

# A Depth Resolved Insight into Benzoyl CoA Reductase and Benzoate Dioxygenase Gene Copy Numbers within a Marine Sediment Associated with Methane Seepage

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## ABSTRACT

A marine sediment core obtained from a methane seepage site off the northern coast of Ireland was analysed, at 3 depths, for catabolic genes associated with the aerobic and anaerobic degradation of aromatic compounds. Catabolic gene copy numbers varied through the core peaking at 2.1 meters below sediment surface (mbsf)-just above the sulphate boundary. Beyond the sulphate boundary gene copy numbers fell considerably, suggesting the boundary may be a critical factor in the degradation of aromatic compounds within marine sediments. At the 2.1 mbsf depth our data also suggest that known benzoyl CoA reductase utilizing bacteria are readily detectable.

**Keywords:** Aromatic Compounds; qPCR; *bcr*; *bdo*

## 1. Introduction

The importance of marine sediments, in terms of bioremediation, has grown significantly in recent times [1]. Various studies show that marine sediments, and the indigenous microorganisms therein, possess the potential to degrade a wide array of aromatic compounds [2]. Benzene, a classical, persistent aromatic compound [3], has been shown to be degraded in laboratory microcosms under anaerobic conditions where marine sediments were used as a source of microorganisms in the presence of nitrate or sulphate as an electron acceptor [3-5]. Both monocyclic and polycyclic aromatic compounds, have been shown to be degraded under a variety of conditions when marine sediment is used as a source of microorganisms in enrichment studies [2].

The discovery of catabolic gene sequences associated with the degradation of aromatic compounds from marine sediments via metagenomic research consolidates the fact that eubacteria associated with these sediments are capable of carrying out diverse aromatic compound degradation under both aerobic and anaerobic conditions [6]. This is of particular relevance as aromatic substrates

commonly occur in marine sediments, and are derived from both natural and anthropogenic sources. Hydrophobic aromatic compounds commonly migrate and exhibit recalcitrance in marine sediments [7].

However, despite the importance of microbes associated with the degradation of ubiquitous aromatic compounds, little is known about their actual distribution in marine sediments and subsequently their role in the degradation of these substrates.

In recent work we characterised a 6 metre marine sediment core from a methane seepage site [8] and found that chemistry and microbial activity in the core are influenced by the presence of methane and sulphate. In this study we wanted to investigate the quantities of benzoyl CoA reductase (*bcr*) and benzoate dioxygenase (*bdo*)-catabolic genes associated with the anaerobic and aerobic degradation of various aromatic compounds—at defined depths—as markers for microbial activity associated with aromatic degradation. We then wanted to consider the significance of these data in conjunction with the nature of the overall eubacterial population from within the methane seepage site. We wanted to see if heterotrophic aromatic substrate-degrading bacteria also played a potential role in the carbon cycle in these methane-rich sites.

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## 2. Methods

### 2.1. Sample Collection and Nucleic Acid Isolation

Sediment samples were collected, characterised and stored as described in our previous work [8]. Frozen sediment was taken at 0.2, 2.1 and 5.9-metre depths from a single core. Total DNA was extracted using a combination of heat and chemical lysis to disrupt microbial cells [9]. Total DNA was then subjected to purification with chloroform: isoamyl alcohol (Sigma). Finally DNA was precipitated with 0.6 volumes of isopropanol and eluted in 100  $\mu$ L of sterile TE buffer. Extracts of DNA were quantified spectrophotometrically as described in the literature [9] and stored at  $-20^{\circ}\text{C}$ .

### 2.2. 16S rRNA Gene Amplification

16S bacterial rRNA polymerase chain reactions were carried out using DNA Engine DYAD<sup>TM</sup> Peltier Thermal Cycler. 16S bacterial rRNA sequences were amplified from genomic DNA using universal 63f (5'-CAGGCCTAACACATGCAAGTC-3') forward primer and 1387r (5'-GGGCGWGTGTACAAGGC-3') reverse primer [10]. PCR was performed as follows: denaturation step of  $95^{\circ}\text{C}$  for 5 min; followed by 33 cycles of  $94^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s and finally  $72^{\circ}\text{C}$  for 1 min 30 s.

