Environmental Microbiology (2008) 10(6), 1557–1570 doi:10.1111/j.1462-2920.2008.01571.x

Novel uncultured *Chloroflexi* dechlorinate perchloroethene to *trans*-dichloroethene in tidal flat sediments

Sandra Kittelmann and Michael W. Friedrich*
Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch Strasse, D-35043 Marburg, Germany.

Summary

The marine environment represents a rich source of bio- and geogenically produced organohalogens, including the common pollutant perchloroethene (PCE). However, diversity and function of marine chloroethene-dechlorinating microorganisms are largely unknown. Here, we have studied the activity and composition of a tidal flat sediment bacterial and archaeal community from the North Sea exposed to low concentrations of PCE. After 2 weeks of incubation, PCE was rapidly dechlorinated via trichloroethene to dichloroethene (DCE). Unexpectedly, these microcosms produced 3.5-fold more *trans*-DCE than *cis*-DCE. The actively dechlorinating microbial populations were traced by stable isotope probing of rRNA with ^{13}C-labelled acetate for 4 days. Terminal restriction fragment length polymorphism fingerprinting and clone libraries of isotopically enriched, 'heavy' ^{13}C-labelled bacterial 16S rRNA revealed the populations potentially involved in reductive dechlorination. Major clone groups belonged to the *Proteobacteria* (50.0%; 22.4% δ-, 12.1% γ-, 6.9% α-, 6.9% β- and 1.7% ε-subgroup) and *Chloroflexi* (29.3%). Populations represented by the two dominant terminal restriction fragments were affiliated with the *Dehalococcoidetes* (subphylum II of the *Chloroflexi*), and were exclusively detected in the heavy fraction of the PCE-dechlorinating incubation. The phylogenetically novel, larger population, designated Tidal Flat *Chloroflexi* Cluster, was closely related to the recently discovered PCE-dechlorinating Lahn Cluster bacteria from anoxic river sediment but more distantly related to canonical *Dehalococcoides* spp. (92–94% sequence identity). The second population was closely related to '*Dehalobium chlorocoercia* DF-1'. Both populations appear to be responsible for reductive dechlorination of highly chlorinated ethenes to predominantly *trans*-DCE in tidal flat sediment incubations.

Received 4 October, 2007; accepted 24 January, 2008. *For correspondence. E-mail michael.friedrich@mpi-marburg.mpg.de; Tel. (+49) 6421 178 430; Fax (+49) 6421 178 429.

Introduction

Halogenated organic compounds represent one of the largest groups of environmentally hazardous chemicals. Highly chlorinated ethenes such as tetrachloroethene (perchloroethene, PCE) and trichloroethene (TCE) have been excessively used as solvents in dry-cleaning, metal-degreasing and paper-milling applications (Abelson, 1990). Inappropriate discharge has resulted in widespread dispersal and environmental contamination, with rivers, estuaries and coastal marine sediments as significant temporary sinks (De Rooij *et al*., 1998; Mazur and Jones, 2001; Christof *et al*., 2002).

While resistant to aerobic degradation, under anoxic conditions, PCE can be reductively dechlorinated via TCE, dichloroethene (DCE) isomers (*cis*-, *trans*- and 1,1-DCE), and vinyl chloride (VC) to the non-chlorinated benign end-product ethene (for reviews see Holliger *et al*., 1998; Smidt and de Vos, 2004). Some bacteria are even capable of coupling reductive PCE dechlorination to energy conservation in a process called 'dehalorespiration' (Holliger *et al*., 1998) or 'chlororespiration' (Löffler *et al*., 1996). Chloroethene dehalorespirers are highly substrate-specific and gain energy from the transfer of electrons to PCE and/or its less chlorinated congeners (Holliger and Schraa, 1994).

Past research efforts have largely focused on remediation of contaminated ground water aquifers and industrial sites (US Environmental Protection Agency, 2006), and thus, only little is known to date about reductive dechlorination of PCE in marine habitats. The marine environment, however, is an important source of naturally occurring halogenated compounds, including highly chlorinated ethenes. Marine organisms, such as marine algae, produce PCE and TCE as secondary metabolites (Abrahamsson *et al*., 1995; Häggblom *et al*., 2003). Furthermore, subsurface volcanic emissions and pyrogenic activity contribute highly chlorinated ethenes to marine waters (Gribble, 1994; 2003). Nonetheless, the diversity

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd

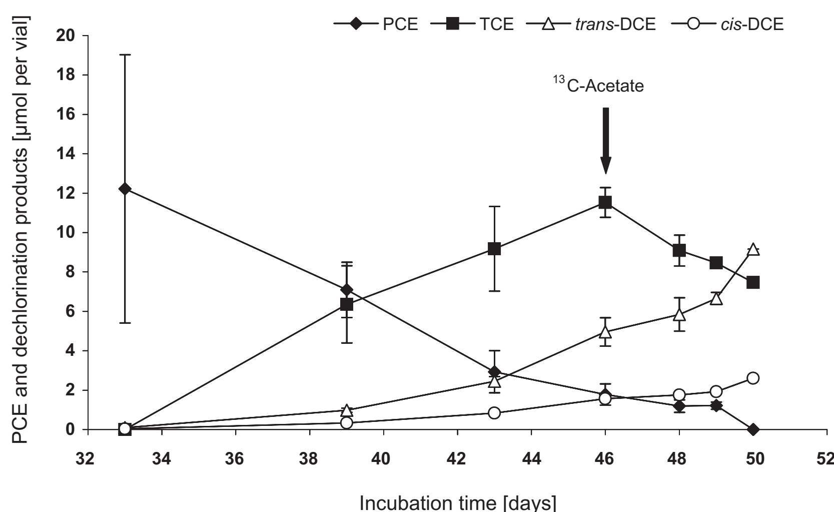

Fig. 1. Time-course of PCE dechlorination in tidal flat sediment incubations. Perchloroethene (◆) dechlorination led to accumulation of TCE (■), which was then further transformed to predominantly *trans*- (△) and small amounts of *cis*-DCE (○) in four parallels of tidal flat sediment microcosms incubated at 20°C. The arrow indicates the time point of [^{13}C]-acetate addition.

and structure of dechlorinating microbial communities in marine habitats are yet poorly understood. A thermophilic enrichment culture inoculated with chloroethene-contaminated Rotterdam harbour sediments was dominated by *Dehalobacter restrictus* and *Desulfotomaculum thermosapovorans*-related populations, both potentially involved in PCE dechlorination (Kengen *et al*., 1999). A highly enriched culture containing the PCB-dechlorinating strain '*Dehalobium chlorocoercia* DF-1' from estuarine sediments (Wu *et al*., 2000) also dechlorinated PCE to DCE, however, at a ratio of *trans*-DCE to *cis*-DCE of 1.5 (Miller *et al*., 2005). Most known isolates capable of PCE dechlorination to DCE preferentially produce the *cis*-isomer (Holliger *et al*., 1993; Neumann *et al*., 1994; Krumholz *et al*., 1996; Sharma and McCarty, 1996; Chang *et al*., 2000; De Wever *et al*., 2000).

