RESEARCH LETTER

Accelerated methanogenesis from aliphatic and aromatic hydrocarbons under iron- and sulfate-reducing conditions

Michael Siegert[1], Danuta Cichocka[2], Steffi Herrmann[2], Friederike Gründger[1], Stefan Feisthauer[3], Hans-Hermann Richnow[3], Dirk Springael[2] & Martin Krüger[1]

[1]Bundesanstalt für Geowissenschaften und Rohstoffe, Hannover, Germany; [2]Katholieke Universiteit Leuven, Afdeling Bodem- en Waterbeheer, Heverlee, Belgium; and [3]Helmholtzzentrum für Umweltforschung – UFZ, Leipzig, Germany

Correspondence: Michael Siegert, Bundesanstalt für Geowissenschaften und Rohstoffe, Stilleweg 2, 30655 Hannover, Germany. Tel.: +49 511 643 3172; fax: +49 511 643 2304; e-mail: michael@siegert.org

Received 5 July 2010; revised 28 October 2010; accepted 11 November 2010.
Final version published online 6 December 2010.

DOI:10.1111/j.1574-6968.2010.02165.x

Editor: Elizabeth Baggs

Keywords
methanogenic hydrocarbon degradation; microbially enhanced oil recovery; metal reduction; bioremediation; *Geobacter*; anaerobic oxidation of methane.

Abstract

The impact of four electron acceptors on hydrocarbon-induced methanogenesis was studied. Methanogenesis from residual hydrocarbons may enhance the exploitation of oil reservoirs and may improve bioremediation. The conditions to drive the rate-limiting first hydrocarbon-oxidizing steps for the conversion of hydrocarbons into methanogenic substrates are crucial. Thus, the electron acceptors ferrihydrite, manganese dioxide, nitrate or sulfate were added to sediment microcosms acquired from two brackish water locations. Hexadecane, ethylbenzene or 1-^{13}C-naphthalene were used as model hydrocarbons. Methane was released most rapidly from incubations amended with ferrihydrite and hexadecane. Ferrihydrite enhanced only hexadecane-dependent methanogensis. The rates of methanogenesis were negatively affected by sulfate and nitrate at concentrations of more than 5 and 1 mM, respectively. Metal-reducing *Geobacteraceae* and potential sulfate reducers as well as *Methanosarcina* were present *in situ* and *in vitro*. Ferrihydrite addition triggered the growth of *Methanosarcina*-related methanogens. Additionally, methane was removed concomitantly by anaerobic methanotrophy. ANME-1 and -2 methyl coenzyme M reductase genes were detected, indicating anaerobic methanotrophy as an accompanying process [Correction added 16 December after online publication: 'methyl coenzyme A' changed to 'methyl coenzyme M' in this sentence]. The experiments presented here demonstrate the feasibility of enhancing methanogenic alkane degradation by ferrihydrite or sulfate addition in different geological settings.

Introduction

Roughly, one third of oil in reservoirs remains inaccessible (US Department of Energy, 2006). Since Zengler *et al.* (1999) reported the conversion of hexadecane to methane, it has been suggested that remaining energy can be recovered as methane gas (Anderson & Lovley, 2000; Head *et al.*, 2003). Moreover, the conversion of hydrocarbons to carbon dioxide (CO_2) or methane represents a useful tool for bioremediation of oil-impacted ecosystems. The overall reaction kinetics of hydrocarbon biodegradation are controlled by the initial attack on hydrocarbons, where hydrocarbon biodegradation with oxygen as an electron acceptor is the energetically most favorable process. However, microbial methanogenesis usually requires anoxic conditions and methanogenesis, including the conversion of hexadecane to methane, is a slow process (Zengler *et al.*, 1999; Feisthauer *et al.*, 2010).

The initial anaerobic activation of hexadecane may be irreversible and the removal of reaction products is unlikely to accelerate the initial steps or the overall degradation (Cravo-Laureau *et al.*, 2005; Callaghan *et al.*, 2006). However, β-oxidation and the release of electrons are essential steps in hydrocarbon biodegradation pathways (Fig. 1; Kniemeyer *et al.*, 2003; Rabus, 2005; Callaghan *et al.*, 2006). It is commonly accepted that the removal of reducing power from the reaction system drives β-oxidation. Examples for this are fermentative hydrogen (H_2)-releasing microorganisms, which require a low H_2 partial pressure to effectively unload electrons from the system. One can deduce that electron acceptors are required to accelerate the oxidation of hydrocarbons and their intermediate reaction products to