16S PCR products were visualised by UV transillumination (Biorad) and bands were removed and purified prior to cloning using a DNA purification kit (Fermentas).

### 2.3. Cloning and Sequence Analysis

Cloning of 16S rRNA genes was carried out as described previously [11] at depths of 2.1 m and 5.9 m as these depths were found on either side of the sulphate boundary [8]. Operational taxonomic units (OTUs) were grouped by restriction analysis using Fastdigest *RSaI* and *HaeIII* (Fermentas) restriction enzymes. Restriction profiles were analysed on 1.4% agarose gel and each gel was standardized to allow comparison between gels. Clones showing identical restriction profiles were assigned to the same OTU group using a total of 60 clones from 2.1 mbsf and 52 clones from 5.9 mbsf. The nucleotide sequence data reported in this study were deposited in the GenBank nucleotide sequence database under the accession numbers JQ349446 to JQ349503.

### 2.4. Phospholipid Fatty Acid (PLFA) Analysis

Lyophilized sediment samples were extracted using the modified Bligh-Dyer method, according to White & Ringelberg [12]. Total lipid extracts (TLEs) were desulphurised by mixing with activated Cu powder overnight. TLEs were fractionated into neutral, glycolipids and polar lipids using solid phase extraction on solvent-cleaned

aminopropyl cartridges (Alltech 500 mg Ultra-Clean, USA) according to Pinkart *et al.* [13]. Phospholipids were transesterified by mild alkaline methanolysis using 0.5 M sodium methoxide (Sigma Aldrich) for 30 min at  $50^{\circ}\text{C}$ . Fatty acid methyl esters (FAMES) were analysed using an Agilent 6890 gas chromatograph (GC) coupled to Agilent 5973 N quadrupole mass selective detector (MSD). The GC was fitted with a HP-5MS capillary column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m). Splitless injection mode was used while other GC and MSD operating conditions were as described in Otto *et al.* [14]. Unsaturation position on monounsaturated FAMES were identified according to Nichols *et al.* [15]. Quantification was performed using relative response factors for a model FAME (tetradecanoic acid methyl ester) to 5- $\alpha$ -cholestane internal standard (100  $\mu\text{g}\cdot\text{mL}^{-1}$ ).

### 2.5. qPCR

The abundance of catabolic genes was quantified by qPCR using primer pair *bdoF/R* [16] for the detection of the *bdo* gene or *BZAQF* [17] coupled to *PFR1* (5'-TCCTGMCCGCCSATGTCSAG'3) for the detection of the *bcr* gene. The thermal cycling program consisted of an initial hotstart at  $95^{\circ}\text{C}$  for 10 min, followed by 35 cycles of  $95^{\circ}\text{C}$  for 20 s,  $58^{\circ}\text{C}$  for 20 s and  $72^{\circ}\text{C}$  for 20 s. Plate reads were taken following each extension step at  $72^{\circ}\text{C}$ . Gel electrophoresis (Biorad) of products was performed to ensure specificity of product and in the construction of standards. At all other times melting curves were performed at the end of a qPCR run to confirm reaction specificity. All Quantitative PCR reactions were performed in an Opticon 3 real-time PCR machine (Biorad) using the Maxima SYBR Green MasterMix (Fermentas). Each 25  $\mu$ L reaction contained 1  $\mu$ L of template DNA and a final primer concentration of 0.35  $\mu\text{M}$  each.

Standard curves, for each target gene, were generated using purified PCR products from environmental samples [18]. Briefly, PCR products, specific to each target gene, from environmental samples were excised from an agarose gel and purified using an agarose gel PCR purification kit (Fermentas). DNA concentrations were then measured spectrophotometrically at 260 nm using a microcell cuvette (Hellma). Gene copy numbers were calculated according to the size of the amplicon [19]. The standard curves were linear over 5 orders of magnitude with an  $r^2$  value above 0.95. Amplification efficiency ranged from 91% to 105% with samples analyzed in triplicate. Samples of qPCR products were chosen at random, cloned, as described previously [11] and sequenced to confirm product identity.

