Biodegradation (2005) 16: 435–448

Degradation of organic pollutants by methane grown microbial consortia

Martin Hesselsoe[1,*], Susanne Boysen[2], Niels Iversen[1], Lars Jørgensen[2], J. Colin Murrell[3], Ian McDonald[3], Stefan Radajewski[3], Helle Thestrup[2] & Peter Roslev[1]
[1]*Aalborg University, Department of Life Sciences, Section of Environmental Engineering, Aalborg, Denmark;* [2]*Norferm Denmark AS, Odense, Denmark;* [3]*Department of Biological Science, University of Warwick, Coventry, UK (*author for correspondence: e-mail: mahe@bio.aau.dk)*

Accepted 13 October 2004

Key words: activated sludge, benzene, BTX, consortia, degradation, di-butyl-phthalate, methanotrophic bacteria, stable isotopes

Abstract

Microbial consortia were enriched from various environmental samples with methane as the sole carbon and energy source. Selected consortia that showed a capacity for co-oxidation of naphthalene were screened for their ability to degrade methyl-tert-butyl-ether (MTBE), phthalic acid esters (PAE), benzene, xylene and toluene (BTX). MTBE was not removed within 24 h by any of the consortia examined. One consortium enriched from activated sludge (''AAE-A2''), degraded PAE, including (butyl-benzyl)phthalate (BBP), and di-(butyl)phthalate (DBP). PAE have not previously been described as substrates for methanotrophic consortia. The apparent K_m and V_{max} for DBP degradation by AAE-A2 at 20 °C was 3.1 ± 1.2 mg l^{-1} and 8.7 ± 1.1 mg DBP (g protein × h)$^{-1}$, respectively. AAE-A2 also showed fast degradation of BTX (230 ± 30 nmol benzene (mg protein × h)$^{-1}$ at 20 °C). Additionally, AAE-A2 degraded benzene continuously for 2 weeks. In contrast, a pure culture of the methanotroph *Methylosinus trichosporium* OB3b ceased benzene degradation after only 2 days. Experiments with methane mono-oxygenase inhibitors or competitive substrates suggested that BTX degradation was carried out by methane-oxidizing bacteria in the consortium, whereas the degradation of PAE was carried out by non-methanotrophic bacteria co-existing with methanotrophs. The composition of the consortium (AAE-A2) based on polar lipid fatty acid (PLFA) profiles showed dominance of type II methanotrophs (83–92% of biomass). Phylogeny based on a 16S-rRNA gene clone library revealed that the dominating methanotrophs belonged to *Methylosinus*/*Methylocystis* spp. and that members of at least 4 different non-methanotrophic genera were present (*Pseudomonas, Flavobacterium, Janthinobacterium* and *Rubivivax*).

Introduction

Methanotrophic bacteria have a unique capacity for co-oxidation of certain types of organic pollutants (Hanson and Hanson 1996; Higgins et al. 1980; Sullivan et al. 1998). In particular, several types of reactions initiated by enzymes produced in methanotrophic bacteria have been described: aliphatic hydroxylation of alkanes (Burrows et al. 1984), oxidation of aromatics (oxidation of monohalobenzenes) (Colby et al. 1977) and oxidation of olefins (e.g., halogenated alkenes) (Oldenhuis & Janssen 1993). For the halogenated alkenes, previous work has intensively focused on the oxidation of trichloroethylene (TCE) (Brusseau et al. 1990; Burrows et al. 1984; Dalton et al. 1993; Oldenhuis & Janssen 1993). Co-oxidation reactions by methanotrophs are mainly initiated by the soluble methane mono-oxygenase (sMMO), which can be expressed by some type I and type II methanotrophs in the absence of copper (Hanson & Hanson 1996; Murrell et al. 2000).

Many previous studies on bioremediation and degradation of organic pollutants by methanotrophs have focused on well-described pure cultures, in particular *Methylosinus trichosporium*

OB3b (Aziz et al. 1999; Bowman & Sayler 1994; Phelps et al. 1992) or *Methylococcus capsulatus* Bath. (Stanley & Dalton 1992; Stanley et al. 1992). Pure cultures can be regarded as artificial microbial communities and exclusive focus on pure cultures may prohibit exploitation of a large fraction of the methane driven degradation potential in natural environments. Additionally, the co-metabolic transformation of organic pollutant compounds carried out by methanotrophs, may lead to accumulation of toxic and inhibitory degradation products in pure cultures.

Microorganisms involved in degradation of organic pollutants may generate new toxic products as well as detoxifying potentially harmful substances (Nunes-Halldorson et al. 2004). A severe toxic response has been reported for mixed methanotrophic cultures after TCE degradation (Alvarez-Cohen & McCarthy 1991; Henry & Grbic-Galic 1991) most probably explained by product toxicity (Chu & Alvarez-Cohen 1999). Methanotrophs are recognized as important organisms for initiating biodegradation, making the substrate more susceptible to further transformation by heterotrophs (Higgins et al. 1980). Hence, stable consortia of methane oxidizers and other heterotrophs may efficiently mineralize organic pollutants and their metabolites (Haigh 1996; Hrsak 1995; Hrsak & Begonja 2000).

The degradation of large and more complex organic molecules by methane grown consortia has only been documented in a few studies e.g. degradation of linear alkylbenzene sulfonates (Hrsak 1995; Hrsak & Begonja 2000; Hrsak & Grbic-Galic 1995). The purpose of this study was to investigate the degradation of compounds, which have never previously been described as substrates for methanotrophs or methanotrophic consortia: phthalic acid esters (PAE) and methyl tert-buthyl ether (MTBE). Additionally, benzene co-oxidation, previously described as a co-substrate for methanotrophs (Colby et al. 1977; Dalton et al. 1993), was investigated to elucidate differences between methanotrophic pure cultures and methane grown consortia.

Materials and methods

Dilution cultures of methantrophic consortia

Soil and water samples were collected from various environments in Europe and Greenland (Table 1). The samples from Greenland represent pristine environments (soil and fresh water sediments). Sediment and soil samples from Denmark and Germany were collected at polluted sites. Activated sludge samples originated from Aalborg East municipal wastewater treatment plant. Groundwater samples (Skagen, Denmark) were saturated with methane when pumped from the subsurface reservoir, due to a high subsurface flux of biogenic methane.

Table 1. Sample sites and screening of naphthalene co-oxidation

Environment	Sample site	Enrichment temperatures (°C)	Screening of co-oxidation potential: No. of naphthalene-oxidizing consortia	
			10 °C	30 °C
Activated sludge	Aalborg, Denmark	30 and 10	11	23
Brakish sediment	Rihne, Germany	30 and 10	3	5
Fresh water sediment	Greenland (Rivitut)	10	1	ND
Fresh water sediment	Greenland (Mellemsoe)	10	2	ND
Soil	Greenland (Diska)	10	0	ND
Peat soil	Vildmose, Denmark	30	ND	27
Soil	Elling, Denmark	30 and 10	3	0
Ground water	Skagen, Denmark	30 and 10	10	14

ND: Not determined.

Environmental samples were suspended (1:10, weight:vol) in nitrate minimal medium (NM-medium) containing: 0.8 mM Na_2SO_4; 0.2 mM $MgSO_4 \cdot 7H_2O$; 0.05 mM $CaCl_2 \cdot 2H_2O$; 10 mM KNO_3; 1.0 μM $ZnCl_2$; 0.5 μM $Na_2MoO_4 \cdot 2H_2O$; 1.0 μM $MnCl_2 \cdot 4H_2O$; 1.0 μM NaI; 1.0 μM H_3BO_3; 0.5 μM $CoCl_2$; 2.0 μM $CuSO_4 \cdot 5H_2O$; 5 μM EDTA-di sodium; 50 μM $FeSO_4 \cdot 7H_2O$ in 5 mM phosphate-buffer pH 7.0. To promote enrichment of the most abundant organisms, we carried out 5-fold serial dilution of the environmental samples in sterile 96-well microtiter plates (250 μl/well, NUNC, Roskilde, Denmark). Eight replicates in each dilution were applied. The microtiter plates were incubated at 10 °C or 30 °C in a sealed gas-tight container with 10% CH_4 (v/v) and 1% CO_2 (v/v) in the headspace.