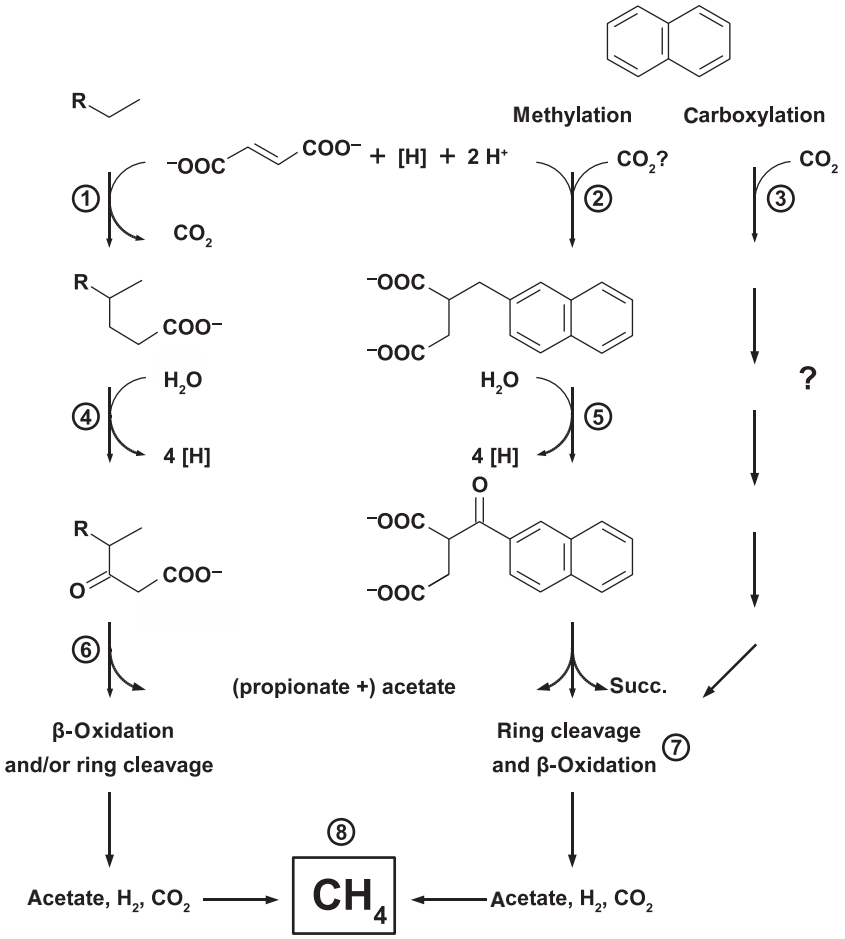

Fig. 1. Conceptual figure depicting the proposed pathways of hydrocarbon degradation. A removal of electrons [H] by adding electron acceptors such as Fe(III), Mn(IV), nitrate or sulfate may accelerate the overall reactions to yield substrates for methanogens. This may accelerate all β-oxidation reactions, for example at numbers 4–7. R may be an aliphatic or an aromatic residue. Note that besides fumarate addition, hydroxylation was shown for R = phenyl (Kniemeyer & Heider, 2001). 1, Fumarate addition to the hydrocarbon [e.g. hexadecane (Cravo-Laureau *et al.*, 2005; Callaghan *et al.*, 2006) or ethylbenzene (Kniemeyer *et al.*, 2003)]. 2, fumarate addition to methylnaphthalene after methylation (Annweiler *et al.*, 2000; Safinowski & Meckenstock, 2005). This may possibly be achieved by CO_2 reduction/acylation in a reversed carbon monoxide dehydrogenase pathway (Safinowski & Meckenstock, 2005). Intermediate succinate adducts and carbon skeleton rearrangements (Callaghan *et al.*, 2006) are not shown because they may be indirectly driven by electron acceptor addition. 3, Carboxylation and further ring reduction (Zhang & Young, 1997). 4 and 5, Proposed β-oxidation yielding four electrons (Annweiler *et al.*, 2000; Callaghan *et al.*, 2006). 6, β-Oxidation yielding acetate. Propionate would only be released when R = aliphatic. 7, Ring cleavage would precede further β-oxidation to yield acetate and CO_2 analogous to a proposed ring cleavage of toluene (Boll & Fuchs, 1995). Steps 4–7 and all subsequent β-oxidations may be accelerated by electron acceptor addition. 8, The substrates acetate and CO_2/H_2 are finally converted to methane by methanogenic *Archaea*. Question marks indicate debated mechanisms. Succ., succinate.

transform them into substrates for methanogens, for example acetate, CO_2 and H_2 (Fig. 1; Zhang *et al.*, 2010). For activation and processing of biological hydrocarbon degradation, the presence of oxidants is not necessary (Zengler *et al.*, 1999). However, it is plausible to indirectly stimulate the activity of the methanogenic community by providing oxidants other than oxygen to hydrocarbon-degrading microorganisms (Zengler *et al.*, 1999; Zhang *et al.*, 2010).

Sulfate reduction is well described in oil spills and oil field souring, where the latter can result in substantial economic losses (Sunde & Torsvik, 2005). Research on trivalent iron reduction by hydrocarbon oxidation emerged during the last 20 years (Lovley, 2000; Rabus, 2005; Kunapuli *et al.*, 2007), but was not studied in detail in conjunction with hydrocarbon-induced methanogenesis. Hydrocarbon-associated manganese reduction has only been described in few reports so far (Greene *et al.*, 1997, 2009; Langenhoff *et al.*, 1997a, b). Alkane biodegradation to methane is well documented and some reports for methanogenesis from aromatics and polyaromatics are available (Grbić-Galić & Vogel, 1987; Kazumi *et al.*, 1997; Zengler *et al.*, 1999; Townsend *et al.*, 2003; Chang *et al.*, 2006; Jones *et al.*, 2008;

Feisthauer *et al.*, 2010; Herrmann *et al.*, 2010). However, detailed research on the impact of electron acceptors on hydrocarbon-dependent methanogenesis remains elusive. Our central hypothesis is that electron acceptors can accelerate hydrocarbon-dependent methanogenesis. Thus, we tested their stimulating effect on the rates of hydrocarbon-dependent methanogenesis in different sediments.

Materials and methods

Site descriptions and sampling

Sediment samples were obtained from two different sites. One sampling site was contaminated by hydrocarbons (Zeebrugge) and the other site was pristine (Eckernförde Bay, Supporting Information, Appendix S1).

The sea port of Zeebrugge (Belgium; NW: 51°19′59N 3°11′57E, SE: 51°19′55N 3°12′12E, approximately 0.1 km^2) comprised several sediment sections with anoxic conditions and was contaminated with hydrocarbons and heavy metals (Ministerie van de Vlaamse Gemeenschap, 2002). The water depth was 3 m during ebb. A constant freshwater influx was maintained by the irrigation system of Brugge. In September 2008, samples were obtained from three locations within the harbor basin using a manual sediment grabber. Sample bottles were filled completely and closed using butyl rubber stoppers and screw caps. Surface water samples were also collected.

Chemical analyses were performed by SGS, Mol, Belgium. Typical contaminants in the harbor mud originated from protective boat paints and fuel leakages. Besides metals such as nickel, zinc, lead, copper, mercury and chromium, the concentrations of mineral oil ranged from 5 to 400 $\mu g\,cm^{-3}$ sediment. Iron, manganese and sulfate were detected in concentrations of up to 85, 0.1 or 2 $\mu mol\,cm^{-3}$, respectively. The pH was between 8.0 and 8.5 and the *in situ* water temperature was 14 °C.

Preparation of microcosms

For incubations established from the Zeebrugge samples, filter-sterilized harbor water (using 0.2-μm membrane filters) served as a medium to mimic *in situ* conditions. However, the harbor water naturally contained 2 mM sulfate and sediment microcosms without electron acceptors were therefore impossible to prepare. Basal salts were not added. Dissolved oxygen was removed by nitrogen gassing of 1 L filtered water. All additional manipulations were performed in an anaerobic glove box. To homogenize the sediment sample, a 1/1 mix of sediment and medium was stirred. The slurry was sampled for DNA extraction and 20 mL was used to inoculate 40 mL medium in 120-mL serum bottles. These were sealed with butyl rubber stoppers and aluminum crimp caps. Triplicate microcosms were incubated under a nitrogen headspace at atmospheric pressure at 25 °C.

Before inoculation, 2.5 mM ferrihydrite, 1.25 mM manganese dioxide, 1 mM potassium nitrate or 20 mM sodium sulfate was added to the medium. Ferrihydrite was precipitated by neutralization of an $FeCl_3$ solution (Lovley & Phillips, 1986) and manganese dioxide was obtained by oxidation of an $MnCl_2$ solution with $KMnO_4$ (Lovley & Phillips, 1988). To determine indigenous methanogenesis, controls without additional hydrocarbons and electron acceptors were prepared. Controls without hydrocarbons, but with electron acceptors were set up as single incubations.

The final hexadecane or ethylbenzene concentrations were 0.1% v/v in 60 mL total liquid volume. To test polyaromatic hydrocarbon (PAH) degradation, 1.6 mg 1-^{13}C-naphthalene or ^{12}C-naphthalene was added to 100 mL medium containing 20 mL sediment in 120-mL serum bottles sealed with butyl rubber stoppers and aluminum crimp caps. Manganese dioxide was not used in the case of naphthalene. To examine the activity of anaerobic methanotrophs, the headspace of separate microcosms was flushed with a 1/1 methane–nitrogen mix without additional higher hydrocarbons.