## 3. Results

### 3.1. 16S rRNA Cloning

An overview of the composition of the 2.1 mbsf and 5.9

mbsf bacterial clones libraries is given in **Table 1**. Clone library analysis indicates that proteobacteria are the dominant phylum within this particular marine sediment, representing 93.4% and 98.1% of the bacterial population at 2.1 mbsf and 5.9 mbsf respectively. Also evident in **Table 1** is the presence of Sequences sharing homology with *Psychrobacter* spp and *Sulfitobacter* spp which are apparent at both the 2.1 m and 5.9 m depths—both known marine sediment inhabitants [20]. Some minor components of the community at 2.1 m but not 5.9 m were shown to be similar to facultative anaerobes previously shown to utilise the benzoate CoA reductase enzyme for aromatic hydrocarbon biodegradation. A notable example here is the OUT group 8—comprising 1.6% of the community at 2.1 mbsf.

### 3.2. Distribution of Catabolic Genes

For both catabolic genes tested it is evident that variations in copy numbers of the genes occur throughout the sediment core (**Figure 1**). The highest abundance of both *bcr* and *bdo* genes is noted at the 2.1 m depth of the sediment (**Figure 1**) with  $3.3 \times 10^5$  and  $1.8 \times 10^5$  copies

detected respectively. The lowest abundance of both catabolic genes is observed at the 5.9 m depth where  $5.2 \times 10^4$  and  $9.9 \times 10^4$  copies of *bcr* and *bdo* are detected. Also evident at the 2.1 m depth is an almost 2:1 abundance of *bcr* relative to *bdo*. In total 10 clones of each gene were sequenced, to confirm qPCR product identity, with sequences sharing homologies of 85% - 91% and 87% - 92% to known *bcr* and *bdo* genes held within the NCBI database respectively.

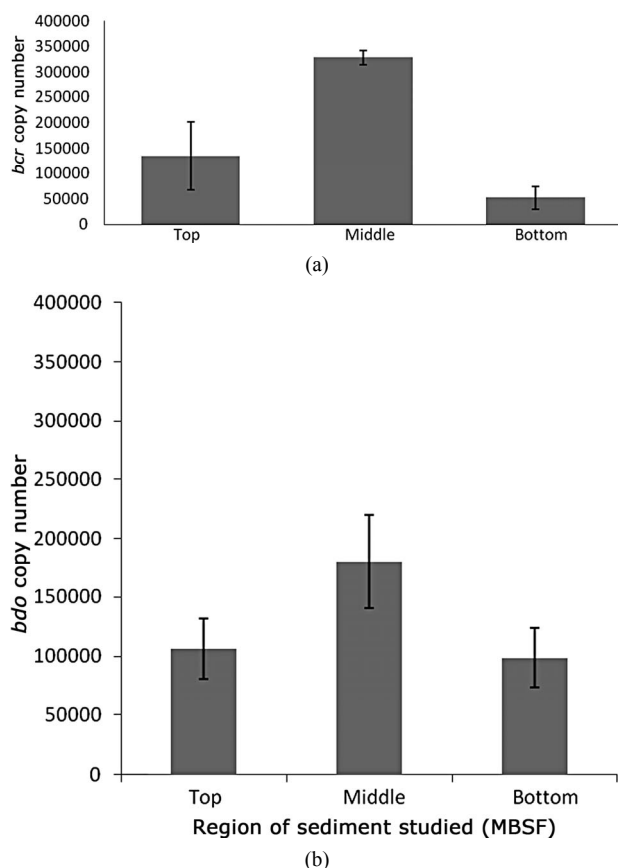
### 3.3. Phospholipid Fatty Acid (PLFA) Analysis

PLFA profiles were overall similar down-core, dominated by saturated fatty acids from C<sub>14</sub> to C<sub>24</sub> (31% to 80% of total PLFA), C<sub>16</sub> and C<sub>18</sub> monounsaturated fatty acids (10% to 15% of total PLFA) and methyl-branched fatty acids from C<sub>14</sub> to C<sub>16</sub> (2.5% to 7.5% of total PLFA.) and are likely derived from bacteria [12,21,22]. PLFA abundance has been used to estimate the number of viable cells in environmental samples, based on the estimation that the average bacterium the size of *E. Coli* contains approximately  $1.7 \times 10^{-17}$  mol PLFA per bacterial cell [23,24]. This corresponds to between 1.9 and  $4.7 \times 10^7$