Cultures

Methylosinus trichosporium OB3b was obtained from the NCIMB (strain # 11131) and *Methylomonas methanica* S1 was obtained from University of Warwick (UK). These strains belong to the two well-described methanotrophic families *Methylococcacea* (*Methylomonas*, Type I) and *Methylocystaceae* (*Methylosinus*, Type II), respectively (Hanson & Hanson 1996).

Screening of co-oxidation

A co-oxidation test based on naphthalene was adapted from Wackett & Gibson (1983) with relevant modifications to customize for a microtiter plate format. Replica-microtiter plates were inoculated with 10% of the volume transferred from the original dilution-culture plates. These replica plates were prepared for co-oxidation of naphthalene without copper in the NM-medium to induce activity of sMMO (Burrows et al. 1984). Before exposure to naphthalene, the replica plates were incubated for 2 weeks in a sealed gas-tight container with 10% CH_4 and 1% CO_2 at 10 °C or 30 °C. Replica-plates were then exposed (24 h) to naphthalene evaporating from crystals in a gas-tight container. A parallel replica-plate was not exposed to naphthalene. After naphthalene exposure, 20-μl *O*-dianisidine, tetrazotized zinc chloride complex (Sigma, St. Louis, MO, USA) solution in water (0.01 g ml^{-1}) was added to each well in the microtiter plate. The red complex formation between α-naphthol and O-dianisidine was measured in a plate reading spectrophotometer at 540 nm (Biotek, Winooski, USA). The difference between the absorbance at 540 nm measured before and after addition of *O*-dianisidine was regarded as a measure of the production of α-naphthol. No production of α-naphthol was observed in control plates, which had not been exposed to naphthalene.

MTBE and PAE degradation

Enrichment cultures with high co-oxidation of naphthalene were investigated further for co-oxidation capacity of MTBE and PAE. The selected enrichment cultures were inoculated from the dilution-culture plates into gas-tight bottles containing NM-medium without copper (10% CH_4 and 1% CO_2 in the headspace). The enrichments were incubated at 10 °C or 30 °C on a shaker at 150 rpm in the dark. When visible growth was observed ($OD_{600\ nm}$ 0.1–0.2), the cultures were harvested and resuspended in fresh NM-medium at a slightly higher cell density ($A_{600\ nm} = 0.3$). In order to enhance growth, enrichments at 10 °C were transferred to 20 °C prior to screening for PAE and BTX degradation.

MTBE was added to a final concentration of 10 mg l^{-1} in the water phase, considering a dimensionless Henry's law constant at 25 °C of 0.02 (Robbins et al. 1993). Parallel samples without cells were included as controls. MTBE was analyzed after 24 h incubation (150 rpm) by injection of 0.3 ml headspace gas into a gas chromatograph (Packard 438S Chrompack, Delft, NL) equipped with a FID detector and a silica column (Ø 0.53 mm, length 30m, CP-select 624 CP, Chrompack). Nitrogen (300 kPa) was used as carrier.

Cells were grown and harvested for PAE degradation as described above. For screening purposes, a mixture of three PAEs in acetone were prepared: DMP (di-methyl-phthalate), DEHP di-(2-ethylhexyl)-phthalate and DBP (di-butyl-phthalate). The phthalate solution in acetone (10–15 μL) was added to 0.5 ml NM-medium in glass vials. Acetone was evaporated for 1 h while shaking the samples under an air-fan. Subsequently, harvested cells were added (2.5 ml) to obtain an optical density ($OD_{600\ nm}$) of approx. 0.3. The bottles were then incubated for 24 h at

20 °C in the dark under three different conditions: No methane, addition of 1% methane or addition of 5 mM formate. NM-medium without cells or with autoclaved cells was used as controls. After incubation, PAEs were extracted by adding 2 ml *n*-hexane:ethylacetate (1:1) containing internal standards as suggested previously (Zurmühl 1990). For liquid–liquid extractions, samples were shaken for 30 min and phases separated overnight. Then 0.5 ml from the organic phase was transferred to sealed GC autosampler vials. one micro liter from each sample was automatically injected into a gas chromatograph (HP 5890 series II) equipped with a FID detector and a HP-5MS column (length 60 m; Ø 0.25 mM). H_2 (200 kPa) was used as carrier. Removal of PAE in the samples was expressed relative to the control without cells. No removal of PAE was observed in samples with autoclaved cells.

Additional experiments with PAE degradation were carried out for one of the investigated consortia (named ''AAE-A2''), which showed significant removal of PAE. The same experimental setup as described above was used, in combination with addition of other PAEs (butylbenzyl-phthalate, BBP and di-octyl-phtalate, DOP), different starting concentrations of PAE, addition of acetylene as an irreversible inhibitor of methane mono-oxygenase and/or addition of di-chloromethane (DCM) as a general toxic substance inhibitory towards most microorganisms. All samples were prepared in triplicate.

Mineralization of PAE was examined using [^{14}C]DBP (carbonyl labeled, 21.2 mCi mmol^{-1}; Sigma, St. Louis, USA). [^{14}C]DBP was dissolved in acetone and added as described for the non-labeled PAEs. Non-labeled PAE was added to obtain a total concentration of 5 mg l^{-1} PAE. After incubation in sealed glass vials, the samples were fixed by addition of 2.5% NaOH. Radioactive $^{14}CO_2$ was evacuated after acidification with 40% H_3PO_4 and trapped in ethyleneglycol monomethylether:ethanolamine (7:1) as described previously (Kofoed 1976). Trapped CO_2 was transferred to 20-ml polyethylene scintillation vials (Packard, Groningen, NL). The radioactivity was quantified by liquid scintillation counting (Packard 1600 TR, Packard) after addition of 10 ml scintillation cocktail (Hionic-Flour, Packard). The residual isotope, not evacuated as $^{14}CO_2$ (biomass and non-degraded ^{14}C-labeled compound), was quantified by liquid scintillation counting using Insta-Gel (Packard) as scintillation cocktail. The counts were corrected for quenching using quench curves obtained from internal standard addition. Samples fixed with NaOH immediately after isotope additions were used as controls. All samples were prepared in triplicate subsamples from each of the tested cultures.

BTX degradation

The methane grown consortia selected for screening were harvested (20,000 × g for 10 min) and cells were resuspended in 50 mM phosphate buffer (50 mM $K_2HPO_4 \cdot 3H_2O$, 50 mM $NaH_2PO_4 \cdot 2H_2O$) to a optical density ($OD_{600\ nm}$) of 0.1–0.15. The cell suspension (10 ml) was transferred to 20-ml GC vials and benzene, toluene or *o*-xylene was added to a final concentration of 40 mg l^{-1}. Formate was added as a source of reducing power for methanotrophs to a final concentration of 20 mM. The vials were closed with gas tight rubber septa and incubated (150 rpm) for 1 h at room temperature. BTX degradation was terminated by injection of 0.4 ml of 2 mM phosphoric acid. The BTX concentration was analyzed by headspace injection (autosampler HS40XL, Perkin Elmer, Wellesley, MA, USA) with an injection time of 0.02 min (pressure time 2.0 min, oven temp. 35 °C, needle temp 110 °C, transfer temp 110 °C) coupled to a gas chromatograph (Autosystem XL, Perkin Elmer). The gas chromatograph was equipped with a FID detector and silica column (Ø 0.25 mm, L 25 m, CP-porabond Q, Chrompack, Delft, NL). Helium (99.99%, 300 kPa) was used as carrier. The temperatures in all samples were carefully kept constant during analysis to avoid changes in headspace concentration of BTX. The BTX measured in controls (no cells) were compared to samples with cells.