Methane and CO_2 measurements

Methane and CO_2 in headspace samples were analyzed using a GC–FID (+nickel catalyst methanizer, SRI 8610C, SRI Instruments) equipped with a 6-foot Hayesep D column (SRI Instruments) running continuously at 60 °C. Methane and CO_2 formation from ^{12}C- and 1-^{13}C-naphthalene was also measured using a Thermo Fisher MAT252 GC–IRMS (Herrmann *et al.*, 2010). The rates were calculated based on the formation of $^{13}CH_4$ measured in the headspace and subtracted from the $\delta^{13}C_{CH_4}$ of indigenously produced methane. δ^{13}C values are expressed as ‰ vs. Vienna Pee Dee Belemnite (VPDB)

The rates in unamended control experiments, hexadecane, ethylbenzene and methane incubations were calculated for a timeframe of 178 days with an intermediate measurement at day 155. For naphthalene incubations, the rates were calculated in a timeframe of 435 days without an intermediate measurement.

DNA analytical methods

Sediment DNA was extracted using a FastDNA Spin Kit for Soil DNA extraction kit (MP Biomedicals). Genes of interest were quantified using an Applied Biosystems StepOne thermocycler. 16S rRNA gene copy numbers of *Archaea* and *Bacteria* were determined as described previously (Takai & Horikoshi, 2000; Nadkarni *et al.*, 2002). The concentrations of *mcrA* and *dsrA* genes were investigated according to

Nunoura *et al.* (2006) and Schippers & Nerretin (2006), respectively. Members of the *Geobacteraceae* were quantified using the method described by Holmes *et al.* (2002). Copy numbers are expressed as copies cm^{-3} sediment.

Members of the microbial community in the Zeebrugge sediment were identified by the incorporation of 16S rRNA gene sequence fragments of a clone library into an existing maximum-parsimony tree (version 102) provided by Pruesse *et al.* (2007). Fragments of 16S rRNA genes were obtained using the modified primer sets Ar109f (5′-ACKGCTCAGTAACACGT) and Ar912r (5′-CTCCCCC GCCAATTCCTTTA) for *Archaea* and 27f (5′-AGAGTTTG ATCCTGGCTCAG) and 907r (5′-CCATCAATTCCTTT RAGTTT) for *Bacteria* (Liesack & Dunfield, 2004). Subsequently, cloning was performed using the pGEM-T vector system according to the manufacturer's instructions (Promega). All sequencing was conducted at Seqlab Göttingen (Germany). Sequences were deposited at the GenBank online database under accession numbers HM598465–HM598629.

Results

Aliphatic hydrocarbon-dependent methanogenesis and CO_2 release

Methanogenesis was observed in all Zeebrugge microcosms after 178 days. Without added hydrocarbons, the methanogenesis rates were 2.9, 0.8, 0.6, 0.3 or 0.8 nmol methane $cm^{-3}\,day^{-1}$ for ferrihydrite, manganese dioxide, nitrate, 2 or 22 mM sulfate-amended microcosms, respectively. The respective CO_2 release rates in these controls ranged from 35.5 nmol CO_2 $cm^{-3}\,day^{-1}$ for ferrihydrite to 73.8 nmol CO_2 $cm^{-3}\,day^{-1}$ for nitrate.

In microcosms containing Zeebrugge sediment with hexadecane, a significant increase of methanogenesis was observed compared with control experiments without hexadecane (Fig. 2a). Moreover, hexadecane-dependent methanogenesis rates were significantly different between microcosms with and without an added electron acceptor (Fig. 2a). Most prominently, ferrihydrite accelerated hexadecane-dependent methanogenesis to 87.3 ± 2.3 nmol methane $cm^{-3}\,day^{-1}$ compared with 37.8 ± 6.6 nmol methane $cm^{-3}\,day^{-1}$ in 2 mM sulfate incubations (natural harbor water). The increase of methanogenesis in manganese dioxide incubations to 45.9 ± 1.9 nmol methane $cm^{-3}\,day^{-1}$ was insignificant compared with 2 mM sulfate incubations (Fig. 2a). Adding 20 mM sulfate decreased methanogenesis to 2.1 ± 1.1 nmol methane $cm^{-3}\,day^{-1}$. Nitrate inhibited methanogenesis completely. However, the addition of hexadecane triggered CO_2 release from the microcosms (Fig. 2a). The CO_2 release rates ranged from 64.6 ± 5.8 nmol CO_2 $cm^{-3}\,day^{-1}$ for 2 mM sulfate to 139.6 ± 3.0 nmol CO_2 $cm^{-3}\,day^{-1}$ for 22 mM sulfate.

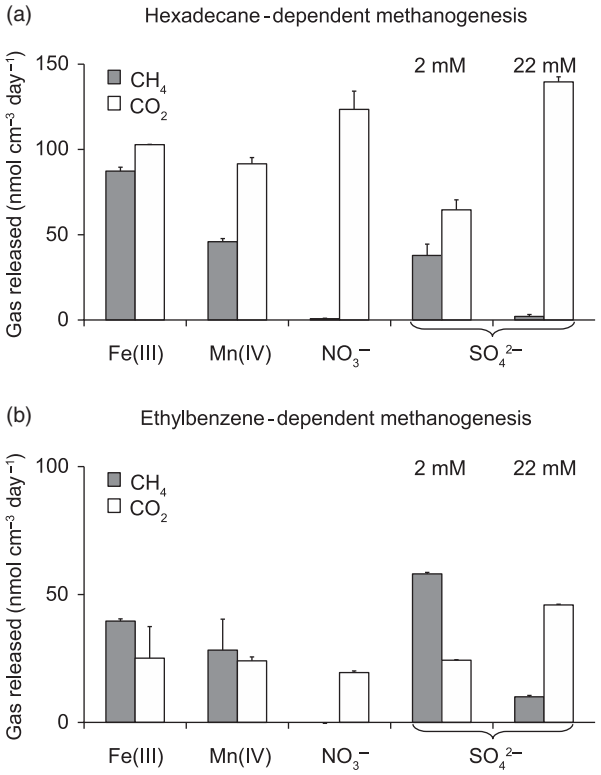

Fig. 2. Effect of the type of electron acceptor on hydrocarbon-dependent methanogenesis in Zeebrugge sediments. Hexadecane (a) and ethylbenzene (b) were used as substrates. Ninety-five percent confidence intervals of the triplicate regression slopes against time were calculated. SEs within this confidence limit are shown.

The addition of 1 mM nitrate or 10 mM sulfate almost completely inhibited methanogenesis in Eckernförde Bay microcosms (Fig. 3a). Hexadecane-dependent methanogenesis (46.5 ± 3.5 nmol methane $cm^{-3}\,day^{-1}$) was higher than naturally occurring methanogenesis without hexadecane of no more than 10 nmol methane $cm^{-3}\,day^{-1}$ in the sediment layer of the highest methanogenesis (Fig. 3a; Treude *et al.*, 2005). While hexadecane-dependent methanogenesis occurred without additional electron acceptors at a rate of 24.5 ± 1.7 nmol methane $cm^{-3}\,day^{-1}$, the process was significantly slower than that in incubations with 2 mM sulfate concentrations 46.5 ± 3.5 nmol methane $cm^{-3}\,day^{-1}$ (Fig. 3b).