Conversely, *trans*-DCE appears to be frequently present at contaminated sites, for example at 39% of current or former US Environmental Protection Agency National Priority List Sites compared with 10% for *cis*-DCE (Agency for Toxic Substances and Disease Registry, 1996). Source contamination or abiotic dechlorination mechanisms have been implicated for these sites (Griffin *et al*., 2004). However, a few enrichment studies have proven that reductively dechlorinating bacteria are capable to produce *trans*-DCE. A 1,2-dichloropropane-dechlorinating enrichment culture from Red Cedar creek sediment dechlorinated PCE to *trans*- and *cis*-DCE at a ratio of 2.5 (Löffler *et al*., 1997); and enrichment cultures from river sediments containing *Dehalococcoides*-related species, produced threefold more *trans*- than *cis*-DCE (Griffin *et al*., 2004).

Evidence is accumulating that the diversity of dehalorespiring microorganisms in the environment is largely underestimated (Kittelmann and Friedrich, 2008). Recently, we traced dehalorespiring bacteria as a functional guild by RNA-based stable isotope probing (SIP; Manefield *et al*., 2002; Dumont and Murrell, 2005; Friedrich, 2006) with ^{13}C-labelled acetate as carbon source. RNA-based SIP allows linking the function of certain populations to phylogeny by incorporation of the heavier carbon isotope (^{13}C) into RNA of metabolically active populations. The major actively PCE-respiring and acetate assimilating population in a complex underlying river sediment microbial community was a novel bacterial cluster (Lahn Cluster) only distantly related to dehalorespiring *Dehalococcoides* spp. (Kittelmann and Friedrich, 2008).

In the present study, we identified chloroethene-dechlorinating bacterial populations from a diverse tidal flat sediment microbial community that produced *trans*-DCE as major end-product at a ratio of ~3.5 compared with the *cis*-isomer.

Results

Dechlorination of PCE to predominantly trans-*DCE*

Incubations with pristine North Sea tidal flat sediments were initiated to evaluate the PCE dechlorination potential in a marine habitat. Microcosms were kept at 20°C, reflecting the temperature average of the sampling site during the summer season (Köpke *et al*., 2005). A non-aqueous liquid phase was used to maintain PCE at low aqueous concentration (≤ 5 µM), and exogenous electron donors were not added. Perchloroethene dechlorination activity was detected in tidal flat samples already after 12 days of incubation (data not shown). After transfer into microcosms and renewed amendment with PCE (after 33 days of incubation), TCE was dechlorinated at high rate (~750 nmol vial^{-1} day^{-1}), and TCE rapidly accumulated in microcosms (Fig. 1). In parallel, TCE was turned

© 2008 The Authors

over after a lag phase of 12 days to predominantly *trans*-DCE (960 nmol vial^{-1} day^{-1}) and only small amounts of *cis*-DCE (250 nmol vial^{-1} day^{-1}). Up to 3.5-fold more *trans*-DCE was produced over the *cis*-isomer (Fig. 1). Sediment endogenous electron acceptors other than CO_2 had been depleted almost completely (residual sulfate concentration ~330 μM) at the end of the pre-incubation. In the PCE-amended microcosms, methane was formed at lower rate (~1 μmol vial^{-1} day^{-1}) than in non-PCE-amended control microcosms (2.3 μmol vial^{-1} day^{-1}). Final CH_4 pool sizes were ~12 μmol in the incubation with PCE and ~27 μmol in the incubation without PCE (data not shown).

Stable isotope probing with [^{13}C]-acetate

Sediment slurries with and without PCE were incubated for altogether 47 days before SIP of rRNA was initiated by the addition of $^{13}C_2$-labelled acetate (0.5 mM) as carbon source and potential electron donor. [^{13}C]-acetate, an important intermediate of the anaerobic food chain in marine sediments (Jørgensen, 1978; Ansbaek and Blackburn, 1980; Sansone and Martens, 1982), was rapidly turned over to [^{13}C]-CO_2 and [^{13}C]-methane (data not shown). While in both incubation types, large fractions of total methane were formed from [^{13}C]-acetate (31% and 26%, respectively, after 4 days of incubation) the total amount of $^{13}CH_4$ produced in the microcosm with PCE (~1.2 μmol) was lower compared with the non-PCE-amended control (~2.4 μmol).

Addition of ^{13}C-labelled acetate stimulated the formation of *trans*-DCE from mainly TCE, which was the predominant electron acceptor during SIP. Acetate was not limiting (130 μM residual acetate) and was the predominant carbon source either assimilated or metabolized to CO_2 and CH_4 (3.4 μmol consumed). In the microcosm with PCE, small amounts of formate (0.4 μmol) were consumed (36 μM residual formate) and small amounts of propionate were produced (0.5 μmol; 125 μM residual propionate). In the control without PCE, acetate was consumed in similar amounts (2.2 μmol; 148 μM residual acetate), but formate concentrations did not change during SIP (73 μM residual formate).

In PCE-amended tidal flat sediment microcosms and in the controls, hydrogen partial pressures (pH_2) were 749 and 686 Pa, respectively, indicating that syntrophic acetate oxidation was thermodynamically unfavourable.

Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of bacterial populations

Perchloroethene-dechlorinating bacteria were identified in tidal flat sediment incubations by RNA-based SIP combined with terminal restriction fragment length polymorphism (T-RFLP) analysis, cloning and sequencing of bacterial 16S rRNA. After 4 days of incubation in the presence of ^{13}C-labelled acetate, total RNA was extracted from the incubation with PCE (TfC20, tidal flat contaminated) and the control without PCE amendment (TfP20, tidal flat pristine), and ^{13}C-enriched, 'heavy' RNA was separated from unlabelled, 'light' RNA by isopycnic centrifugation. Density resolved bacterial rRNAs from gradient fractions were analysed by polymerase chain reaction (PCR)-based T-RFLP fingerprinting to detect specific members of the tidal flat sediment bacterial community that had incorporated ^{13}C-label into their rRNA and, therefore, had potentially been dechlorinating PCE (Fig. 2).

Terminal restriction fragment length polymorphism analysis revealed that distinct populations were involved in label assimilation under the experimental conditions prevailing (Fig. 2A). Fully labelled rRNA is typically found in fractions with buoyant densities (BD) between 1.81 and 1.82 g ml^{-1} (Lueders *et al.*, 2004a). A shift from highly diverse light fractions (BD $<$ 1.80 g ml^{-1}) to a much lower diversity in heavy gradient fractions (BD $>$ 1.80 g ml^{-1}) was observed only in the incubation amended with PCE as electron acceptor. The differential incorporation of ^{13}C-label by active bacterial populations resulted in the presence of only two major (513 and 143 bp) and two minor terminal restriction fragments (T-RFs) (~164 and 498 bp) in the high density fractions. These populations accounted for altogether 70% of the total bacterial amplicon pool based on peak heights. Two of these four populations (T-RFs of 143 and 513 bp) were predominant in the high-density fractions of the PCE-amended tidal flat sediment incubation but were not present in the control (Fig. 2B). Instead, in the control incubation the 513 bp T-RF was represented by different populations as revealed by cloning and sequencing (Table 1). The presence of only a few characteristic populations in the heavy-gradient fraction of the PCE-amended microcosm indicated an effective enrichment of populations able to utilize the ^{13}C-labelled acetate from the total bacterial community; these populations either were not present in the light fractions at all as suggested by the clone libraries (Table 1, T-RFs of 513 and 143 bp) or accounted for only a small proportion of the total amplicon pool (T-RFs of ~164 and 498 bp) as indicated by low proportions of the total heights of all T-RFs (Table 1). In the control, a specific incorporation of label into bacterial rRNA was not detected. All fractions showed very similar bacterial community profiles (Fig. 2B).