Lipid analysis

Lipids were extracted and divided into lipid classes as described previously (Roslev et al. 1998). After lipid extraction, polar lipids (PL) were subjected to mild alkaline methanolysis to form fatty acid methyl esters (FAME). PL-FAMEs were analyzed by GC. Phospholipid fatty acids (PLFA) were identified based on a retention time index calculated relative to internal standards 13:0 and 19:0. The

retention times were compared to those obtained from methanotrophic type strains and reference standards (Supelco 37 standard mixture, Supelco, Bellfonte, PA, USA). *Methylomonas methanica* S1 and *Methylosinus trichosporium* OB3b were used as type strains representing type I and type II methanotrophs, respectively. Fatty acids were named based on standard nomenclature as described previously (Roslev & Iversen 1999).

Fluorescent in situ *hybridization*

Fluorescent *in situ* hybridization (FISH) was carried out after fixation (1 h) in 3% paraformaldehyde as described previously (Manz et al. 1992). The following probes were applied for the FISH analysis: EUB 338, which is a general eubacterial probe (Amann et al. 1990), and specific probes targeting type I methane oxidizers (Mα450) and type II (Mγ84 and Mγ705) (Eller et al. 2001). Stringency conditions were controlled with formamide during hybridization and NaCl during washing using the same levels of stringency as described by Eller et al. (2001). After hybridization, slides were stored in the dark at 4 °C and microscopy was carried out within 1 week to avoid fading of the fluorescence from the specimen. Epifluorescence microscopy was performed with an Axioplan microscope (Zeiss, Oberkochen, Germany).

Cloning and phylogenetic analysis

DNA was extracted for phylogenetic analysis from 20 ml samples using two different methods; a chemical lysis method (Marmur 1961) and a bead beating method (Yeates & Gillings 1998). The bacterial 16S rRNA gene was PCR amplified using primers 27f/1492r (DeLong 1992). PCR products were cloned with the TOPO TA cloning kit (Invitrogen, UK). Plasmid inserts were screened for restriction fragment length polymorphisms (RFLPs) with *Eco*RI/*Rsa*I, and clones with identical RFLPs were grouped into an operational taxonomic unit (OTU). One representative from each OTU was sequenced (>500 bp) using the sequencing primer 357f (Lane 1991). DNA sequences recovered in this study have been assigned the GenBank numbers AY692234, AY692235 AY692236, AY692237, AY692238, AY692239, AY692240, AY692241 and AY692242.

Stable isotope analysis

To monitor the carbon flow in the consortium, AAE-A2 grown in absence of copper ions was inoculated (10%) to fresh growth medium and incubated with 1% $^{13}CH_4$ and 9% unlabeled CH_4 in the headspace. Four samples were collected for lipid-extraction (see above) during growth with $^{13}CH_4$ (lag-phase, early log-phase, late log-phase and stationary phase determined from the optical density at 600 nm). ^{13}C-labeled phospholipid fatty acid (^{13}C-PLFA) methyl esters were then analyzed on a Finnigan Delta Plus XL gas chromatograph combustion isotope ratio mass spectrometer [GC-C-IRMS] (ThermoQuest, Bremen, Germany). The gas chromatograph (Hewlett-Packard 6890) was equipped with a HP-5MS column (L 60 m, Ø 0.25 mm), and a GC/C III combustion interface. Helium was used as carrier gas. δ^{13}C values were determined based on authentic standards certified relative to PeeDee Belemnite.

Results

Screening of co-oxidation, MTBE, PAE and BTX degradation

Methane-grown consortia enriched from the investigated sites were screened for co-oxidation capacity using the naphthalene-oxidation assay. This test showed that dilution cultures from nearly all of the investigated sites oxidized naphthalene to α-naphthol (Table 1). The dilution cultures with the highest α-naphthol production were selected for further screening (MTBE, PAE and BTX degradation). In this context, the term "degradation" is used to indicate a transformation of the substrate, i.e. removal of the parent compound. Fifty-two consortia representing all investigated sites were screened for MTBE degradation, however, no consortia degraded MTBE and no accumulation of MTBE degradation products such as tert-butyl-alcohol was detected. Thirteen consortia were screened for PAE degradation, and among those one consortium showed significant degradation of PAE. This consortium (AAE-A2) had

Table 2. Screening of BTX degradation among naphthalene-oxidizing methane-grown consortia. Copper was added/omitted during growth. BTX degradation was measured in a 1-h assay. Data are shown with standard deviation between replicates ($n = 3$)

Consortium/culture name	Environment	Copper addition	Benzene	Toluene	Xylene
			nmol (mg protein × min)$^{-1}$		
Methylosinus trichosporium OB3b	Pure culture	no Cu	57 ± 35	16 ± 8	0
Methylosinus trichosporium OB3b	Pure culture	2 μM Cu	4	0	0
AAE-A2	Activated sludge	no Cu	**233 ± 34**	**106 ± 21**	**40 ± 20**
AAE-A2	Activated sludge	2 μM Cu	**24 ± 9**	**5 ± 5**	0
AAE-D1	Activated sludge	no Cu	93 ± 39	34 ± 24	95 ± 13
AAE-B5	Activated sludge	no Cu	0	0	0
Rihne-B1	Brakish sediment	no Cu	65 ± 34	14 ± 8	33 ± 23
Rivitut-F2	Fresh water sediment	no Cu	24 ± 11	31 ± 20	13 ± 8
Elling-F2	Soil	no Cu	46 ± 26	23 ± 3	29 ± 6
Skagen-A	Groundwater	no Cu	57 ± 25	11 ± 9	35 ± 23
Skagen-E3	Groundwater	no Cu	94 ± 31	0	0

been enriched from activated sludge collected at Aalborg East wastewater treatment plant.

Eight of the naphthalene-oxidizing consortia were screened for BTX degradation (Table 2). The screening results were compared with degradation by the methanotrophic type strain *M. trichosporium* OB3b. Most of the consortia included in the BTX screening degraded benzene and toluene with approximately the same maximum rate as *M. trichosporium* OB3b. However, one consortium (AAE-A2, mentioned above) showed significantly greater benzene and toluene degradation capacity than other cultures in this study ($p < 0.01^{*}$, student *t*-test). The consortium AAE-A2, which showed PAE degradation and the greatest benzene and toluene degradation, was selected for further investigation.

BTX degradation by AAE-A2

After the first screening of BTX degradation, AAE-A2 was transferred to NM-medium with copper (2 μM). After this transfer, the capacity to degrade all BTX compounds was largely diminished (Table 2). However, the copper only affected benzene degradation when added during growth of the cells; BTX degradation was not affected by addition of copper during the 1 h BTX exposure. Copper addition during growth also reduced the benzene degradation by the type strain *M. trichosporium* OB3b (Table 2).

Benzene degradation was followed for a prolonged period (28 days) in a closed batch culture in the presence of formate as source of reducing power and energy for methane oxidizers (Figure 1). Benzene degradation ceased after 2 days in cultures with *M. trichosporium* OB3b, whereas the consortium AAE-A2 continued benzene degradation for 15 days (Figure 1). *M. trichosporium* OB3b developed pronounced reddish coloration in

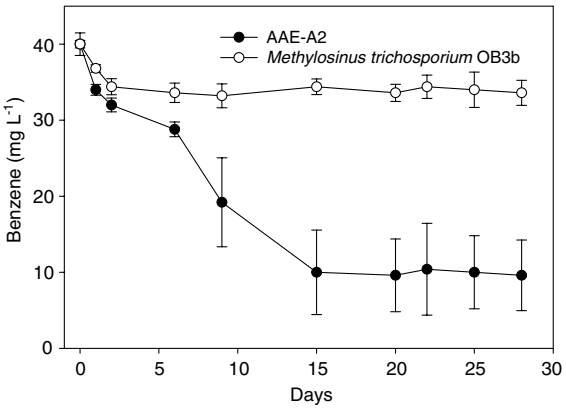

Figure 1. Benzene degradation by *M. trichosporium* OB3b and methane-grown consortium AAE-A2 incubated in the absence of copper and in the presence of formate (20 mM). Error bars indicate standard deviation between replicates (n = 3).

the medium, indicating accumulation of benzene degradation products (e.g. catechol).