Aromatic hydrocarbon-dependent methanogenesis and CO_2 release

Also, the addition of ethylbenzene significantly increased methanogenesis in microcosms containing Zeebrugge sediment (Fig. 2b). Compared with 2 mM sulfate, the addition

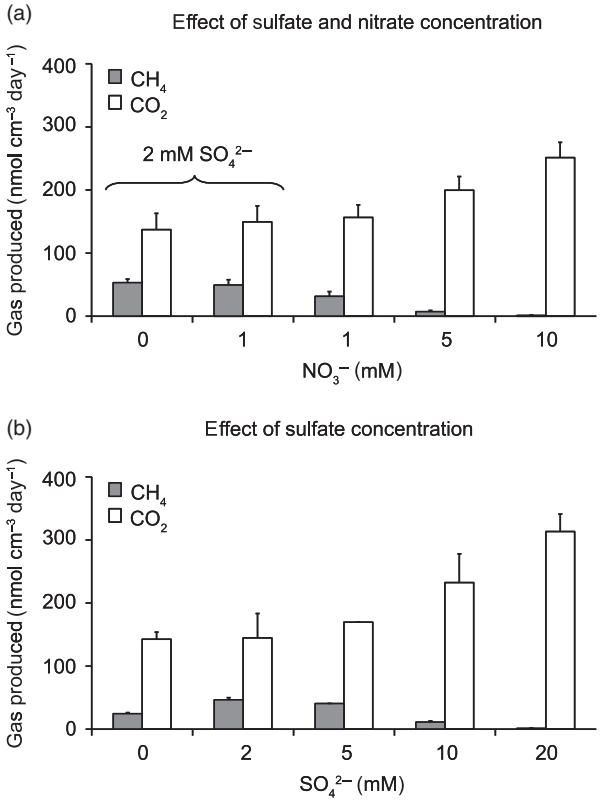

Fig. 3. Shift from methane production towards CO_2 upon hexadecane addition depending on increased sulfate and/or nitrate concentrations in Eckernförde Bay microcosms. Error bars indicate SEs of three incubations. (a) Nitrate concentrations from 1 to 10 mM are displayed on the *x*-axis. Additionally, 0 and 1 mM nitrate were tested with 2 mM sulfate present (left). All other microcosms were incubated without sulfate. (b) Sulfate concentrations are displayed on the *x*-axis. Nitrate was not added.

of ferrihydrite or manganese dioxide reduced methanogenesis from 58.1 ± 0.6 to 39.6 ± 0.9 or 28.2 ± 12.1 nmol methane cm^{-3} day^{-1}, respectively (Fig. 2b). Like in hexadecane incubations, an increase of sulfate concentrations to 22 mM decreased the methanogenesis rate to 10.0 ± 0.5 nmol methane cm^{-3} day^{-1}. Nitrate inhibited methanogenesis completely. The addition of ethylbenzene inhibited CO_2 release (Fig. 2b) compared with unamended controls. The lowest CO_2 production rate was detected with nitrate (19.5 ± 0.6 nmol CO_2 cm^{-3} day^{-1}), while 22 mM sulfate led to an increase in CO_2 release to 45.9 ± 0.3 nmol CO_2 cm^{-3} day^{-1}.

Methanogenesis depending on 1-^{13}C-naphthalene commenced between days 124 and 235 in 2 mM sulfate incubations, with maximum rates of 12.5 ± 0.3 pmol methane cm^{-3} day^{-1} (Table 1). At the same time, the $\delta^{13}C_{CH_4}$ was -37.1 ± 1.6‰ (unamended control: $\delta^{13}C_{CH_4} = -43.2 \pm 1.1$‰; Fig. 4d). At day 435, 1-^{13}C-naphthalene-derived $^{13}CH_4$ formation was also detected as indicated by the elevated $\delta^{13}C_{CH_4}$ values compared with unamended controls. Methanogenesis rates were, however, within the same order of magnitude in all microcosms (Table 1). Furthermore, a strong enrichment in $^{13}CO_2$ was observed already after 42 days of incubation in all setups amended with 1-^{13}C-naphthalene (Fig. 4e–h). The $\delta^{13}C_{CO_2}$ values ranged from $+34.9 \pm 2.6$‰ (nitrate addition) to $+68.4 \pm 23.5$‰ (iron addition), which was significantly different from the $\delta^{13}C_{CO_2}$ values produced in microcosms amended with unlabelled naphthalene (total mean -26.6 ± 0.2‰). In the 1-^{13}C–naphthalene-degrading cultures, the $\delta^{13}C_{CO_2}$ values further increased to a maximum at day 235 (total mean $\delta^{13}C_{CO_2}$ $+419 \pm 21$‰; Fig. 4e–h). The CO_2 release rates were at least 200 times higher than the methane formation rates (Table 1). Ferrihydrite addition resulted in relatively low CO_2 formation rates from 1-^{13}C-naphthalene of 236.7 ± 3.4 pmol CO_2 cm^{-3} day^{-1}, while the highest rate was observed with nitrate (499.4 ± 0.5 pmol CO_2 cm^{-3} day^{-1}).

Anaerobic methanotrophy

In parallel experiments, anaerobic oxidation of methane (AOM) was observed in Zeebrugge microcosms. Incubations with 22 mM sulfate showed the highest AOM rates (1216.0 ± 135.3 nmol methane cm^{-3} day^{-1}), while cultures with ferrihydrite or manganese dioxide displayed slightly lower rates (1117.3 ± 0.2 or 1070.9 ± 37.8 nmol methane cm^{-3} day^{-1}, respectively). The AOM rates were lower with nitrate (881.3 ± 0.7 nmol methane cm^{-3} day^{-1}) or with 2 mM sulfate (479.0 ± 6.4 0.0 nmol methane cm^{-3} day^{-1}).

Hydrocarbon-degrading microbial community

The original Zeebrugge sediment contained 16S rRNA gene copy numbers of 2.6×10^9 copies cm^{-3} for *Bacteria* and 3.1×10^8 copies cm^{-3} for *Archaea* (Fig. S1 in Appendix S1). Compared with the sediment used as an inoculum, a significant increase of the methanogenic (*Methanosarcina mcrA*) and the methanotrophic (ANME-1 and -2 *mcrA*) populations was observed in microcosms with ferrihydrite and hexadecane (Fig. 5). With sulfate and methane, only the number of ANME-2 copies increased. The growth of *Geobacteraceae* – although present in significant numbers – was not initiated by the addition of hexadecane or electron acceptors compared with the inoculum (Fig. 5). In contrast, the addition of sulfate and/or ferrihydrite stimulated the growth of the sulfate-reducing community in the microcosms. Experiments with ethylbenzene, naphthalene, nitrate or manganese were not monitored by real-time PCR.