Identification of dechlorinating bacterial populations

Bacteria found to specifically incorporate ^{13}C-label under PCE- and TCE-dechlorinating conditions (Fig. 1), were identified by cloning and sequencing analysis; libraries

© 2008 The Authors

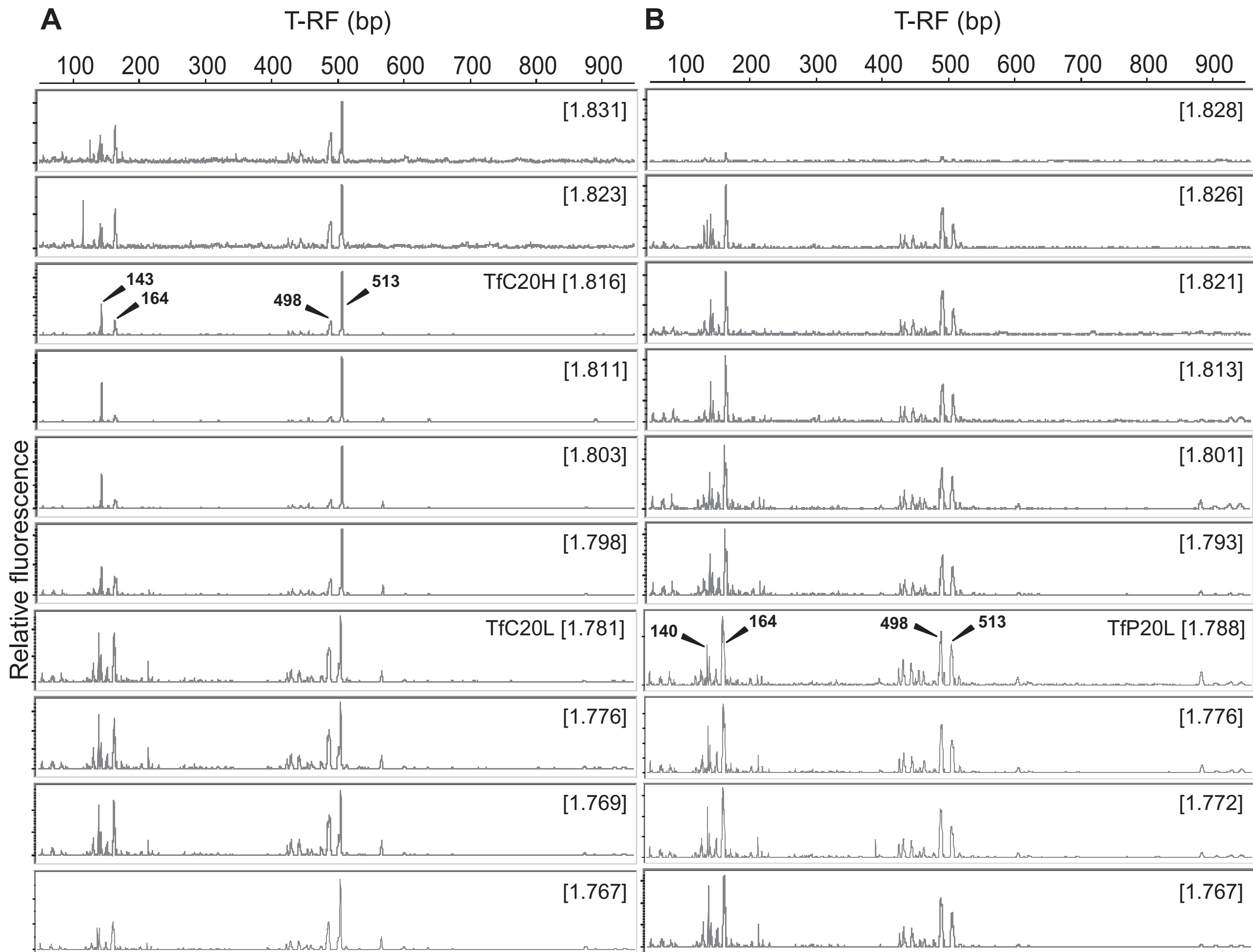

Fig. 2. Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of density resolved bacterial 16S rRNA from (A) a *trans*-DCE-forming microcosm with PCE, and (B) a control microcosm without PCE. CsTFA buoyant densities of fractions (BDs in grams per millilitre) are given in brackets. Clone libraries were generated from fractions TfC20H, TfC20L and TfP20L. Lengths of major T-RFs are indicated.

with rRNA from a heavy (TfC20H, BD = 1.816 g ml^{-1}) and a light (TfC20L, BD = 1.781 g ml^{-1}) gradient fraction of the incubation with PCE as well as a light fraction of the control microcosm (TfP20L, BD = 1.788 g ml^{-1}) were generated. In total, 136 clones were analysed from all libraries.

The majority of clones obtained from the heavy-gradient fraction (TfC20H) belonged to the *Proteobacteria* (50.0%) and *Chloroflexi* (29.3%). The two major [^{13}C]-acetate-assimilating populations that were exclusively found in the heavy fraction under PCE- and TCE-dechlorinating conditions (represented by the T-RFs of 513 and 143 bp) were both associated with the '*Dehalococcoidetes*', subphylum II of the *Chloroflexi*. The dominant clone cluster, designated Tidal Flat *Chloroflexi* Cluster [TFCC; Accession No. for representative partial 16S rRNA sequence: EU362187 (clone TfC20H06)] with a T-RF of 513 bp, was closely related to Lahn Cluster bacteria (98% sequence identity) previously identified to dechlorinate PCE to *cis*-DCE in anoxic river sediments incubated at 15°C (Kittelmann and Friedrich, 2008) and was only distantly related to *Dehalococcoides* isolates (92–94% sequence identity, Fig. 3). The second major group of clones was represented by a characteristic T-RF of 143 bp length and was closely related to the dehalorespiring '*D. chlorocoercia* DF-1' (98% sequence identity; Fig. 3; Miller *et al*., 2005). Among the *Proteobacteria* representatives belonging to the δ-subgroup were most abundant (22.4%). Nine clones (15.5%) were closely related to sulfate-reducing δ-*Proteobacteria*; however, similar clones were abundantly present in the clone library of the control incubation without PCE (Table 1, Fig. 4). Three clones (T-RFs of 133 and 162 bp) were related to the PCE-dechlorinating *Desulfuromonas michiganensis* strains BB1 and BRS1 (97% sequence identity; Fig. 4), and to the dehalorespiring *Desulfomonile tiedjei* (1 clone, T-RF of 129 bp, 97% sequence

© 2008 The Authors

Table 1. Relative abundances (in per cent) of major phylogenetic bacterial and archaeal groups in the analysed fractions of the microcosm incubated with PCE at 20°C (TfC20H and TfC20L) and the control (TfP20L) based on frequencies in 16S rRNA clone library and T-RFLP analysis.