PAE degradation by AAE-A2

The capacity of AAE-A2 to degrade five different PAEs was investigated. It was seen that AAE-A2 degraded 30–35% of the added DBP and BBP, resulting in a removal rate of 7 mg PAE (g protein $\times$ h)$^{-1}$ for both compounds (at an initial concentration of 4 mg l^{-1}). The effect of different initial concentrations of DBP on the removal rate is illustrated in Figure 2. Based on the data in Figure 2, apparent kinetic parameters and confidence limits were calculated using non-linear regression of Michaelis-Menten type kinetics resulting in a $K_{m(apparent)}$ of 3.1 $\pm$ 1.2 mg l^{-1} DBP and a V_{max} of 8.7 $\pm$ 1.1 mg DBP (g protein $\times$ h)$^{-1}$. Hence, close to maximum degradation was obtained at 5 mg l^{-1} DBP. This concentration was used in the following experiments.

A time series was prepared to compare the degradation of DBP and the mineralization of [^{14}C]DBP to $^{14}CO_2$ (Figure 3). It was shown that the mineralization of the carbonyl labeled DBP proceeds considerably slower than the initial degradation. Nevertheless, a significant amount of the carbonyl labeled [^{14}C]DBP was mineralized within 44 h (59%). It was also shown that toxic concentrations of dichloromethane completely inhibited the DBP degradation, whereas addition of substrates for methane-oxidizing bacteria (methane and formate) did not affect the degradation of DBP (data not shown).

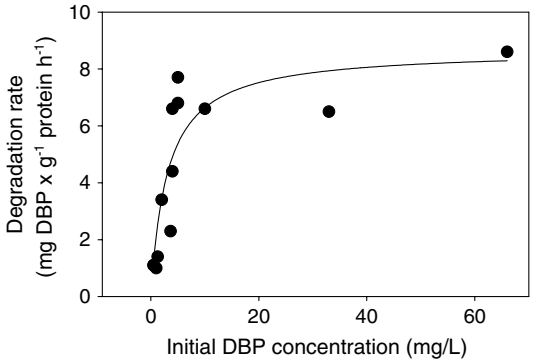

Figure 2. Degradation of di-butyl-phthalate (DBP) by the methane-grown consortium AAE-A2 at different initial DBP concentrations. Trend line represents non-linear regression of Michaelis–Menten kinetics.

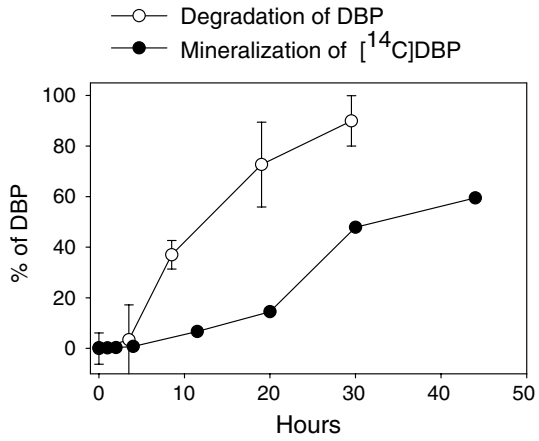

Figure 3. Comparison of di-butyl-phthalate (DBP) degradation (open symbols) and mineralization of carbonyl labeled [^{14}C]DBP (closed symbols) by the methane-grown consortium AAE-A2. The initial concentration of DBP was 5 mg l^{-1}. Error bars indicate standard deviation between replicates ($n = 3$). Some error bars are contained within the symbol.

The capacity to mineralize carbonyl labeled [^{14}C]DBP was reproduced after 5 months of culturing AAE-A2 at 20 °C on methane. The type II methanotroph *M. trichosporium* OB3b did not mineralize [^{14}C]DBP.

Effect of physical and chemical parameters

Temperature: Benzene degradation by the consortium AAE-A2 was tested at different temperatures and compared to the activity of *M. trichosporium* OB3b (Figure 4a). At ambient temperature (20 °C), AAE-A2 degraded benzene faster than *M. trichosporium* OB3b. It must be emphasized that the data presented in Figure 4a for *M. trichosporium* OB3b and AAE-A2 were generated from the same experiment using the same incubators for all samples. The mineralization of carbonyl labeled [^{14}C]DBP by AAE-A2 was also tested at different temperatures between 5 and 35 °C, and the greatest mineralization capacity was also observed at 20 °C (Figure 4b).

Formate: Formate is a source of reducing power and energy for methane-oxidizing bacteria. Unlike methane, formate is not a competitive substrate for methane mono-oxygenase. BTX degradation was stimulated by addition of formate, and all BTX degradation experiments were carried out at 20 mM formate in the absence of

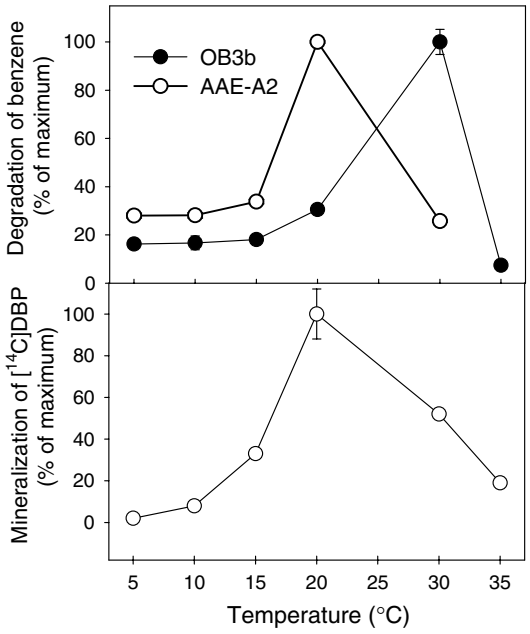

Figure 4. Panel A: Degradation of benzene by *M. trichosporium* OB3b and the methane-grown consortium AAE-A2 at different temperatures. The initial benzene concentration was 40 mg l^{-1}. Panel B: Mineralization of carbonyl labeled [^{14}C]DBP by the methane-grown consortium AAE-A2 at different temperatures. The initial DBP concentration was 5 mg l^{-1}. All data are expressed relative to the maximum activity observed for each culture. Error bars indicate standard deviation between replicates. Some error bars are contained within the symbol.

methane. However, neither formate nor methane affected the degradation of PAE by AAE-A2.

Acetylene: Acetylene is an inhibitor of MMO activity (Knowles 1990). Addition of acetylene (1% of headspace) inhibited the degradation of benzene, however, no effect of acetylene was observed on PAE degradation by AAE-A2.

Characterization of the methanotrophic consortium AAE-A2

The growth rate of AAE-A2 was measured at 10, 20 and 30 °C based on measurements of optical density ($OD_{600\ nm}$). The optimal growth was at 20 °C with a generation time of 35 h, whereas generation time was more than 80 h at 10 and 30 °C.

Colony forming units (CFU) of heterotrophic bacteria on R2A agar (DIFCO, Detroit, USA) were enumerated during different growth phases of AAE-A2 and made up approximately 2–4% of all cells in the consortium. We observed a comparable increasing number of heterotrophic CFU and total cell number (measured as optical density at 600 nm) during logarithmic growth phase (data not shown). No change of CFU in AAE-A2 was observed during prolonged stationary phase incubations (up to 25 days).

FISH analysis with specific probes targeting methanotrophs indicated the presence of both type I and type II methanotrophs in AAE-A2. The FISH results also indicated that type II methanotrophs quantitatively dominated the consortium.

The PLFA composition of the consortium AAE-A2 was compared to the PLFA profiles of two known methanotrophic bacteria *M. methanica* S1 and *M. trichosporium* OB3b (Figure 5b). The PLFA profile of the AAE-A2 consortium showed a similarity to the profile of the type II methanotroph *M. trichosporium* OB3b (Figure 5a and b). Considering the PLFAs specific for many type I (16:1ω8) and type II (18:1ω8) methanotrophs (Bowman et al. 1991), the consortium consisted of 83–92% type II methanotrophs and 8–17% other organisms. Among the other organisms was approximately 1–5% type I methanotrophs.

