plates (20–100 μm) (Harvey and Burzell, 1972), rotating drums (60–100 μm) (Harvey, 1965) and mesh screens (Garrett screen) (150–400 μm) (Sieburth, 1965; Garrett, 1967). It is therefore difficult to make direct comparisons with other studies that used different sampling techniques. Franklin *et al.* (2005), who were the first to use polycarbonate membranes for such molecular biological studies, collected SML samples from a site in the North Sea approximately 10 km off the coast of Northumberland (UK), and hence in coastal waters close to the Blyth River estuary. The sampling strategy and subsequent sample processing used by Franklin *et al.* (2005) was almost identical to the approach used in this study (see Materials and methods). Direct comparisons can therefore be made with the bacterial 16S rRNA gene clone libraries generated by Franklin *et al.* (2005) and the bacterial 16S rRNA gene clone libraries generated in this study. The North Sea bacterioneuston was dominated by two groups, *Vibrio* spp. (68% of clones) and *Pseudoalteromonas* spp. (21% of clones). The estuarine bacterioneuston 16S rRNA gene clone library produced in this study was not dominated by any group, but instead was very diverse compared to those found in the North Sea SML (Franklin *et al.*, 2005). High diversity is common in estuarine bacterioplankton communities, with *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Bacteriodetes* being widespread taxa (Crump *et al.*, 1999, 2004; Kisand and Wikner, 2003). As this study was performed on an estuary in the winter and the Franklin *et al.* (2005) study collected samples from an area of coastal sea in the summer, the differences in results are possibly due to differences in these two types of marine system or seasonal effects. This clearly warrants future detailed investigation.

In the Blyth estuary, microbial community structure in the SML was not the same as in subsurface water, as determined here by DGGE. Specific populations, detected as either novel DGGE bands or novel bacterial 16S rRNA gene clone library OTUs, were found only in the SML at both sampling sites. Agogue *et al.* (2005a) sampled the SML at two coastal stations in the Mediterranean Sea using several different sampling techniques. Single-strand conformation polymorphism analysis of the bacterioneuston and the underlying bacterioplankton at one sampling station showed very similar patterns; however, some differences were noted in community structure at the other sampling site (Agogue *et al.*, 2005a).

16S rRNA gene sequence analysis of *Bacteria* and *Archaea* detected in the Blyth estuary showed closest homology to 16S rRNA gene sequences associated with a range of marine, estuarine and freshwater habitats. Similar results were reported for *Bacteria* cultivated from the Mediterranean Sea SML (Agogue *et al.*, 2005b), indicating that populations present in the SML are found in other environments and are not exclusively neustonic. However, as only two other studies have addressed bacterial diversity in the marine SML using modern molecular techniques (Franklin *et al.*, 2005; Agogue *et al.*, 2005b) and no other study has addressed *Archaea* diversity in the SML, the existence of SML-specific populations cannot be dismissed. Indeed, the freshwater bacterium *Nevskia ramosa* is found in the freshwater SML and has niche adaptations for the neustonic environment (Sturmeyer *et al.*, 1998; Pladdies *et al.*, 2004).

Interestingly, in our study, a *Crenarchaeota* population was detected with its closest known affiliate having previously been found in an estuary in the United States (Crump and Baross, 2000). Recent studies have suggested that mesophilic *Crenarchaeota* are involved in nitrification by performing ammonia oxidation particularly in estuarine environments (Francis *et al.*, 2005; Beman and Francis, 2006). A functional role for the *Crenarchaeota* was not determined in this study; however, since SML-specific populations were detected, future work will try to establish a functional role for populations of archaea in the SML.