Analysed fraction BD (g ml^{-1})	TfP20L (1.784)		TfC20L (1.781)		TfC20H (1.816)		
Phylogenetic lineage	Clone library	T-RFLP	Clone library	T-RFLP	Clone library	T-RFLP	Characteristic T-RF lengths (bp)[a]
Bacteria							
Actinobacteria	5.6	–	4.8	–	5.2	–	178
Bacteroidetes	–[b]	–	2.4	1.6	–	–	89
Chloroflexi							
Anaerolinea	8.3	7.9	4.8	14.1	6.9	2.6	513[c]
Dehalobium spp.	–	–	–	–	6.9	16.5	**143**
TFCC bacteria	–	–	–	–	15.5	25.8[d]	**513**
Cyanobacteria	11.1	6.7	2.4	5.7	6.9	4.8	**496–498**
Deferribacteres	–	–	–	–	1.7	–	153
Firmicutes	–	–	–	–	1.7	–	148
Planctomycetes	11.1	–	2.4	–	3.4	–	Diverse
Proteobacteria							
α-*Proteobacteria*	2.8	–	–	–	6.9	–	130, 152, 439
β-*Proteobacteria*	2.8	2.6	–	2.2	6.9	1.9	**432**
γ-*Proteobacteria*	11.1	14.9	31.0	14.0	12.1	12.2	82, 141, 432, 494
δ-*Proteobacteria*	36.1	34.2	47.6	28.7	22.4	18.4	**163–168**, 513
ε-*Proteobacteria*	8.3	–	2.4	–	1.7	–	466, 470
Spirochaetes	–	–	2.4	2.7	–	–	162
Verrucomicrobia	2.8	–	–	–	–	–	144
Archaea							
Crenarchaeota	4.7	–	2.2	–	n.d.[e]	n.d.	186, 687
Methanomicrobiales							
Methanocorpusculaceae	7.0	–	–	–	n.d.	n.d.	186
Methanomicrobiaceae	18.6	12.0	42.2	28.7	n.d.	n.d.	186, **393**
Methanosarcinales							
Methanosarcinaceae	37.2	42.7	26.7	34.3	n.d.	n.d.	**186**, 681, 794/5
Methanosaetaceae	25.6	34.2	15.6	19.1	n.d.	n.d.	**228**, **284**, 495
Thermoplasmatales	4.7	5.6	6.7	8.0	n.d.	n.d.	381, 792
Diverse	–	–	–	–	1.7	–	

a. Terminal restriction fragments detected for a major number of clones within a lineage.
b. No determination of relative abundance due to the lack of a distinct assignable T-RF.
c. Terminal restriction fragment shared by few clones from light fractions.
d. Relative abundance corrected by fractions of δ-proteobacterial clones possessing the same T-RF.
e. Not determined, as no archaeal 16S rRNA was obtained from this fraction.
Only T-RFs with a peak height > 1% of the total peak height of the electropherogrammes were considered. Major T-RFs are highlighted in bold.

identity; Fig. 4). Smaller clone groups ($n \leq 4$ clones) of the heavy-gradient fraction belonged to the *Cyanobacteria* (Fig. 4), *Actinobacteria*, *Firmicutes*, *Planctomycetes* and *Deferribacteres*.

The light fraction of the incubation with PCE mainly consisted of γ- and δ-*Proteobacteria* (78.6%), *Actinobacteria* (4.8%) and *Chloroflexi* (4.8%), whereas major phylogenetic groups in the light fraction of the non-PCE-amended control were γ- and δ-*Proteobacteria* (61.1%), *Cyanobacteria* (11.1%), *Planctomycetes* (11.1%) and *Chloroflexi* (8.3%). *Chloroflexi*-associated clones obtained from the light-gradient fractions did not cluster within subphylum II, *Dehalococcoidetes*, which is formed by various chloroethene-dechlorinating isolates and environmental sequences, but within subphylum I, *Anaerolineae* (Table 1). Single clones retrieved from the light fractions belonged to the *Bacteroidetes*, *Cyanobacteria*, *Planctomycetes* and *Spirochaetes* (TfC20L), and to the *Actinobacteria* and *Verrucomicrobia* (TfP20L).

Relative abundances in T-RFLP patterns and relative clone frequencies were compared by the assignment of the majority of clone sequences to their corresponding T-RFs (Table 1). Differences were found with respect to the frequencies of 16S rRNA amplicons related to the *Actinobacteria*, *Planctomycetes* and α- and ε-*Proteobacteria*, which were apparently 5–8% over-represented in clone libraries, whereas those of *Dehalobium* spp. appeared to be under-represented. Based on peak height, the 513 bp T-RF of the putatively chloroethene-dechlorinating TFCC bacteria accounted for 25.8% of the total amplicon pool; however, only 15.5% of clone sequences belonged to the TFCC (Table 1).

T-RFLP fingerprinting of archaeal populations

After SIP, the active archaeal populations in the incubations with and without PCE amendment were analysed by T-RFLP fingerprinting of all density gradient frac-