Forty 16S rRNA gene clones from the library constructed using DNA obtained by the Marmur method were grouped by RFLP analysis, and sequences representing 36 clones were obtained (Figure 6). The 16S rRNA gene library constructed using DNA obtained from a bead-beating method consisted of 20 clones and 18 of these had the same RFLP as clones from the Marmur library. Sequences of the methanotrophic genera *Methylosinus*/*Methylocystis* were obtained. Among non-methanotrophic bacteria, sequences were recovered from the following genera: *Pseudomonas, Janthinobacterium, Rubrivivax* and *Flavobacterium*.

Growth of non-methanotrophs in the consortium AAE-A2

Assimilation of methane-derived carbon into non-methanotrophs was examined during growth of AAE-A2 on $^{13}CH_4$. Cells from the consortium were harvested for ^{13}C-PLFA analysis in different growth phases after 1, 3, 7 and 10 days (Figure 7a). At least 13 different PLFAs were enriched

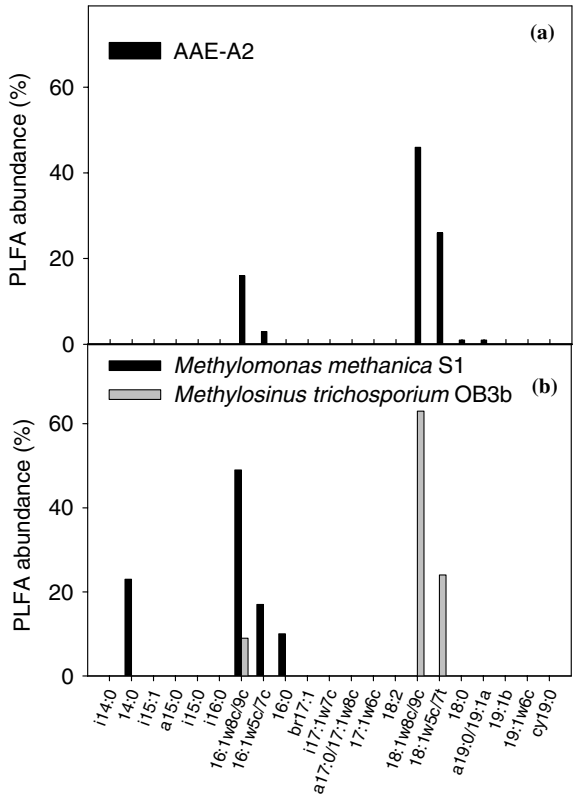

Figure 5. Panel A: Relative distribution of PLFAs in the methane-grown consortium AAE-A2. Panel B: Relative distributions of PLFA in methanotrophic type strains *M. methanica* S1 (Type I methanotroph) and *M. trichosporium* OB3b (Type II methanotroph).

in ^{13}C after 10 days of growth on $^{13}CH_4$ (early stationary phase). PLFAs specific for type I methanotrophs (16:1ω8c), and type II methanotrophs (18:1ω8c) showed a major increase in $\delta^{13}C$ during the initial 3 days of growth (Figure 7b). In contrast, several PLFAs including 18:2 and fatty acids with carbon chain length exceeding 20 did not show significant labelling until after day 3 of the incubation (Figure 7c). These PLFAs represent fatty acids that have not been reported previously from methanotrophic bacteria (Bowman et al. 1991; Guckert et al. 1991). Hence, the 18:2 and >20:0 PLFAs may represent growth of non-methanotrophs in the consortium. A total of 5 apparent non-methanotrophic PLFAs including 4 long chain species (>C20) were enriched in ^{13}C between day 3 and 10 ($\delta^{13}C$ values after 10 days were between −0.85 and +37.8). To the best of our knowledge, PLFAs with chain length longer than C19 are not known from any methanotrophic bacteria.

Discussion

Negative feed-back inhibition during biodegradation

As a negative feed-back mechanism, accumulation of toxic degradation products may inhibit the activity of organisms involved in biodegradation processes. For example, it is known that significant toxicity may remain after biological degradation of benzene (Nunes-Halldorson et al. 2004). Boyd et al. (1997) observed that a primary benzene degradation product, catechol, was far more toxic towards lux-modified *Pseudomonas fluorescens* than the parent compound. Other benzene degradation products have also been reported more toxic than the parent compound, e.g. phenol (Burback et al. 1994) and glutathionyl hydroquinone (Rao 1996). Our result showed (Figure 1) that the benzene co-oxidizing pure culture *M. trichosporium* OB3b ceased benzene degradation after few days, probably due to accumulation of inhibitory benzene degradation products: a distinct reddish dis-coloration of growth medium was visible (e.g., catechol). In contrast, the consortium AAE-A2 continued to degrade benzene for 15 days. This indicated that the consortium either prevented accumulation of toxic degradation products or had a lower sensitivity towards inhibitory degradation products than the pure culture. Although formate was continuously added as a reducing power and energy source, the lack of a carbon source and micronutrients may explain why benzene degradation by AAE-A2 ceased after 15 days.

BTX degradation and chemical factors

BTX degradation was heavily retarded when AAE-A2 and *M. trichosporium* OB3b was grown in the presence of excess copper ions (Table 2). BTX have previously been described as substrates for sMMO (Burrows et al. 1984; Green & Dalton 1989; Higgins et al. 1980). Although methanotrophs expressing sMMO constitutively have been isolated recently (Dedysh et al. 2000, 2002), it is well known that most sMMO containing methanotrophs express the pMMO rather than the sMMO in the

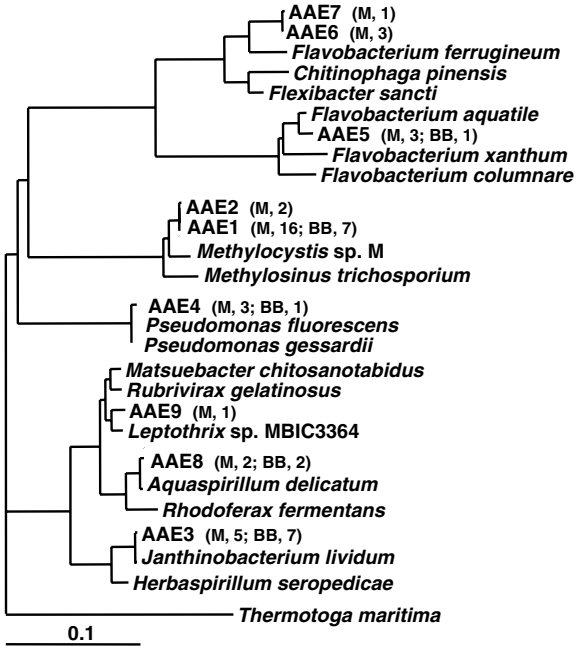

Figure 6. Phylogeny of 16S rRNA gene sequences obtained from the methane-grown consortium AAE-A2. Nine different OTUs were identified and named AAE1–9. The number of clones from Marmur lysis (M) and Beat-Beating (BB) libraries in each OTU are shown in parentheses. Evolutionary distance (Jukes Cantor correction) analysis was based on 546 nucleotide positions and clustered by FITCH. Scale bar represents 10 inferred substitutions per 100 nucleotide positions.

presence of copper (Bowman & Sayler 1994; Brusseau et al. 1990; Graham et al. 1992; Murrell et al. 2000). Additionally, it is known that the pMMO has a significant lower capacity for co-oxidation than the sMMO. This explains why copper ions in the growth medium consistently retarded BTX degradation by *M. trichosporium* OB3b and AAE-A2. Furthermore, this observation supports the notion that methane-oxidizing bacteria in the consortium AAE-A2 carried out the initial steps in the degradation of BTX. The inhibition of BTX degradation in AAE-A2 with acetylene also indicated that MMO enzymes and methane oxidizers were essential for the BTX degradation observed. Other organisms in the consortium were probably involved in the degradation of potential toxic co-oxidation products (e.g. catechol).