16S rRNA gene clone libraries of *Bacteria* ($n = 82$) and *Archaea* ($n = 93$) of the Zeebrugge sediment revealed a broad microbial diversity (Figs S2–S4 in Appendix S1). Among *Bacteria*, *Alpha-*, *Gamma-* and *Deltaproteobacteria* 16S rRNA

Table 1. Change of the $\delta^{13}C_{CH_4}$ and $\delta^{13}C_{CO_2}$ values during 435 days of incubation with 1-^{13}C-naphthalene, ^{12}C-naphthalene or without naphthalene

	Day 0		Day 435							
	1-^{13}C-naphthalene		1-^{13}C-naphthalene		^{12}C-naphthalene				MFR or CFR (pmol cm^{-3} day^{-1})	
Electron acceptor	Mean	Error	Mean	Error	Mean	Error	Without naphthalene		Mean	Error
$\delta^{13}C_{CH_4}$ [‰ *VPDB*]										
Ferrihydrite	−48.4	±1.5	−35.5	±0.1	−52.4	±2.1	−52.1		8.3	±1.0
Nitrate	−48.6	±0.4	−30.7	±0.4	−50.1	±1.0	−53.5		11.8	±0.3
2 mM sulfate	−48.7	±0.5	−30.0	±0.1	−58.5	±4.0	−53.6		12.5	±0.3
22 mM sulfate	−48.7	±0.1	−30.5	±0.4	−50.2	NA	−55.3		12.4	±0.3
$\delta^{13}C_{CO_2}$ [‰ *VPDB*]										
Ferrihydrite	−24.8	±0.3	374.5	±5.8	−30.3	±0.0	−27.7		236.7	±3.4
Nitrate	−24.8	±0.1	363.4	NA	−28.9	±0.1	−26.6		499.4	±0.5
2 mM sulfate	−24.5	±0.2	336.9	±3.6	−29.4	±0.3	−27.2		285.0	±2.9
22 mM sulfate	−24.3	±0.5	317.3	NA	−28.7	±0.0	−27.8		338.6	±0.1

Errors are SDs from the mean of samples within 95% confidence intervals. Methane formation rates (MFR, top) and CO_2 formation rates (CFR, bottom) were calculated based on the difference between the isotopic ratios of days 0 and day 435 related to the total amount of methane in the headspace measured by GC-FID. Of the two $\delta^{13}C$ errors (days 0 and 435), the greater error was selected for the calculation of rate errors. NA, not available.

gene sequences were recovered as well as sequences associated with *Campylobacterales*, *Planctomycetes*, *Clostridia*, *Actinobacteria* and *Chloroflexi*. 16S rRNA gene sequences associated with potential pathogens, such as *Neisseria* and *Coxiella*, were also found as well as sequences associated with *Geobacteraceae*. Seven potential aerobic iron oxidizers of the family *Acidithiobacillaceae* and another seven of the *Acidimicrobinea* could be identified. Some clones were closely related to sequences recovered in other potentially hydrocarbon influenced environments such as the Victoria Harbour in Hong Kong, China (Zhang *et al.*, 2008), the Belgian coast off Zeebrugge (Gillan & Pernet, 2007), the Milano mud volcano (Heijs *et al.*, 2005) as well as the Gullfaks and Tommeliten oil fields of the North Sea (Wegener *et al.*, 2008; Fig. S2 in Appendix S1). The phylogenetic diversity of *Archaea* comprised *Crenarchaeota* and *Euryarchaeota*. In the latter, members of the *Methanosarcina* prevailed.

Discussion

Electron acceptors may accelerate hydrocarbon degradation, thus providing an increased substrate supply for methanogenesis. In this work, we evaluate the hypothesis that the addition of electron acceptors leads to accelerated hydrocarbon-dependent methanogenesis. This process may be useful to stimulate the recovery of oil-related carbon as methane from reservoirs or for bioremediation of contaminated sites. Our aim was to stimulate the initial steps in hydrocarbon degradation and thus the formation of methanogenic substrates such as acetate, CO_2 and H_2. Four different electron acceptors were added to sediment microcosms. Two different ecosystems – contaminated harbor mud and pristine marine sediment – were investigated to show that this approach is generally applicable.

Hydrocarbon-dependent methanogenesis

Methane evolved upon hexadecane, ethylbenzene or naphthalene addition in different sediment microcosms (Fig. 2 and Table 1). In most cases, conversion of hexadecane to methane was faster compared with aromatic hydrocarbons (Fig. 2 and Table 1). Exceptions were ethylbenzene microcosms with 2 mM sulfate, in which the conversion to methane was faster (58.1 ± 0.6 nmol methane cm^{-3} day^{-1}) than that in the respective hexadecane incubation (37.8 ± 6.6 nmol methane cm^{-3} day^{-1}). The observed rates were approximately one order of magnitude lower than those reported in a study of an inoculated oil field sediment core (Gieg *et al.*, 2008). Apparently, inoculation using an enriched consortium was more efficient than the stimulation of indigenous hydrocarbon degraders. In another study of a sediment-free methanogenic hexadecane-degrading enrichment culture, hexadecane-dependent methanogenesis was lower (13 nmol methane mL^{-1} day^{-1}) than the rates