© 2008 The Authors

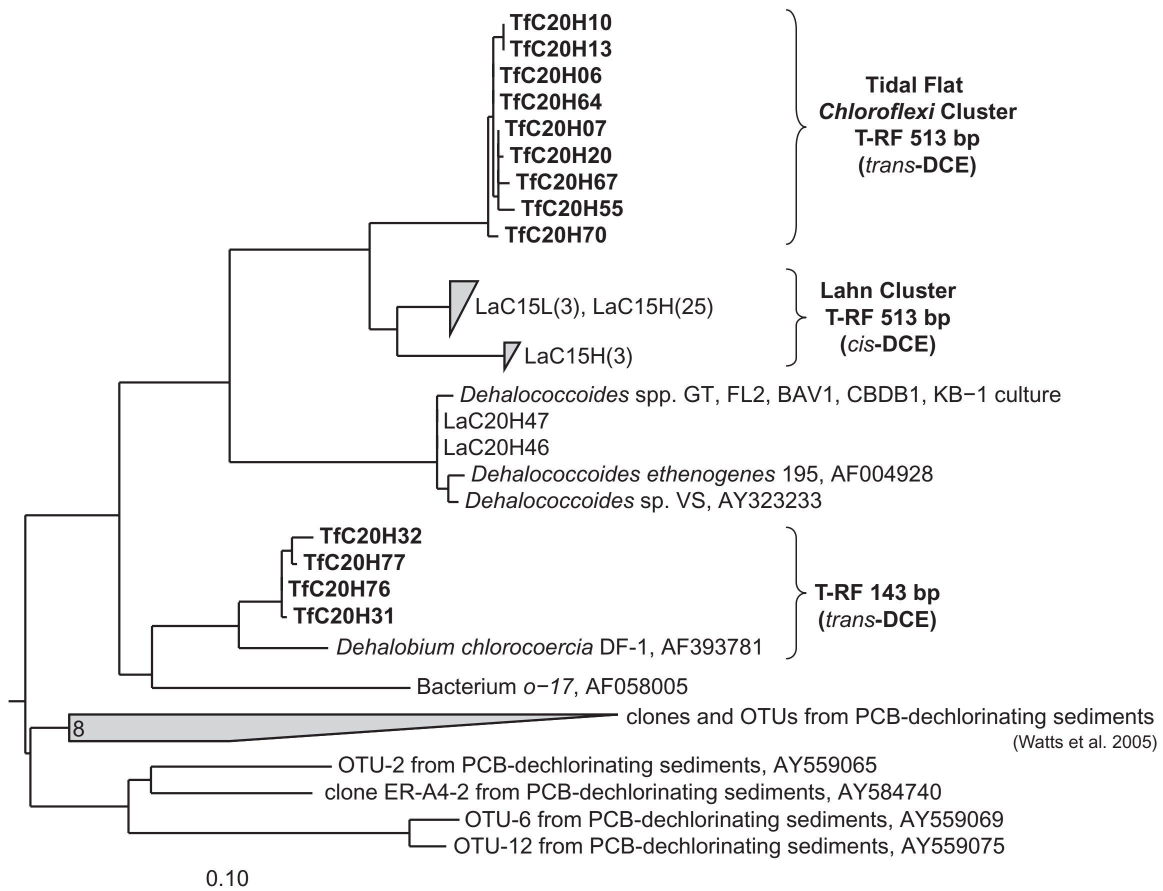

Fig. 3. Distribution of 16S rRNA clone sequences and phylogenetic placement of 'Tidal Flat *Chloroflexi* Cluster' bacteria as novel clone cluster within the *Dehalococcoidetes.* The scale bar represents 10% sequence divergence. *Thermomicrobium roseum* (M34115) and *Sphaerobacter thermophilus* (X53210) served as outgroup.

tions (Fig. 5). As methanogens are known anaerobic acetate degraders, we analysed the influence of PCE-dechlorinating conditions on the composition of the archaeal community. Gradient fractions with a BD > 1.81 g ml^{-1} did not yield sufficient PCR product (concentrations were below 10 ng μl^{-1}) for performing T-RFLP analysis. The remaining lighter fractions (BD < 1.81 g ml^{-1}) of both the PCE-amended incubation and the control showed four major T-RFs of 186, 228, 284 and 393 bp. The 186 bp T-RF was dominant in both incubations. In contrast, the T-RF of 393 bp was abundant only in the presence of PCE and the T-RF of 284 bp was only detected in the control microcosm without PCE.

Phylogenetic affiliation of archaeal populations

To identify the archaeal populations present in the tidal flat sediment incubations, clone libraries were constructed of a light fraction of both the PCE-amended microcosm [TfC20L, 1.781 g ml^{-1}] and the control [TfP20L, 1.788 g ml^{-1}]. A total of 88 clones were phylogenetically analysed. Clone sequences belonged to four different classes within the *Euryarchaeota*, and to a class represented only by sequences of yet uncultivated *Crenarchaeota* (Fig. 6, Table 1). The majority of clones from the incubation with PCE fell into the *Methanomicrobiales* (42.2%; T-RF of 393 bp) and *Methanosarcinales* (42.2%; T-RFs of 186, 228 and 284 bp). In the control, however, the *Methanosarcinales* were predominant (62.8%) and the *Methanomicrobiales* only played a minor role (25.6%). The proportion of *Methanosarcinaceae* (~60%; 186 bp) and *Methanosaetaceae* (~40%; 228 and 284 bp) from total *Methanosarcinales* clones was almost equal in both incubations. A small number of clones clustered within the euryarchaeal *Thermoplasmatales* and *Crenarchaeota* (Fig. 6, Table 1). Terminal restriction fragment abundance and clone frequencies observed in the libraries were in good agreement except for the *Methanomicrobiales*. Their high frequency according to the clone library was not confirmed by relative abundances in T-RFLP due to the ambiguity of the 186 bp T-RF, which is representative of mostly *Methanosarcinaceae* but also some *Methanocorpusculaceae* and *Methanomicrobiaceae* (Table 1, Fig. 6).

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd,

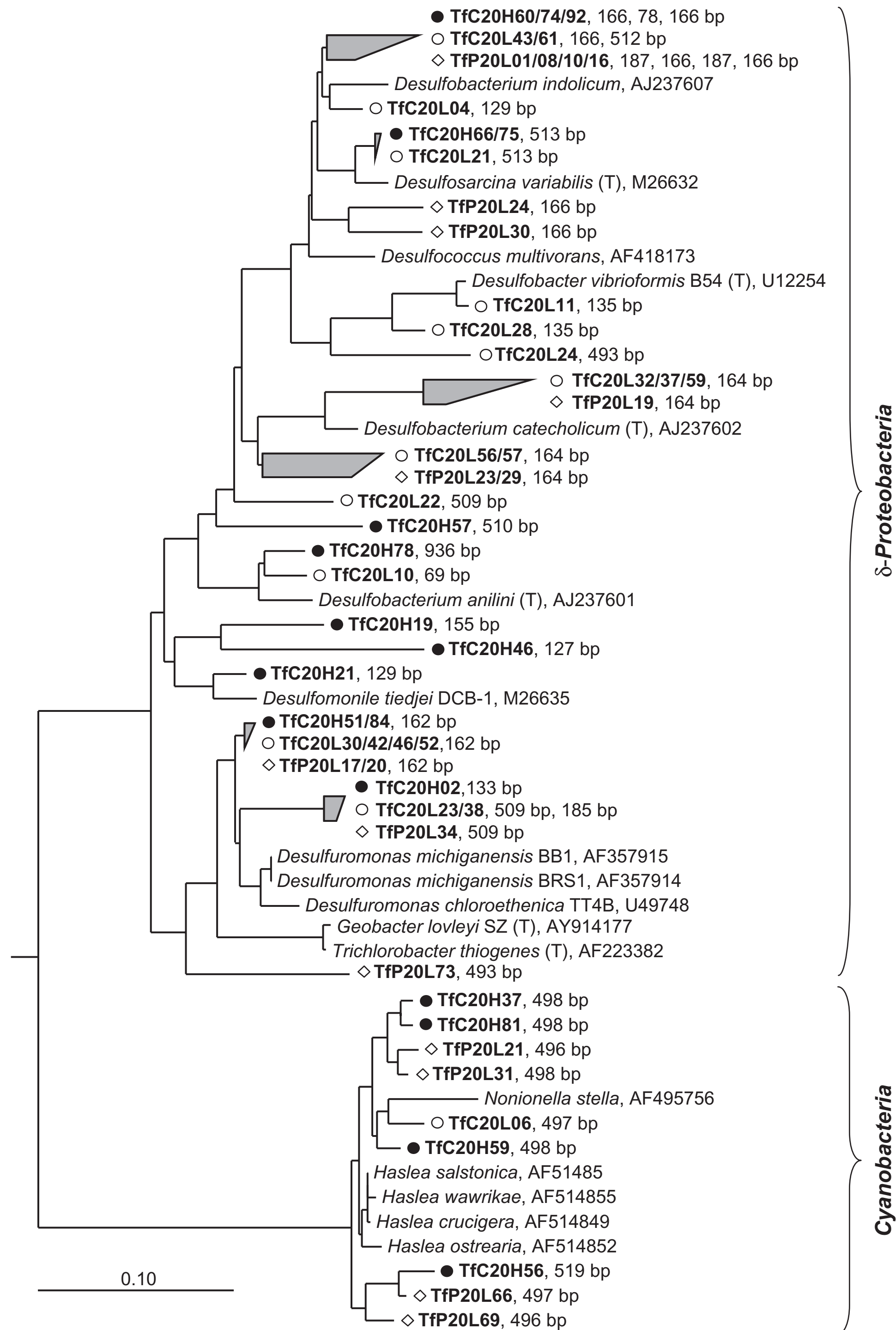

Fig. 4. Phylogeny of clones within the δ-subgroup of the *Proteobacteria* and *Cyanobacteria* based on 16S rRNA. Clone libraries from which the clones were obtained are marked as follows: TfC20H (●), TfC20L (○), TfP20L (◇). The scale bar indicates 10% sequence divergence. Archaeal sequences of *Methanosarcina* spp. and *Methanogenium* spp. served as outgroup for tree construction.