PAE degradation by methane-derived biomass

There is an increasing focus on man-made chemicals, which may act as hormone analogues, hence confusing the natural function of hormones in animals and human. PAE, which are commonly used as additives in plastics (Staples et al. 1997), are among the most important hormone analogues suspected to have estrogenic activity (''xenoestrogens'') (Fromme et al. 2002). Staples et al. (1997) reported in a review degradation of DBP in aerobic environments ranging from no degradation to 100% degradation within 1–180 days at start concentrations ranging from 1600 mg l^{-1} to 0.00325 mg l^{-1} DBP. Most previous studies on DBP degradation have focused on bacterial isolates and enrichments grown on DBP as the sole energy and carbon source (Jianlong et al. 1995, 1999) or environmental degradation of DBP without any enrichment (Chauret et al. 1996; Yan et al. 1995; Yuan et al. 2002). However, none of the previous reports on DBP degradation have included microbial biomass derived from methane as the sole carbon and energy source as reported in this study for the consortium AAE-A2. Together with recent reports on the capacity of methanotrophic consortia to degrade linear alkylbenzene sulfonates (Hrsak 1995; Hrsak & Begonja 2000; Hrsak & Grbic-Galic 1995), our finding emphasize the potential importance of methane derived biomass in the biodegradation of complex organic pollutants.

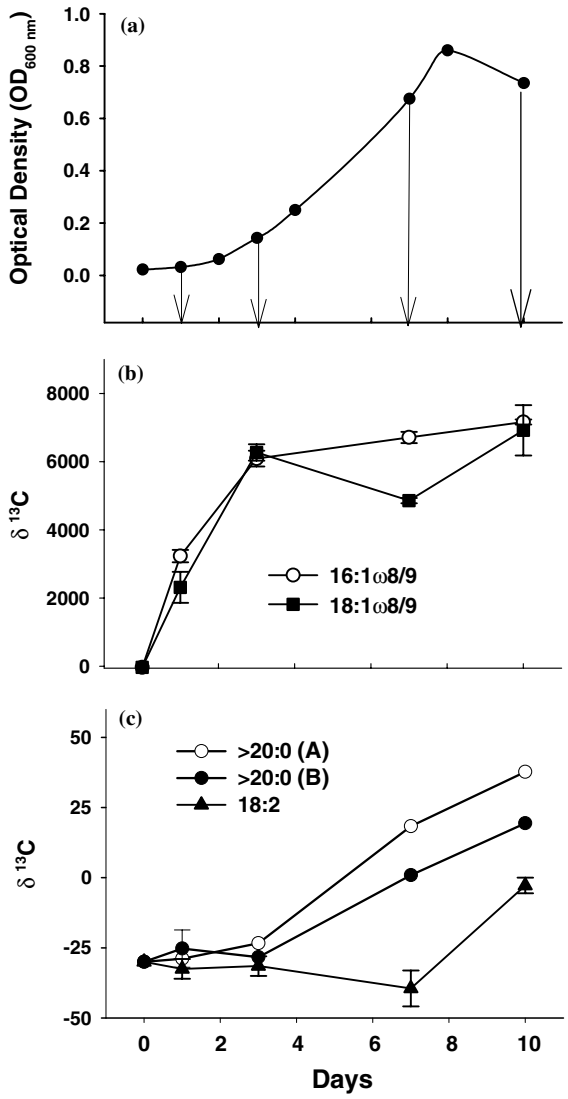

Figure 7. Panel A: Growth of the consortium AAE-A2 with $^{13}CH_4$ measured as optical density ($OD_{600\ nm}$). Arrows indicate sampling of biomass for analysis of ^{13}C-PLFA. Panel B: $\delta^{13}C$-values for selected PLFAs specific for methane-oxidizing bacteria. Panel C: $\delta^{13}C$-values for selected non-methanotrophic PLFAs. Error bars indicate standard deviation between replicates (n = 3).

Previous kinetics studies of DBP degradation by microbial biomass enriched on DBP revealed a K_s value of 100 μM (Wang et al. 1998). A somewhat higher K_m value for DBP of 215 μM was reported for purified enzymes extracted from a PAE degrading *Pseudomonas fluorescens* FS1 (Zeng et al. 2000). This is far above the apparent K_m value for AAE-A2 of 3.1 mg l^{-1} (11.2 μM). This clearly indicated that the methane grown consortium described in this study had a much higher affinity for environmentally relevant (lower) concentrations of DBP than some previous enrichments or pure cultures utilizing DBP as primary growth substrate. The relatively low optimal temperature for the mineralization of carbonyl labeled [^{14}C]DBP (around 20 °C, Figure 4), and the significant mineralization capacity between 10 and 15 °C (Figure 4) demonstrate the potential adaptability of AAE-A2 to environmental conditions. Only few reports on DBP degradation at lower temperatures are available in the literature, e.g. Chauret et al. (1996).

The first step in PAE degradation is cleavage of alkyl chains by hydrolysis of the ester bonds (Staples et al. 1997). The [^{14}C]DBP applied in this study was labeled in the carbonyl group. Hence, production of $^{14}CO_2$ would be slightly delayed compared to the initial hydrolysis of the ester bonds. This may explain the observed difference between DBP degradation and DBP mineralization (Figure 3). Acetylene, methane and formate did not affect PAE degradation by AAE-A2. Since acetylene is a well-known inhibitor of MMO (Bedard & Knowles 1989), this observation suggests that the active site of MMO is not involved in PAE degradation by AAE-A2. Hence, the methane-oxidizing bacteria in the consortia are probably not essential for the observed PAE degradation *per se*. However, it must be emphasized that the AAE-A2 consortium maintains this capacity for PAE degradation while grown on methane as the sole carbon and energy source.

Composition and carbon transfer in AAE-A2

Repetitive PLFA analysis during several month showed that AAE-A2 was a stable mixed culture dominated by type II methane-oxidizing bacteria. Phylogenetic analysis of 16S rRNA gene sequences (Figure 6) confirmed the domination of type II methanotrophs belonging to the genera *Methylosinus*/*Methylocystis* spp. Additionally, 16S rRNA gene cloning and sequencing revealed the presence of at least four different non-methanotrophic genera in AAE-A2 including *Janthinobacterium, Rubivivax, Flavobacterium* and *Pseudomonas*.

Enumeration of heterotrophic CFU in AAE-A2 showed that culturable heterotrophs made up a relative constant proportion of the consortium.

In turn, this indicated that the methanotrophs probably excreted methane-derived metabolites and/or lysis products, which supported the non-methanotrophic heterotrophs in the consortium. Comparable co-existence of heterotrophs and methanotrophs has previously been described for a denitrifying bioreactor fed with methane as the only carbon source and electron donor (Costa et al. 2000), and for linear alkylbenzene sulfonate degrading methanotrophic-heterotrophic groundwater communities (Hrsak & Begonja 2000).

The transfer of metabolites and/or lysis products from methanotrophs to non-methanotrophs was further supported by the pronounced ^{13}C enrichment of PLFA's representing non-methanotrophic organisms in the consortium (Figure 7c). This ^{13}C enrichment was delayed in time as compared to the enrichment of methanotrophic PLFA's (Figure 7b), indicating that non-methanotrophs grow on ^{13}C-labeled organic material produced by the methanotrophs. To our knowledge, neither 18:2 nor any PLFA's longer than C20 have been described from methane-oxidizing bacteria (represented by the unidentified PLFA's "$>$20:0 A" and "$>$20:0 B" in Figure 7c). However, 18:2ω6 was previously reported as PLFA specific for fungi (Boschker & Middelburg 2002). This PLFA (which may be included in the 18:2 group, Figure 7c) was not enriched with ^{13}C until the last sampling point after 10 days, which may represent a minor transfer of carbon from methanotrophs to unidentified fungi.