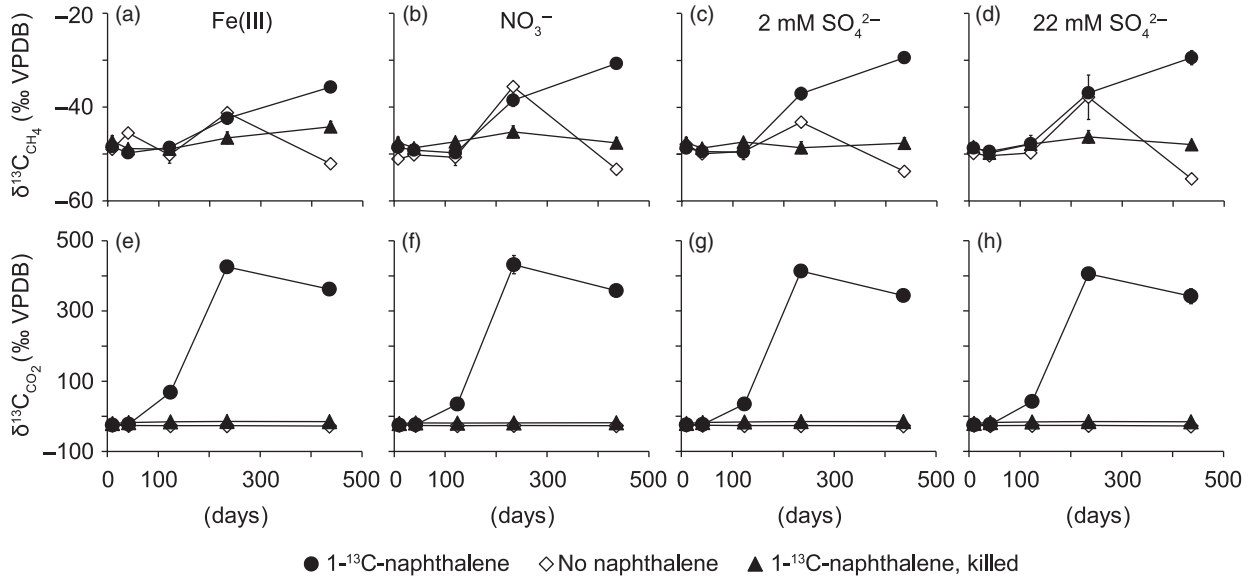

Fig. 4. Time course of $^{13}CH_4$ (top, a–d) and $^{13}CO_2$ (bottom, e–h) formation upon 1-^{13}C-naphthalene addition to microcosms prepared from contaminated Zeebrugge harbor mud. ●, 1-^{13}C-naphthalene; ▲, 1-^{13}C-naphthalene, killed; ◇, without naphthalene. Error bars are SDs from the mean of three parallel microcosms. Error bars of control experiments (no naphthalene, dead controls) are SDs from the mean of two parallel microcosms. Dead controls were killed with 8% final concentration formaldehyde.

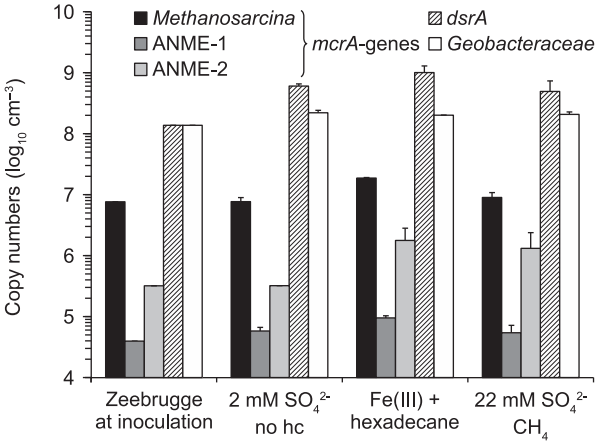

Fig. 5. Logarithmic plots of the community composition in microcosms of contaminated harbor mud of Zeebrugge. DNA was extracted from the sediment microcosms after 178 days of incubation with 2 mM sulfate without any additional hydrocarbon (hc), ferrihydrite and hexadecane or methane and 20 mM sulfate. ANME-1, ANME-2 and *Methanosarcina*-specific *mcrA* genes were quantified. Sulfate reducers were detected targeting their *dsrA* gene and *Geobacteraceae* were quantified by amplification of their 16S rRNA genes. When given, error bars were calculated from SDs of the mean of two extracted incubations, each determined in three parallel PCR reactions.

observed in our experiments (Feisthauer *et al.*, 2010). Presumably, a sediment-free enrichment culture never reaches cell densities of sediments (approximately 10^9 cells cm^{-3} sediment, Fig. S2 in Appendix S1), resulting in lower volume-related rates.

Methanogenesis from naphthalene was in a picomolar range while other hydrocarbons induced methane release in nanomolar ranges (Fig. 2 and Table 1). The time lag between $^{13}CO_2$ and $^{13}CH_4$ evolution as well as the significant difference in δ^{13}C-signature shifts (Fig. 4) indicate that methanogenesis played a minor role in naphthalene-degrading microcosms. Primarily, naphthalene seems to have been mineralized to CO_2. Anaerobic oxidation of naphthalene and subsequent formation of CO_2 was demonstrated under nitrate- (Bregnard, 1996) and sulfate-reducing conditions (Langenhoff *et al.*, 1989; Coates *et al.*, 1996; Hayes *et al.*, 1999; Musat *et al.*, 2009). Nevertheless, methanogenesis occurred in our naphthalene-degrading microcosms, a process that was suggested (Sharak Genthner *et al.*, 1997; Chang *et al.*, 2006), but hitherto never confirmed.

Sharak Genthner *et al.* (1997) observed an inhibition of methanogenesis after naphthalene addition and concluded that naphthalene may be toxic to methanogens. In our microcosms, this seems unlikely because they were naturally exposed to various mineral oil compounds found in the sediments (Ministerie van de Vlaamse Gemeenschap, 2002). Regardless of naphthalene toxicity, methanogens possibly had better access to degradation products of hexadecane and ethylbenzene than to those of naphthalene. We therefore postulate that methanogens themselves were directly involved in the degradation chain of hexadecane and ethylbenzene, but not of naphthalene degradation. The observed increase in the

methanogenic population and the finding of a rich methanogenic community in 16S rRNA gene clone libraries support this assumption (Fig. 5; Fig. S4 in Appendix S1).

Impact of electron acceptors on hydrocarbon-dependent methanogenesis

We studied the impact of ferrihydrite, manganese dioxide, nitrate and sulfate on hydrocarbon-dependent methanogenesis. Ferrihydrite accelerated hexadecane-dependent methanogenesis compared with sulfate or nitrate. Nitrate almost completely inhibited methanogenesis from hexadecane and ethylbenzene (Figs 2 and 3a). This is not surprising because nitrate is a well-known inhibitor of methanogenesis (Klüber & Conrad, 1998). Furthermore, nitrate and high sulfate concentrations negatively influenced the conversion rates of hexadecane to methane (Figs 2 and 3a). However, in the presence of 2 mM sulfate, nitrate was not inhibitory (Fig. 3a), indicating that a sulfate-reducing hexadecane-degrading community prevailed.

Adding sulfate in concentrations up to 5 mM to the sediment microcosms of Eckernförde Bay resulted in a significant increase of hexadecane-dependent methanogenesis (Fig. 3b). In contrast, concentrations higher than 5 mM strongly inhibited hexadecane-dependent methanogenesis. Possibly, sulfate addition stimulated the growth of new or other sulfate reducers, dominating substrate competition for intermediates with methanogens. In contrast, a previous study reported no inhibition of methanogenesis by sulfate of up to 10 mM (Gieg *et al.*, 2008). The inhibitory effect of 22 mM sulfate on ethylbenzene-dependent methanogenesis was less pronounced compared with hexadecane. For naphthalene, neither the inhibition nor the stimulation of methanogenesis was found with either electron acceptor (Fig. 4 and Table 1). This agrees with a recent study of contaminated sediments, where no stimulating effect of Fe(III) on PAH degradation was observed (Li *et al.*, 2010).

The impact of electron acceptors on hydrocarbon-dependent methanogenesis demonstrates that (1) the concentration of the added electron acceptor is crucial for hexadecane-fed methanogenesis and (2) the solubility of the electron acceptor appears to be important. Indeed, insoluble electron acceptors such as ferrihydrite or manganese dioxide had a stimulating effect on hexadecane-dependent methanogenesis (Fig. 2a). However, these electron acceptors are only locally bioavailable, which may result in microscale compartment formation. In contrast, theoretically possible products of hexadecane degradation, such as carbonate, acetate and H_2, can freely diffuse and become available for methanogens in niches where other electron acceptors are depleted.