Diversity of bacterial and archaeal communities

The diversity of the microbial communities in microcosms with and without PCE was compared using standard indices of diversity based on T-RF lengths and peak heights. Despite the fact that the PCE-amended microcosm was incubated differently for a considerable period of time (51 days), the underlying overall bacterial diversity was not affected much, as indicated by Simpson's dominance index (SDI), a statistical means for the estimation

© 2008 The Authors

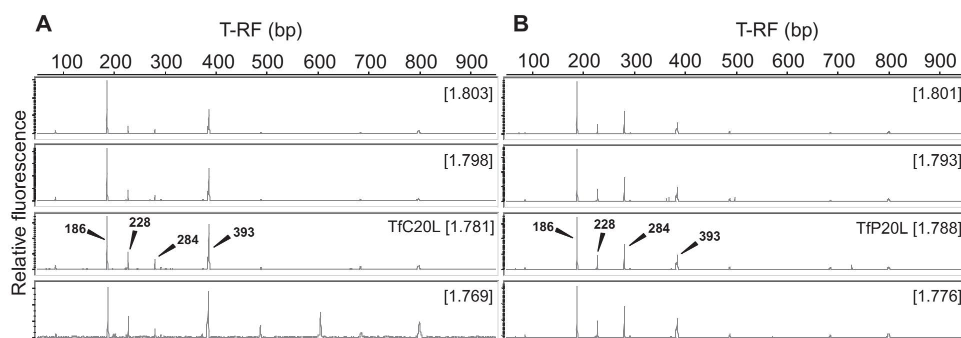

Fig. 5. Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of density resolved archaeal 16S rcRNA from (A) a *trans*-DCE forming microcosm amended with ^{13}C-labelled acetate, and (B) a control microcosm only amended with ^{13}C-labelled acetate. CsTFA buoyant densities (BDs) of fractions (in grams per millilitre) are given in brackets, and clone libraries were constructed from fractions TfC20L and TfP20L. Terminal restriction fragment lengths of major peaks are indicated.

of diversity in an environmental sample (Dollhopf *et al*., 2001). Both light fractions TfC20L and TfP20L were highly diverse bacterial communities (SDI of 0.06). Archaeal diversity was considerably lower but similar in both analysed fractions with SDI of 0.23 for TfC20L and 0.26 for TfP20L. The Morisita index of community similarity (I_M), which takes into account not only diversity but also species abundance, revealed that despite the long preincubation bacterial communities of the two different treatments still shared a similarity of 78%. Archaeal communities of the incubations with and without PCE were even more identical (88%).

Screening for known reductive dehalogenase genes

In order to evaluate whether reductive dechlorination of PCE in tidal flat incubations was catalysed by known reductive dehalogenases (RDases) such as PceA- or TceA-related enzymes, we carried out a PCR targeting reductive dehalogenase genes. The degenerate primers used are mainly based on amino acid sequences encoding for two highly conserved iron-sulfur (FeS) cluster-binding motifs typically found in reductive dehalogenases of *Sulfurospirillum multivorans* (*pceA*), *Desulfitobacterium dehalogenans* (*cprA*) and *Dehalococcoides ethenogenes* 195 (*tceA*; Rhee *et al*., 2003). No amplicons were obtained for the tidal flat sediments incubated in the presence of PCE and TCE; however, despite their high degree of sequence degeneracy the used primer combination obviously did not cover the full range of so far known reductive dehalogenases. Alternative RDase gene-specific primers available may allow for a more thorough screening of our samples in future.

Discussion

Using SIP of rRNA, we identified novel bacteria within the phylum *Chloroflexi* that are apparently dechlorinating PCE reductively in anoxic tidal flat sediment incubations. The predominant population, designated TFCC bacteria, is phylogenetically novel, yet closely related to previously identified Lahn Cluster bacteria (98% 16S rRNA sequence identity; Kittelmann and Friedrich, 2008) and only distantly related to the well-studied *Dehalococcoides* isolates (92–94% sequence identity). A second, albeit smaller population, shares 98% sequence identity with '*D. chlorocoercia* DF-1' (Miller *et al*., 2005). Both populations are likely responsible for dechlorination, because they were exclusively detected in incubations amended with PCE but were undetectable in the control and in the light fraction of the PCE-amended incubations. Unexpectedly, the *trans*-isomer of DCE was the major end-product of PCE dechlorination in tidal flat incubations at very high *trans*- to *cis*-DCE ratios (compare Griffin *et al*., 2004; Miller *et al*., 2005), whereas typically *cis*-DCE has been reported as the predominant intermediate or end-product for most enrichments studied so far (Smidt and de Vos, 2004).

Stable isotope probing of chloroethene-reducing bacteria in marine sediment

Recently, we have shown that dehalorespiring bacteria can be targeted as a functional guild by RNA-based SIP with [^{13}C]-acetate (Kittelmann and Friedrich, 2008). As chlorinated ethenes are not assimilated into biomass of dehalorespiring bacteria, ^{13}C-labelled acetate was used to trace dehalorespiring bacteria, when highly chlorinated ethenes were the predominant electron acceptors in Lahn river sediment incubations. This labelling strategy capitalizes on the fact that dissimilatory acetate oxidation is thermodynamically unfavourable under anoxic conditions in the absence of a suitable electron acceptor. Under these conditions, the novel Lahn Cluster bacteria became heavily labelled from [^{13}C]-acetate, which suggested that these populations were in fact dechlorinating and most

© 2008 The Authors
Journal compilation © 2008 Society for Applied Microbiology and Blackwell Publishing Ltd,