**Table 1. Operational taxonomic unit (OTU) table of 16S rRNA bacterial clone 2.1 and 5.9 mbsf libraries from the pockmark.**

OTU	Accession Number	Clones Sequenced/Total	Closest Phylotype (Accession No.)		Match	OTU % in library
			2.1 mbsf library			
1	JQ349463	17/35	<i>Psychrobacter nivimaris</i> strain 88/2-7 (NR_028948.1)		≥97	59.0
2	JQ349486	4/11	<i>Sulfitobacter pontiacus</i> ChLG-10 (NR_026418.1)		≥97	18.0
3	JQ349489	1/4	<i>Alcanivorax borkumensis</i> SK2 (NR_029340.1)		98	6.7
4	JQ349490	1/3	Uncultured actinobacterium clone ANTXXIII_706-4_Bac69 (FN429805.1) <sup>2</sup>		98	4.9
5	JQ349494	1/2	<i>Pseudoalteromonas arctica</i> strain C53q-3a (JN681829.1)		98	3.3
6	JQ349491	1/1	Uncultured bacterium clone 3H3M_69 (JN230300.1) <sup>3</sup>		98	1.6
7	JQ349492	1/1	Uncultured bacterium clone Propane SIP20-4-09 (GU584779.1)		98	1.6
8	JQ349493	1/1	<i>Thauera</i> sp. R-28312 (AM084110.1)		96	1.6
9	JQ349496	1/1	<i>Pseudoalteromonas tetraodonis</i> strain IAM 14160 (NR_041787.1)		99	1.6
10	JQ349495	1/1	<i>Pseudomonas</i> sp. PM1 16S (EU768833.1)		99	1.6
OTU	Accession Number	Clones Sequenced/Total	5.9 mbsf library		Match	OTU % in library
1	JQ349448	15/21	<i>Psychrobacter marincola</i> strain KMM 277 (NR_025458.1) <sup>1</sup>		≥97	40.3
2	JQ349479	7/19	<i>Sulfitobacter litoralis</i> strain Iso 3 (NR_043547.1)		≥92	36.5
3	JQ349498	1/4	Uncultured <i>Pseudomonas</i> sp. C-51 (FJ900868.1)		99	7.7
4	JQ349503	1/2	Uncultured bacterium 16S rRNA gene clone D3DH031 (FQ660126.1)		95	3.8
5	JQ349497	1/2	<i>Pseudoalteromonas arctica</i> strain C53q-3a (JN681829.1)		98	3.8
6	JQ349500	1/1	Uncultured bacterium clone Propane SIP20-4-09 (GU584779.1)		99	1.9
7	JQ349499	1/1	Uncultured actinobacterium clone ANTXXIII_706-4_Bac69 (FN429805.1) <sup>2</sup>		98	1.9
8	JQ349501	1/1	<i>Variovorax paradoxus</i> EPS (CP002417.1)		99	1.9
9	JQ349502	1/1	<i>Cobwellia aestuarii</i> strain SMK-10 (NR_043509.1)		98	1.9

Note: <sup>1</sup>OTU group dominated by clones closest to *Psychrobacter marincola* but clones most related to *Psychrobacter celer* strain SW-238 (NR\_043225.1), *Psychrobacter pacifisensis* strain NIBH P2K6 (NR\_027187.1) are also present. <sup>2</sup>Closest cultured relatives are distantly related (~82% match) to sulphate reducing  $\delta$ -proteobacteria *S. palmitis*, *S. svalbardensis*. <sup>3</sup>Closest cultured relatives are distantly related (82% - 84% match) to the sulphate reducing  $\delta$ -proteobacteria *Dessulfobacca acetoxidans*, *Desulfobacterium anilini* and *Desulfacinum subterraneum*.



**Figure 1.** Mean catabolic gene copy numbers of *bcr* (a) and *bdo* (b)/0.5 g sediment were determined by qPCR on DNA isolated from different regions of the Malin core sample. Analysis was conducted on triplicate DNA extractions. Error bars represent standard deviation from the mean copy number.

cells·(g·dry·wt)<sup>-1</sup> at the sediment depths investigated.