It was concluded above, that the methane-oxidizing bacteria in AAE-A2 were not directly involved in PAE degradation. Hence, organisms essential for the PAE degradation are probably found among the non-methanotrophs in the consortium. Fungal biomass may produce enzymes (e.g. esterase) which can contribute to phthalate hydrolysis (Kim et al. 2002, 2003). However, most studies so far, have shown that mainly bacteria carry out phthalate degradation (Jackson et al. 1996; Kurane 1986; Staples et al. 1997). The bacterial rRNA gene phylogenetic analysis (Figure 6) identified 4 non-methanotrophic genera. To our knowledge *Janthinobacterium* and *Rubivivax* have never been described as PAE degraders, whereas *Flavobacterium* related clones have previously been sequenced from a phthalic acid fed consortium (Lecouturier et al. 2003), and isolates belonging to *Flavobacterium* have also been obtained from a PAE enrichment culture (Jackson et al. 1996). In addition, *Pseudomonas* species have also been described as a members of PAE degrading consortia (Wang et al. 2003), and as isolates growing on PAE as sole energy and carbon source (Karpagam & Lalithakumari 1999; Zeng et al. 2000, 2002). Hence, it is likely that PAE degradation in AAE-A2 was carried out by *Flavobacterium* sp. and/or *Pseudomonas* sp., which were able to grow for prolonged periods in close co-existence with methanotrophs on methane derived metabolites and/or lysis products.

Perspectives and conclusions

A co-oxidation test confirmed the wide spread ability of methanotrophs to co-oxidize naphthalene, although only few naphthalene-oxidizing methanotrophic consortia were obtained from pristine environments.

We found one methanotrophic consortium enriched from activated sludge (AAE-A2), which degraded different PAEs, but mainly DBP. It was possible to maintain this consortium on methane for at least 5 month. The dominating type II methane-oxidizing bacterium in AAE-A2 was not directly involved in the degradation of PAE. However, the methane oxidizers in AAE-A2 were essential for the degradation of BTX, and AAE-A2 efficiently degraded benzene at 10–20 °C. Additionally, benzene degradation by AAE-A2 continued for weeks without inhibition by toxic degradation products. These results emphasize the potential importance of focusing on microbial consortia in biodegradation studies with methanotrophs.

Acknowledgements

This study was supported by EU (5th framework program, Grant FP QLK5-CT-2000-01528), post.doc grant # 26-03-0036 (Danish Technical Research Council) and framework program "Activity and Diversity in Complex Microbial Systems" (Danish Technical Research Council). Generous private support to MH from the R98-Foundation is highly appreciated. Kirsten Maagaard and Jacob Aarup provided technical assistance.

References

Alvarez-Cohen L & McCarthy PL (1991) Effects of toxicity, aeration and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol. 57: 228–235

Amann R, Binder BJ, Olson RJ, Chisholm SW, Devereux R & Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analysing mixed microbial populations. Appl. Environ. Microbiol. 56: 1919–1925

Aziz CE, Georgiou G & Speitel GE (1999) Cometabolism of chlorinated solvents and binary chlorinated solvent mixtures using *Methylosinus trichosporium* OB3b. Biotechnol. Bioeng. 65: 100–107

Bedard C & Knowles R (1989) Physiology, biochemistry, and specific inhibitors of CH_4, NH_4^+, and CO oxidation by methanotrophs and nitrifiers. Microb. Rev. 53: 68–84

Boschker HTS & Middelburg JJ (2002) Stable isotopes and biomarkers in microbial ecology. FEMS Microbiol. Ecol. 40: 85–95

Bowman JP & Sayler GS (1994) Optimization and maintenance of soluble methane monooxygenase activity in *Methylosinus trichosporium* OB3b. Biodegradation 5: 1–11

Bowman JP, Skerratt JH, Nichols PD & Sly LI (1991) Phospholipid fatty acid and lipopolysaccharide fatty acid signature lipids in methane-utilizing bacteria. FEMS Microbiol. Ecol. 85: 15—22

Boyd EM, Meharg AA, Wright J & Killham K (1997) Assessment of toxicological interactions of benzene and its primary degradation products (catechol and phenol) using a lux-modified bacterial bioassay. Environ. Toxicol. Chem. 16: 849–856

Brusseau GA, Tsien HC, Hanson RS & Wackett LP (1990) Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. Biodegradation 1: 19–29

Burback BL, Perry JJ & Rudd LE (1994) Effect of environmental pollutants and their metabolites on a soil *Mycobacterium*. Appl. Microbiol. Biotechnol. 41: 134–136

Burrows KJ, Conish A, Scott D & Higgins J (1984) Substrate specificities of the soluble and particulate methane monooxygenases of *Methylosinus trichosporium* OB3b. J. Gen. Microbiol. 130: 3327–3333

Chauret C, Inniss W & Mayfield CI (1996) Biotransformation at 10°C of di-*n*-butyl phthalate in subsurface microcosms. Ground Water 34: 791–794

Chu KH & Alvarez-Cohen L (1999) Evaluation of toxic effects of aeration and trichloroethylene oxidation on methanotrophic bacteria grown with different nitrogen sources. Appl. Environ. Microbiol. 65: 766–772

Colby J, Stirling DI & Dalton H (1977) Soluble methane monooxygenase of *Methylococcus capsulatus* (Bath)- ability to oxygenate normal-alkanes, normal-alkenes, ethers and alicyclic, aromatic and heterocyclic compounds. Biochem. J. 165: 395–402

Costa C, Dijkema C, Friedrich M, Garcia-Encina P, Fernandez-Polanco F & Stams AJM (2000) Denitrification with methane as electron donor in oxygen- limited bioreactors. Appl. Microbiol. Biotechnol. 53: 754–762

Dalton H, Wilkins PC & Jiang Y (1993) Structure and mechanism of action of the hydroxylase of soluble methane monooxygenase. In: Murrell JC & Kelly DP (Eds) Microbial Growth on C1 Compounds (pp 65–80). Intercept Ltd., Hampshire, UK

Dedysh SN, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Liesack W & Tiedje JM (2002) *Methylocapsa acidiphila* gen. nov., sp nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from *Sphagnum* bog. Int. J. Syst. Evolut. Microbiol. 52: 251–261

Dedysh SN, Liesack W, Khmelenina VN, Suzina NE, Trotsenko YA, Semrau JD, Bares AM, Panikov NS & Tiedje JM (2000) *Methylocella palustris* gen. nov., sp nov., a new methane-oxidizing acidophilic bacterium from peat bugs, representing a novel subtype of serine-pathway methanotrophs. Int. J. Syst. Evolut. Microbiol. 50: 955–969

DeLong EF (1992) Archaea in costal marine environments. Proc. Nat. Aca. Sci. USA 89: 5685–5689

Eller G, Stubner S & Frenzel P (2001) Group-specific 16S rRNA targeted probes for the detection of type I and type II methanotrophs by fluorescence *in situ* hybridisation. FEMS Microbiol. Lett. 198: 91–97

Fromme H, Kuchler T, Otto T, Pilz K, Muller J & Wenzel A (2002) Occurrence of phthalates and bisphenol A and F in the environment. Water Res. 36: 1429–1438

Graham DW, Korich DG, LeBlanc RP, Sinclair NA & Arnold RG (1992) Applications of colorimetric plate assay for soluble methane monooxygenase activity. Appl. Environ. Microbiol. 58: 2231–2236

Green J & Dalton H (1989) Substrate-specificity of soluble methane monooxygenase – mechanistic implications. J. Biol. Chem. 264: 17698–17703

Guckert JB, Ringelberg D, White DC, Hanson RS & Bratina BJ (1991) Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the Proteobacteria. J. Gen. Microbiol. 137: 2631–2641

Haigh SD (1996) A review of the interaction of surfactants with organic contaminants in soil. Sci. Total Environ. 185: 161–170