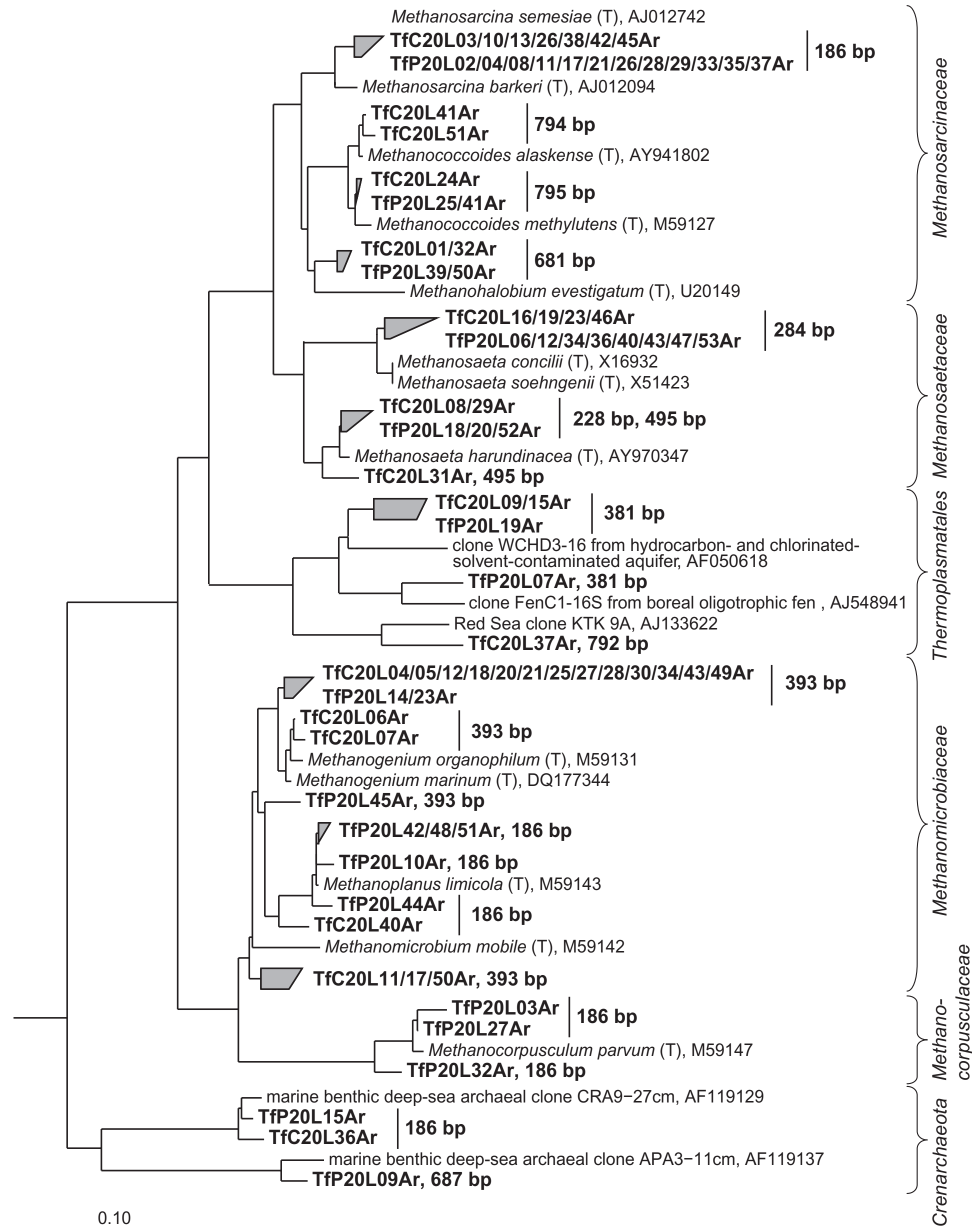

Fig. 6. Phylogenetic distribution of archaeal 16S rRNA clone sequences obtained from fractions TfC20L (with PCE, light) and TfP20L (control, light). The scale bar represents 10% sequence divergence. Sequences of cultivated bacterial dehalorespiring *Dehalococcoides* spp. (AF004928, AY323233, AY914178, AF357918 and AY165308) served as outgroup.

likely also dehalorespiring PCE and TCE (Kittelmann and Friedrich, 2008).

In this study, the same labelling approach with [^{13}C]-acetate was used to identify chloroethene-dechlorinating bacteria in marine tidal flat sediment incubations. During the pre-incubation phase, microbial respiratory processes were exclusively fuelled by sediment endogenous electron donors until endogenous electron acceptors were almost depleted, and chloroethene-reducing bacteria had been enriched sufficiently thriving on sediment endogenous carbon sources. When SIP was started, PCE had been reduced to a large extent and TCE was the most abundant chlorinated ethene (Fig. 1). The addition of ^{13}C-labelled acetate resulted in effective label incorporation by bacterial populations. Tidal Flat *Chloroflexi* Cluster bacteria and *Dehalobium* spp. accounted for a large part of the [^{13}C]-acetate-assimilating populations based on combined T-RFLP and cloning/sequencing analysis (~42.3%) in the heavy-gradient fraction of the microcosm with PCE. Most importantly, however, these populations did not occur in

© 2008 The Authors

the control microcosm, and not even in the light fraction of the microcosm with PCE. Therefore, it is very likely that both populations are involved in catalysing dechlorination in the sediment incubations.

Potentially involved in dechlorination were also small populations related to δ-proteobacterial *Desulfuromonas* and *Desulfomonile* spp.; however, these isolates are known to dechlorinate PCE to TCE and *cis*-DCE, not to *trans*-DCE (Shelton and Tiedje, 1984; Fathepure *et al*., 1987; Krumholz *et al*., 1996; Löffler *et al*., 2000; Sung *et al*., 2003). Moreover, clones retrieved from the light fraction of the PCE-amended microcosm and the non-PCE-amended control fell into the same cluster (Fig. 4).

Other populations involved in ^{13}C-acetate assimilation were detected that are not directly linked to dechlorination activity including sulfate-reducing bacteria (SRB) and methanogens. Typically, tidal flat sediments are dominated by sulfate reduction as terminal electron accepting process in the upper sediment layers (Jørgensen, 1982; Llobet-Brossa *et al*., 2002). In our enrichments, sulfate was depleted almost entirely in the course of the pre-incubation phase (~300 μM at the beginning of SIP); during ^{13}C-probing only a small amount of sulfate (1.74 μmol) was reduced compared with reductive dechlorination of chlorinated ethenes (7.62 μmol of PCE and TCE). Nevertheless, SRB assimilated ^{13}C-labelled acetate to some extent while most likely reducing residual sulfate, and were detected in heavy rRNA (δ-proteobacterial SRB clones; 15.5% frequency).

In tidal flat sediments, methanogens are numerically less important in upper layers due to substrate competition with SRB (Oremland and Taylor, 1978; Wilms *et al*., 2007). In our experiment, a significant amount of [^{13}C]-methane was formed from [^{13}C]-acetate, yet heavy archaeal rRNA was detected only up to intermediate density fractions (BD = 1.80 g ml^{-1}), indicating that the population of methanogens was probably small or not active enough for label incorporation at higher level. It appears that the PCE-amended incubations favoured *Methanosarcinaceae* and *Methanomicrobiaceae*, whereas in the control *Methanosarcinaceae* and *Methanosaetaceae* were assimilating [^{13}C]-acetate to some extent (BDs ~ 1.803; Fig. 5).

Besides microorganisms involved in terminal electron-accepting processes, syntrophically acetate-oxidizing bacteria might have assimilated acetate, and could have become labelled during SIP. When hydrogen partial pressures are thermodynamically permissive, syntrophic acetate-oxidizing bacteria might be involved in interspecies hydrogen transfer to hydrogen-oxidizing populations, e.g. chloroethene-respiring bacteria (Löffler and Sanford, 2005). In fact, syntrophic acetate oxidation was implicated in anaerobic dechlorination in a number of studies (He *et al*., 2002; Becker *et al*., 2005; Kittelmann and Friedrich, 2008). However, in tidal flat incubations the pH_2 was high (~750 Pa), and thus an involvement of syntrophic processes seems unlikely.