#### 4. Discussion

Low growth rates, or intermittent periods of rapid growth interspersed by long periods of non-growth and starvation is the norm for microbes in the environment [25], with bacterial biomass concentration in marine sediments is usually in the region of 10<sup>8</sup> to 10<sup>10</sup> cells·g<sup>-1</sup> [26,27]. In this study the abundance of prokaryotic biomass appears to be at least an order of magnitude lower than typical at the depths investigated supplementing our previous work which suggested a reduction in microbial activity within the marine sediment examined [8].

The lowest abundance of PLFA's is noted at the 2.1 mbsf which, incidentally, is where the highest relative abundance of both catabolic genes is observed. This may imply that the viable bacterial population at this depth is lower than other depths examined but of those viable microorganisms, and indeed previous populations at this depth, a high proportion of eubacteria in this region of

the sediment may harbor the catabolic genes targeted in this study. However this remains a theory as mRNA studies would need to be performed to link viable eubacteria to expressed genes in this marine environment.

*Psychrobacter* spp and *Sulfitobacter* spp are apparent at both the 2.1 m and 5.9 metre depths (**Table 1**). Both genera have been associated with marine sediments in previous studies [20,28,29]. However as this study focuses on different depths within a marine sediment we observed that sequences from both genera tend to cluster according to the depth at which they are found (**Figure 2**) indicating subtle differences within relatively similar eubacterial populations potentially being influenced by the chemistry—such as the sulphate boundary—of the sediment region. Also noted in **Table 1** is the presence of an OTU group (group 8) whose 16S rRNA sequence shares homology with that of *Thauera* spp and *Azoarcus* spp—both facultative, *bcr* harbouring,  $\beta$  proteobacteria capable of aromatic compound degradation under both aerobic and anaerobic conditions [30]. Another OTU group of interest at the 2.1 m depth is group 6 which has highest known species similarity to a *desulfobacterium* spp—a known sulphate reducer [30] and aromatic compound degrader via the *bcr* pathway [31,32].

As stated the highest abundance of both *bcr* and *bdo* genes is noted at the 2.1 m depth of the sediment (**Figure 1**). At this depth aromatic compounds appear to be at the lowest abundance [8]. The aromatic compounds detected within the site are believed to consist of, amongst other groups, benzoate and naturally occurring phenol aromatic derivatives (e.g. vanillic acid). Also evident at the 2.1 m depth is an approximate relevant abundance of 2:1 for *bcr* relative to *bdo* suggesting that facultative anaerobes—such as *Thauera* spp.—may have been metabolic active this region of the sediment. This concept is further enhanced by the detection of sequences sharing homology with known facultative aromatic degraders through clone studies. Intriguingly at 5.9 mbsf more *bdo* copies are detected relative to *bcr*. This could be attributed to the presence of *bdo* harbouring *Psychrobacter* spp at this depth and the potential redundancy of the energy expensive *bcr* beyond the sulphate boundary [30].

Previous studies suggest that microbial populations in marine sediments are shaped in their bioremediation potential by an abundance of sulphate [33]. However this is the first study to show that the distribution of catabolic genes, associated with bioremediation of aromatic compounds, fluctuates through a marine sediment core within a methane seepage site, and varies in response to chemical factors, such as the proximity of a sulphate boundary, within the sediment (3). The highest abundance of catabolic genes associated with the aerobic and anaerobic degradation of aromatic compounds was found above the sulphate boundary at a depth of 2.1 mbsf suggesting that