Hanson RS & Hanson TS (1996) Methanotrophic bacteria. Microb. Rev. 60: 439–471

Henry SM & Grbic-Galic D (1991) Influence of endogenous and exogenous electron donors and trichloroethylene oxidation toxicity on trichlororethylene oxidation by methanotrophic cultures from groundwater aquifer. Appl. Environ. Microbiol. 57: 236–244

Higgins DG, Best DJ & Hammond RC (1980) New findings in the methane-utilizing bacteria highlight their importance in the biosphere and their commercial potential. Nature 286: 561–564

Hrsak D (1995) Aerobic transformation of linear alkylbenzenesulphonates by mixed methane-utilizing bacteria. Arch. Environ. Contam. Toxicol. 28: 265–272

Hrsak D & Begonja A (2000) Possible interactions within a methanotrophic-heterotrophic groundwater community able to transform linear alkylbenzenesulfonates. Appl. Environ. Microbiol. 66: 4433–4439

Hrsak D & Grbic-Galic D (1995) Biodegradation of linear alkylbenzenesulphonates (LAS) by mixed methanotrophic-heterotrophic cultures. J. Appl. Bacteriol. 78: 487–494

Jackson MA, Labeda DP & Becker LA (1996) Isolation for bacteria and fungi for the hydrolysis of phthalate and terephthalate esters. J. Ind. Microbiol. 16: 301–304

Jianlong W, Ping L & Yi Q (1995) Microbial degradation of di-*n*-butyl phthalate. Chemosphere 31: 4051–4056

Jianlong W, Ping L & Yi Q (1999) Microbial metabolism of dibutyl phthalate (DBP) by a denitrifying bacterium. Proc. Biochem. 34: 745–749

Karpagam S & Lalithakumari D (1999) Plasmid-mediated degradation of o- and p-phthalate by *Pseudomonas fluorescens*. World J. Microbiol. Biotechnol. 15: 565–569

Kim YH, Lee JW, Ahn JY, Gu MB & Moon SH (2002) Enhanced degradation of an endocrine-disrupting chemical, butyl benzyl phthalate, by *Fusarium oxysporum* f. sp pisi cutinase. Appl. Environ. Microbiol. 68: 4684–4688

Knowles R (1990) Acetylene inhibition technique: development, advantages and potential problems. In: Revsbech NP & Sørensen J (Eds) Denitrification in Soil and Sediments, vol 1 (pp 151–166). Plenum Press, New York

Kim YH, Lee J & Moon SH (2003) Degradation of an endocrine disrupting chemical, DEHP [di-(2-ethylhexyl)-phthalate], by *Fusarium oxysporum* f. sp pisi cutinase. Appl. Microbiol. Biotechnol. 63: 75–80

Kofoed LH (1976) The feeding biology of *Hydrobia ventrosa* (mantagu). II. Allocation of the components of the carbon-budget and the significance of the secretion of dissolved organic material. J. Exp. Marine Biol. Ecol. 19: 243–246

Kurane R (1986) Microbial degradation of phthalate esters. Microbiol. Sci. 3: 92–95

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebradt E & Goodfellow M (Eds) Nucleic Acid Techniques in Bacterial Systematics (pp 115–175). John Wiley and Sons, New York

Lecouturier D, Godon JJ & Lebeault JM (2003) Phylogenetic analysis of an anaerobic microbial consortium deiodinating 5-amino-2,4,6-triiodoisophthalic acid. Appl. Environ. Microbiol. 62: 400–406

Manz W, Amann R, Ludwig W, Wagner M & Schleifer KH (1992) Phylogenetic oligodeoxynucleotide probes for major subclasses of proteobacteria: problems and solutions. Syst. Appl. Environ. Microbiol. 15: 593–600

Marmur J (1961) A procedure for the isolation of DNA (deoxyribonucleic acid) from microorganisms. J. Mol. Biol. 3: 208–218

Murrell JC, McDonald IR & Gilbert B (2000) Regulation of expression of methane monooxygenases by copper ions. Trends Microbiol. 8: 221–225

Nunes-Halldorson VD, Steiner RL & Smith GB (2004) Residual toxicity after biodegradation: interactions among benzene, toluene, and chloroform. Ecotoxicol. Environ. Safety 57: 162–167

Oldenhuis R & Janssen DB (1993) Degradation of tricloroethylene by methanotrophic bacteria. In: Kelly DP & Murrell JC (Eds) Microbial Growth on C1 Compounds (pp 121–133). Intercept Ltd., Hampshire, UK

Phelps PA, Agarwal SK, Speitel GE & Georgiou G (1992) *Methylosinus trichosporium* OB3b mutants having constitutive expression of soluble methane monooxygenase in the presence of high levels of copper. Appl. Environ. Microbiol. 58: 3701–3708

Rao GS (1996) Glutathionyl hydroquinone: a potent prooxidant and a possible toxic metabolite of benzene. Toxicol. 106: 49–54

Robbins GA, Wang S & Stuart JD (1993) Using the static headspace method to determine Henry's law constant. Anal. Chem. 65: 3113–3118

Roslev P & Iversen N (1999) Radioactive fingerprinting of microorganisms that oxidize atmospheric methane in different soils. Appl. Environ. Microbiol. 65: 4064–4070

Roslev P, Iversen N & Henriksen K (1998) Direct fingerprinting of metabolically active bacteria in environmental samples by substrate specific radiolabelling and lipid analysis. J. Microbiol. Methods 31: 99–111

Stanley SH & Dalton H (1992) The biotransformation of propylene to propylene oxide by *Methylococcus capsulatus* (Bath): 1. Optimization of rates. Biocatalysis 6: 163–175

Stanley SH, Richards A, Suzuki M & Dalton H (1992) The biotransformation of propylene to propylene oxide by *Methylococcus capsulatus* (Bath): 2. A study of the biocatalyst stability. Biocatalysis 6: 177–190

Staples CA, Peterson DR, Parkerton TF & Adams WJ (1997) The environmental fate of phthalate esters: a literature review. Chemosphere 35: 667–749

Sullivan JP, Dickinson D & Chase HA (1998) Methanotrophs, *Methylosisnus trichosporium* OB3b, sMMO, and their application to bioremediation. Crit. Rev. Microbiol. 24: 335–373

Wackett LP & Gibson DT (1983) Rapid method for detection and quantification of hydroxylated aromatic intermediates produced by microorganisms. Appl. Environ. Microbiol. 45: 1144–1147

Wang Y, Fan Y & Gu JD (2003) Aerobic degradation of phthalic acid by *Comamonas acidovoran* Fy-1 and dimethyl phthalate ester by two reconstituted consortia from sewage sludge at high concentrations. World J. Microbiol. Biotechnol. 19: 811–815

Wang JL, Liu P, Shi HC & Qian Y (1998) Kinetics of biodegradation of di-*n*-butyl phthalate in continuous culture system. Chemosphere 37: 257–264

Yan H, Ye CM & Yin CQ (1995) Kinetic of phthalate ester biodegradation by *Chlorella pyrenoidosa*. Environ. Toxicol. Chem. 14: 931–938

Yeates C & Gillings MR (1998) Rapid purification of DNA from soil for biodiversity analysis. Lett. Appl. Microbiol. 27: 49–53

Yuan SY, Liu C, Liao CS & Chang BV (2002) Occurrence and microbial degradation of phthalate esters in Taiwan river sediments. Chemosphere 49: 1295–1299

Zeng F, Cui KY, Fu JM, Sheng GY & Yang HF (2002) Biodegradability of di(2-ethylhexyl) phthalate by *Pseudomonas fluorescens* FS1. Water Air Soil Pollut. 140: 297–305

Zeng F, Fu JM, Sheng GY, Yang HF & Zhang Z (2000) Study on the enzymatic degradability of di-*n*-butyl phthalate. Chin J. Appl. Environ. Biol. 6(5)

Zurmühl T (1990) Development of a method for the determination of phthalate esters in sewage sludge including chromatographic separation from polychlorinated biphenyls, pesticides and polyaromatic hydrocarbons. Analyst 115: 1171–1175