Bacterial functional gene diversity

In this study, the *pmoA* and *mmoX* genes that were retrieved were most closely related to known freshwater methanotroph gene sequences. Several marine methanotrophs have been isolated that were identified as *Methylomonas pelagica* and *Methylobacter marinus* (Sieburth *et al.*, 1987, 1993; Fuse *et al.*, 1998) but none was detected in this study. Sieburth *et al.* (1993) used *M. pelagica*-targeted antisera to identify methanotrophs in CH_4-enriched estuarine (Chesapeake Bay) and ocean (Sargasso Sea) water. From the ocean enrichments, 96% of the isolates were identical to *M. pelagica*; however, none of the estuarine methanotrophs was identical to *M. pelagica* (Sieburth *et al.*, 1993). The estuarine *mmoX* sequences from this study were related to those retrieved from a freshwater lake (AF500304 and AY007290) (Auman *et al.*, 2000; Auman and Lidstrom, 2002) suggesting that sMMO-containing methanotroph recruitment into the estuary comes via freshwater inputs. The *mmoX* gene sequences from the Blyth River estuary form a distinct clade when compared to extant sequences, suggesting the presence of estuarine-specific methanotroph communities. Similar results have also been shown for mesotrophic freshwater lake sediment, with sMMO-containing *Methylomonas* type being the dominant methanotroph populations (Auman and Lidstrom, 2002). It is important to consider that there are very few data available concerning marine methanotroph diversity and gene sequences. Therefore, caution is required when interpreting data.

The potential presence of sMMO-containing methanotrophs in the Blyth estuary SML and

subsurface water may also have wider implications other than CH_4 oxidation, since the SML is known to be enriched with organic pollutants (Wurl and Obbard, 2004) and sMMO is effective at co-oxidizing halogenated organic pollutants such as trichloroethylene (Hanson and Hanson, 1996).

As with *mmoX*, the *pmoA* sequences obtained were most similar to *pmoA* clones retrieved from the freshwater environment (Costello and Lidstrom, 1999), further suggesting that estuarine methanotroph communities develop by colonization of freshwater methanotrophs. The diversity of *pmoA* genes recovered in this study was low, with only three OTUs being detected. Methanotroph diversity in estuarine sediments from Newport Bay in California, determined using *pmoA* as a marker, suggested that distinct estuarine-specific communities were present (McDonald *et al.*, 2005). We did not observe estuarine-specific *pmoA* sequences in the bacterioplankton of this study. The diversity of *pmoA* gene sequences in Newport Bay sediment was greater than the diversity shown here for the Blyth estuary. However, *Methylobacter pmoA* gene sequences were also found to be prevalent in Newport Bay sediment (McDonald *et al.*, 2005) as reported here for the Blyth estuary bacterioplankton.

There was a higher diversity of *coxL* sequences in both the SML and subsurface water. The use of *coxL* as a marker for CO-oxidizing *Bacteria* can however be problematic for two reasons: (1) sequences may be homologous to other genes for example, nicotine dehydrogenase; (2) many *coxL* genes present in GenBank are from genome sequences of bacteria that have not yet been proven to be CO utilizers (King, 2003; Cleave, 2005). Only three *coxL* OTUs were detected in both the SML and subsurface water. A high proportion of bacterioneuston *coxL* sequences detected were similar to *coxL* from *S. pomeroyi*, a known marine CO oxidizer (Moran *et al.*, 2004). *S. pomeroyi* is part of the *Roseobacter* lineage, a physiologically diverse group of *Alphaproteobacteria* that forms a numerically significant component of marine microbial communities (Buchan *et al.*, 2005; Wagner-Döbler and Biebl, 2006). The *Roseobacter* group has been shown to be responsible for a large amount of CO oxidation in coastal marine waters (Tolli *et al.*, 2006). Other *coxL* sequences in both the SML and subsurface were similar to those from freshwater and terrestrial *Bacteria* indicating that in the Blyth estuary, CO-oxidizing communities may arise from a range of habitats.

This study indicates that the estuarine SML is a different microbial ecosystem for some functional groups, for example, methanotrophs. Since only a limited number of SML sites have been studied in depth using molecular tools (Franklin *et al.*, 2005; Agogue *et al.*, 2005b), clearly, further work is needed to determine the importance of bacteria that metabolize atmospheric trace gases in this distinct environment. Further work is needed to address the importance of spatiality on microbial communities in the SML at a broad range of local, regional and global scales. Furthermore, since the SML may be strongly influenced by diurnal cycles, future work will need to address temporal dynamics of SML communities.

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