In Zeebrugge microcosms, the observed increase of the total archaeal community and *mcrA* gene copies suggests that especially *Methanosarcina* species account for iron reduction as demonstrated by van Bodegom *et al.* (2004) (Fig. 5 and Supporting Information). Moreover, neither ferrihydrite or sulfate nor hexadecane or methane addition triggered the growth of *Geobacteraceae*. In conclusion, members of this family are probably less important for the respective processes (Fig. 5). This is not surprising because *Geobacteraceae* are known for their aromatic metabolism, while alkane degradation has not been reported. Instead, other members of the *Proteobacteria*, known for hosting many known hydrocarbon degraders (Widdel & Rabus, 2001), were identified (Fig. S2 in Appendix S1). One sequence was closely related to a clone identified at the Gullfaks and Tommeliten oil field methane seeps of the North Sea (Wegener *et al.*, 2008).

Methanogenesis vs. AOM

AOM rates were determined to assess potential methane losses during incubation time. These rates were in good agreement with those observed typically in methane-fed environments (Knittel & Boetius, 2009). However, methane seepage was apparently not the major energy source of Zeebrugge sediments. Therefore, *in situ* AOM possibly depended on hydrocarbon-derived methane, as indicated by the growth of the AOM community in hexadecane-amended microcosms (Fig. 5). Based on the methane partial pressure-dependent and cell-specific AOM rate constant reported by Thauer & Shima (2008), we calculated a loss of no more than 12% of the produced methane in hydrocarbon-amended microcosms.

Conclusions and possible practical implications

To fully exploit exhausted oil reservoirs, the conversion of residual oil to methane seems to be a viable technique to recover energy that would otherwise be lost. As a possible contribution for this application, our experiments demonstrated that additional sulfate or trivalent iron accelerated methanogenesis in aliphatic and aromatic hydrocarbon (e.g. BTEX)-degrading communities. In contrast, the inhibitory effect of nitrate, commonly used to suppress sulfate reducers in oil fields, most likely prohibits its application for oil recovery as methane. Additionally, we present convincing evidence for the conversion of a PAH to methane.

Consequently, our results also provide novel insights for bioremediation, where the conversion of hydrocarbon contaminants to volatile methane seems to be an option. Nevertheless, methane is a much more potent greenhouse gas than CO_2. Therefore, the addition of high amounts of nitrate or sulfate may be preferred to stimulate biodegradation when methanogenesis is unwanted and oxygen treatment is impossible.

Acknowledgements

Funding was partially provided by the Deutsche Forschungsgemeinschaft (grants KR 3311/5-1 & 6-1), the Bundesministerium für Bildung und Forschung (grant 03G0189A), the Landesanstalt für Altlastenfreistellung Magdeburg and the Flemish Environmental and Technology Innovation Platform (MIP, project '*In situ* conditioning of dredged and mineral sludge'). We thank Dr Axel Schippers for fruitful discussions and for improving the manuscript.

References

Anderson RT & Lovley DR (2000) Hexadecane decay by methanogenesis. *Nature* **404**: 722–723.

Annweiler E, Materna A, Safinowski M, Kappler A, Richnow HH, Michaelis W & Meckenstock RU (2000) Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. *Appl Environ Microb* **66**: 5329–5333.

Boll M & Fuchs G (1995) Benzoyl-coenzyme A reductase (dearomatizing), a key enzyme of anaerobic aromatic metabolism. *Eur J Biochem* **234**: 921–933.

Bregnard TPA, Höhener P, Häner A & Zeyer J (1996) Degradation of weathered diesel fuel by microorganisms from a contaminated aquifer in aerobic and anaerobic microcosms. *Environ Toxicol Chem* **15**: 299–307.

Callaghan AV, Gieg LM, Kropp KG, Suflita JM & Young LY (2006) Comparison of mechanisms of alkane metabolism under sulfate-reducing conditions among two bacterial isolates and a bacterial consortium. *Appl Environ Microb* **72**: 4274–4282.

Chang W, Um Y & Holoman TR (2006) Polycyclic aromatic hydrocarbon (PAH) degradation coupled to methanogenesis. *Biotechnol Lett* **28**: 425–430.

Coates JD, Anderson RT & Lovley DR (1996) Oxidation of polycyclic aromatic hydrocarbons under sulfate-reducing conditions. *Appl Environ Microb* **62**: 1099–1101.

Cravo-Laureau C, Grossi V, Raphel D, Matheron R & Hirschler-Rea A (2005) Anaerobic *n*-alkane metabolism by a sulfate-reducing bacterium, *Desulfatibacillum aliphaticivorans* strain CV2803T. *Appl Environ Microb* **71**: 3458–3467.

Feisthauer S, Siegert M, Seidel M, Richnow HH, Zengler K, Gründger F & Krüger M (2010) Isotopic fingerprinting of methane and CO_2 formation from aliphatic and aromatic hydrocarbons. *Org Geochem* **41**: 482–490.

Gieg LM, Duncan KE & Suflita JM (2008) Bioenergy production via microbial conversion of residual oil to natural gas. *Appl Environ Microb* **74**: 3022–3029.

Gillan D & Pernet P (2007) Adherent bacteria in heavy metal contaminated marine sediments. *Biofouling* **23**: 1–13.

Grbić-Galić D & Vogel TM (1987) Transformation of toluene and benzene by mixed methanogenic cultures. *Appl Environ Microb* **53**: 254–260.

Greene AC, Patel BKC & Sheehy AJ (1997) *Deferribacter thermophilus* gen. nov., sp. nov., a novel thermophilic manganese- and iron-reducing bacterium isolated from a petroleum reservoir. *Int J Syst Bacteriol* **47**: 505–509.

Greene AC, Patel BKC & Yacob S (2009) *Geoalkalibacter subterraneus* sp. nov., an anaerobic Fe(III)- and Mn(IV)-reducing bacterium from a petroleum reservoir, and emended descriptions of the family *Desulfuromonadaceae* and the genus *Geoalkalibacter*. *Int J Syst Evol Micr* **59**: 781–785.

Hayes LA, Nevin KP & Lovley DR (1999) Role of prior exposure on anaerobic degradation of naphthalene and phenanthrene in marine harbor sediments. *Org Geochem* **30**: 937–945.

Head IM, Jones DM & Larter SR (2003) Biological activity in the deep subsurface and the origin of heavy oil. *Nature* **426**: 344–352.

Heijs SK, Sinninghe Damsté JS & Forney LJ (2005) Characterization of a deep-sea microbial mat from an active cold seep at the Milano mud volcano in the Eastern Mediterranean Sea. *FEMS Microbiol Ecol* **54**: 47–56.

Herrmann S, Kleinsteuber S, Chatzinotas A, Kuppardt S, Lueders T, Richnow HH & Vogt C (2010) Functional characterization of an anaerobic benzene-degrading enrichment culture by DNA stable isotope probing. *Environ Microbiol* **12**: 401–411.