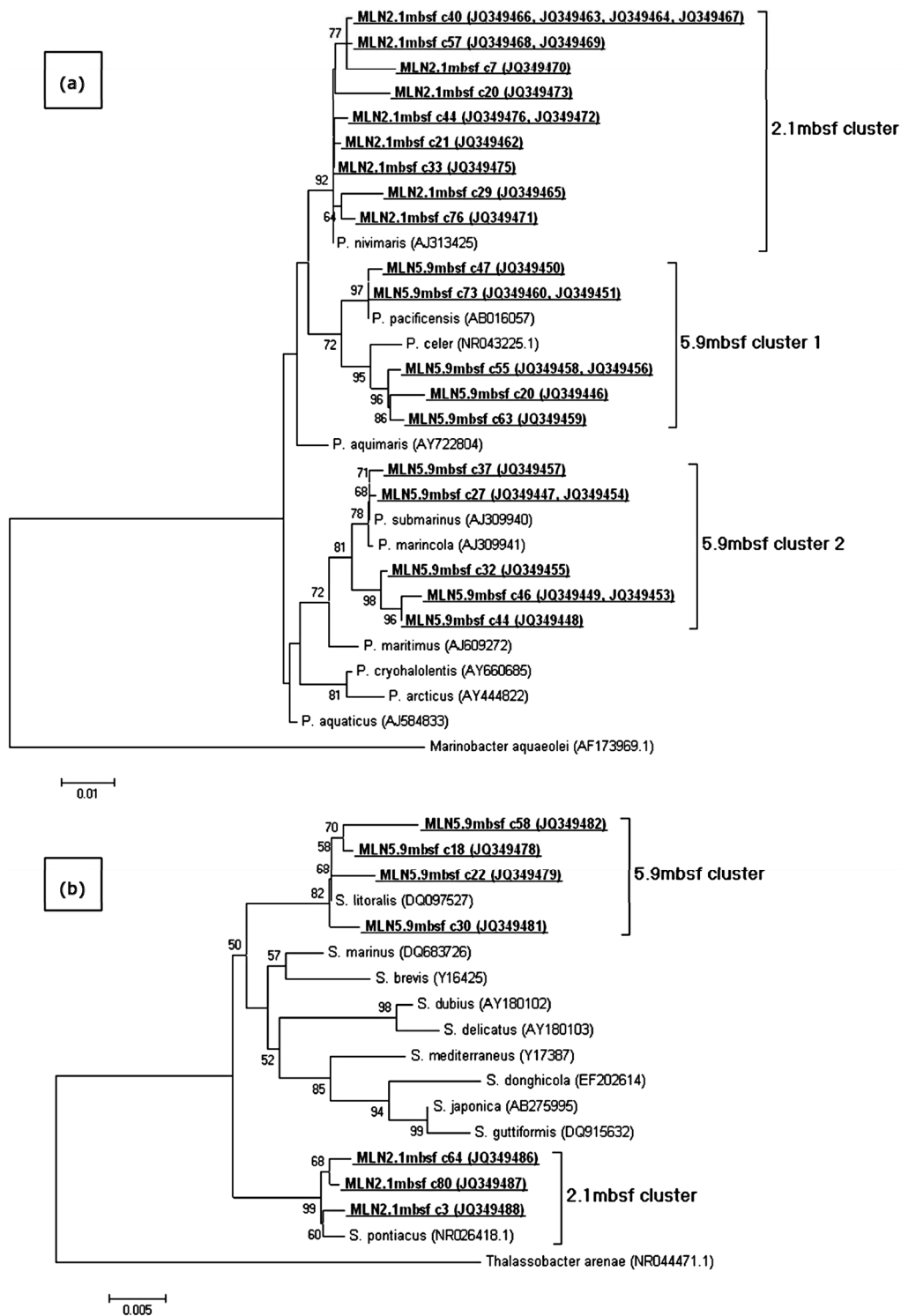


Figure 2. Maximum likelihood phylogenetic tree for selected clones from the *Psychrobacter* (a) and *Sulfitobacter* (b) OTU's from the 2.2 mbsf and 5.9 mbsf clone libraries. Nucleotide sequences from this study are in underlined bold. Accession numbers are in brackets. The scale bars represent the number of base substitutions per site. The tree was subjected to bootstrap analysis ( $n = 1000$ ) to assess confidence intervals. Bootstrap values  $> 50$  are reported. This shows distinct clustering of both bacterial populations at the two depths. At 2.1 mbsf *Psychrobacter* clones ( $n = 14$ ) are phylogenetically most closely related to *P. nivimaris*, while at 5.9 mbsf ( $n = 14$ ) are more diverse and cluster into groups related to *P. celer*, *P. marincola* and *P. submarinus* (Romanenko et al., 2002). *Sulfitobacter* clones at 2.1 mbsf ( $n = 3$ ) are phylogenetically most closely related to *S. pontiacus*, while at 5.9 mbsf ( $n = 6$ ) are most related to *S. littoralis*.

facultative microorganisms, possibly utilizing sulphate as an electron acceptor, play a vital role in the breakdown of these ubiquitous aromatic compounds within marine sediments.

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