Holmes DE, Finneran KT, O'Neil RA & Lovley DR (2002) Enrichment of members of the family *Geobacteraceae* associated with stimulation of dissimilatory metal reduction in uranium-contaminated aquifer sediments. *Appl Environ Microb* **68**: 2300–2306.

Jones DM, Head IM, Gray ND *et al.* (2008) Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature* **451**: 176–180.

Kazumi J, Caldwell ME, Suflita JM, Lovley DR & Young LY (1997) Anaerobic degradation of benzene in diverse environments. *Environ Sci Technol* **31**: 813–818.

Klüber HD & Conrad R (1998) Effects of nitrate, nitrite, NO and N_2O on methanogenesis and other redox processes in anoxic rice field soil. *FEMS Microbiol Ecol* **25**: 301–319.

Kniemeyer O & Heider J (2001) Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron–sulfur/heme enzyme. *J Biol Chem* **276**: 21381–21386.

Kniemeyer O, Fischer T, Wilkes H, Glockner FO & Widdel F (2003) Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. *Appl Environ Microb* **69**: 760–768.

Knittel K & Boetius A (2009) Anaerobic oxidation of methane: progress with an unknown process. *Annu Rev Microbiol* **63**: 311–334.

Kunapuli U, Lueders T & Meckenstock RU (2007) The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. *ISME J* **1**: 643–653.

Langenhoff AA, Brouwers-Ceiler DL, Engelberting JH, Quist JJ, Wolkenfelt JG, Zehnder AJ & Schraa G (1997a) Microbial reduction of manganese coupled to toluene oxidation. *FEMS Microbiol Ecol* **22**: 119–127.

Langenhoff AA, Nijenhuis I, Tan NC, Briglia M, Zehnder AJ & Schraa G (1997b) Characterisation of a manganese-reducing, toluene-degrading enrichment culture. *FEMS Microbiol Ecol* **24**: 113–125.

Langenhoff AAM, Zehnder AJB & Schraa G (1989) Behaviour of toluene, benzene and naphthalene under anaerobic conditions in sediment columns. *Biodegradation* **7**: 267–274.

Li C-H, Wong Y-S & Tam NF-Y (2010) Anaerobic biodegradation of polycyclic aromatic hydrocarbons with amendment of iron(III) in mangrove sediment slurry. *Bioresource Technol* **101**: 8083–8092.

Liesack W & Dunfield PF (2004) T-RFLP analysis: a rapid fingerprinting method for studying diversity, structure, and dynamics of microbial communities. *Environmental Microbiology: Methods and Protocols*, *Vol. 16* (Walker JM, Spencer JFT & Ragout de Spencer AL, eds), pp. 23–37. Humana Press, Totowa, NJ.

Lovley DR (2000) Anaerobic benzene degradation. *Biodegradation* **11**: 107–116.

Lovley DR & Phillips EJP (1986) Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Appl Environ Microb* **51**: 683–689.

Lovley DR & Phillips EJP (1988) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Appl Environ Microb* **54**: 1472–1480.

Ministerie van de Vlaamse Gemeenschap AWK (2002) *Uitvoeren van een waterbodemonderzook in het Albert I dok, het Tijdok an toegang Visartsluis te Zeebrugge: bijkomende studie* (De Vos M & Bogaert G, eds), pp. 12–19. ABO n.v., Gent.

Musat F, Galushko A, Jacob J *et al*. (2009) Anaerobic degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing bacteria. *Environ Microbiol* **11**: 209–219.

Nadkarni MA, Martin FE, Jacques NA & Hunter N (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* **148**: 257–266.

Nunoura T, Oida H, Toki T, Ashi J, Takai K & Horikoshi K (2006) Quantification of *mcrA* by quantitative fluorescent PCR in sediments from methane seep of the Nankai Trough. *FEMS Microbiol Ecol* **57**: 149–157.

Pruesse E, Quast C, Knittel K, Fuchs B, Ludwig W, Peplies J & Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**: 7188–7196.

Rabus R (2005) Biodegradation of hydrocarbons under anoxic conditions. *Petroleum Microbiology* (Ollivier B & Magot M, eds), pp. 277–299. ASM Press, Washington, DC.

Safinowski M & Meckenstock RU (2005) Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Environ Microbiol* **8**: 347–352.

Schippers A & Nerretin LN (2006) Quantification of microbial communities in near-surface and deeply buried marine sediments on the Peru continental margin using real-time PCR. *Environ Microbiol* **8**: 1251–1260.

Sharak Genthner BR, Townsend GT, Lantz SE & Mueller JG (1997) Persistence of polycyclic aromatic hydrocarbon components of creosote under anaerobic enrichment conditions. *Arch Environ Con Tox* **32**: 99–105.

Sunde E & Torsvik T (2005) Microbial control of hydrogen sulfide production in oil reservoirs. *Petroleum Microbiology* (Ollivier B & Magot M, eds), pp. 201–213. ASM Press, Washington, DC.

Takai K & Horikoshi K (2000) Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Appl Environ Microb* **6**: 5066–5072.

Thauer RK & Shima S (2008) Methane as fuel for anaerobic microorganisms. *Incredible Anaerobes*, *Vol. 1125* (Wiegel J, Maier RJ & Adams MWW, eds), pp. 158–170. The New York Academy of Sciences, New York.

Townsend GT, Prince RC & Suflita JM (2003) Anaerobic oxidation of crude oil hydrocarbons by the resident microorganisms of a contaminated anoxic aquifer. *Environ Sci Technol* **37**: 5213–5218.

Treude T, Krüger M, Boetius A & Jørgensen BB (2005) Environmental control on anaerobic oxidation of methane in the gassy sediments of Eckernförde Bay (German Baltic). *Limnol Oceanogr* **50**: 1771–1786.

US Department of Energy (2006) *Undeveloped Domestic Oil Resources*. US Department of Energy, Washington, DC.

van Bodegom PM, Scholten JCM & Stams AJM (2004) Direct inhibition of methanogenesis by ferric iron. *FEMS Microbiol Ecol* **49**: 261–268.

Wegener G, Shovitri M, Knittel K, Niemann H, Hovland M & Boetius A (2008) Biogeochemical processes and microbial diversity of the Gullfaks and Tommeliten methane seeps (Northern North Sea). *Biogeosciences* **5**: 1127–1144.

Widdel F & Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr Opin Biotech* **12**: 259–276.

Zengler K, Richnow HH, Rossello-Mora R, Michaelis W & Widdel F (1999) Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* **401**: 266–269.

Zhang T, Gannon SM, Nevin KP, Franks AE & Lovley DR (2010) Stimulating the anaerobic degradation of aromatic hydrocarbons in contaminated sediments by providing an electrode as the electron acceptor. *Environ Microbiol* **12**: 1011–1020.

Zhang W, Ki JS & Qian PY (2008) Microbial diversity in polluted harbor sediments I: bacterial community assessment based on four clone libraries of 16S rDNA. *Estuar Coast Shelf S* **76**: 668–681.

Zhang X & Young LY (1997) Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl Environ Microb* **63**: 4759–4764.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Eckernförde Bay.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.