Although certain limitations are inherent to SIP (see above and for detailed discussion Kittelmann and Friedrich, 2008), our data showed that SIP with [^{13}C]-acetate specifically allowed for tracing chloroethene-dechlorinating populations in tidal flat sediment incubations.

Microorganisms involved in trans*-DCE production*

In tidal flat sediment incubations from Dangast, North Sea, PCE was dechlorinated via TCE to preferentially *trans*-DCE and only small amounts of *cis*-DCE at a ratio of ~3.5. Tidal Flat *Chloroflexi* Cluster bacteria and *Dehalobium* spp. likely were involved in the formation of predominantly *trans*-DCE. At the beginning of ^{13}C-labelling, PCE had been turned over almost completely to TCE, which therefore was the predominant electron acceptor. Acetate addition stimulated dechlorination of TCE to *trans*-DCE (Fig. 1). The TFCC bacteria were the most abundant active population (with relative abundances of 25.8% and 15.5% according to T-RFLP analysis and clone library respectively). Thus, TFCC bacteria were very likely involved in the production of major amounts of *trans*-DCE in the tidal flat sediment incubations. The second potentially dehalorespiring population detected in tidal flat sediments related to '*D. chlorococercia* DF-1' may contribute to *trans*-DCE production as well; however, strain DF-1 has been found before to form *trans*-DCE at a considerably lower ratio of ~1.5 only. Nevertheless, a possible role of other microorganisms present in the incubations cannot be ruled out completely.

The involvement of methanogens in *trans*-DCE formation is unlikely. Co-metabolic dechlorination of PCE to TCE, *cis*-DCE or even small amounts of VC and ethene by cofactor F_{430} has been observed in *Methanosarcina* spp. (Fathepure and Boyd, 1988; Gantzer and Wackett, 1991; Jablonski and Ferry, 1992); however, cometabolic chloroethene dechlorination is slow and approximately 3–5 less effective than dehalorespiration (El Fantroussi *et al*., 1998). F_{430} has not been found to be involved in production of the *trans*-DCE isomer.

Conclusions

Besides its role as a sink for anthropogenic pollutants, the marine environment is a rich source of naturally produced halogenated compounds. Consequently, microbial capabilities to degrade these compounds under aerobic and anaerobic conditions have evolved, but the diversity of marine chloroethene dehalorespirers is yet largely unknown. Using SIP of RNA we found novel uncultured

© 2008 The Authors

Chloroflexi populations, the TFCC bacteria, capable of reductive chloroethene dechlorination in anoxic tidal flat sediment incubations. Their predominance in the SIP enrichment suggests that TFCC bacteria are mainly responsible for *trans*-DCE formation from TCE at high ratio (~3.5) of *trans*- to the *cis*-isomer of DCE. Enrichment efforts are made in our lab, to elucidate the role of TFCC bacteria in *trans*-DCE formation. A highly enriched or pure culture will also help to gain insight into reductively dechlorinating enzyme complexes that favour *trans*-DCE over *cis*-DCE production. The data are needed to better understand the accumulation of certain chlorinated ethenes at contaminated sites.

Experimental procedures

Materials

All chemicals and analytical standards were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) at the highest purity available.

Sediment sampling and pre-incubation

The sampling site was located in a meso- to macrotidal embayment, the 'Jadebusen' (Jade Bay), which is situated on the North Sea coast of Lower Saxony (Germany) south-west of the Weser estuary. Sediment samples were collected from tidal flats approximately 2 km west of the small town of Dangast in June 2006 (distance from shore-line ~20 m, salinity 26–28‰) (Llobet-Brossa *et al.*, 2002; see also for further geochemical parameters of the sampling site). A total volume of 100 ml sediment slurry was pre-incubated with 1250 μl of a 1:1250 mixture of neat PCE in heptamethylnonane as organic carrier phase (Kittelmann and Friedrich, 2008). A control without PCE was incubated under the same conditions. Upon complete dechlorination of PCE via TCE to DCEs (after ~33 days), 6 ml of sediment slurry was transferred into 13.5 ml serum bottle microcosms, and the headspaces were flushed with N_2:CO_2 (80:20; vol:vol). Four microcosms were again amended with PCE ($\leq 5\ \mu M$ aqueous concentration). From the pre-incubation without PCE four microcosms were set up as described above and further incubated without PCE. All microcosms were kept at 20°C in the dark with occasional shaking. After *trans*- and *cis*-DCE production had resumed (day 47), the PCE-amended microcosms and the non-PCE-amended controls were spiked with 0.5 mM $^{13}C_2$-labelled acetate. Microcosms were sacrificed after 1, 2, 3 and 4 days, immediately transferred into 2 ml reaction tubes and stored at –80°C until further processing.

Analytical measurements

Dechlorination of PCE and formation of the degradation products TCE and *cis*-/*trans*-DCE were tracked gas chromatographically by headspace analysis (Kittelmann and Friedrich, 2008). Total amounts of PCE and its dechlorination products were determined from headspace measurements according to calibration curves of all analysed chlorinated compounds. Standards of chlorinated ethenes were prepared with exactly the same volumes of aqueous, gas and organic phase as used for microcosm set-up. The available Henry constant [Peng and Wan, 1997; corrected by 30% to account for salting-out effects (Schwarzenbach *et al.*, 2003)] and hexadecane-water partitioning coefficient of PCE at 20°C (Abraham *et al.*, 1994) were used to estimate the approximate starting concentration of PCE in our marine system ($\leq 5\ \mu M$).

Methane formation was measured gas chromatographically on a GC-8A gas chromatograph (Shimadzu, Japan) in 100 μl of headspace samples using a packed column (2 m by 1/8″ inner diameter, Porapak Q, Alltech, Germany) at 80°C and an FID heated to 230°C (Shimadzu, Japan). CO_2 was measured upon reduction to CH_4 with a methanizer (Ni-catalyst at 350°C, Chrompack, the Netherlands; Conrad *et al.*, 1987). The proportion of $^{13}CO_2$ and ^{13}CH4 was analysed by gas chromatography combustion isotope ratio mass spectroscopy (GC-c-IRMS; Brand, 1996; Conrad *et al.*, 2000).

Liquid samples for ion chromatography and high-performance liquid chromatography (HPLC) were filtered through 0.2 μm filters (Schleicher & Schuell, Dassel, Germany). Chloride, nitrate, nitrite and sulfate concentrations in the pore waters were analysed by ion chromatography (Bak *et al.*, 1991). Acetate concentrations before and after ^{13}C-probing were measured by HPLC with an Aminex HPX-87H ion exclusion column (Bio-Rad Laboratories, Hercules, CA) with a refraction index detector (RI2000; Sykam, Gilching, Germany) and a UV detector (UVIS 205; Linear Instruments, Reno, NV). Chromatogrammes were analysed with the Peak Simple software (SRI Instruments, Torrance, CA).

Density separation of total RNA and bacterial SSU RNA fingerprinting

Nucleic acids were extracted from [^{13}C]-acetate-amended incubations with and without PCE of day 4 after a published protocol (Lueders *et al.*, 2004b). After DNase I digestion and RNA quantification with RiboGreen stain (Molecular Probes), 500 ng of RNA was mixed with gradient buffer and transferred into gradient medium consisting of cesiumtrifluoroacetate and formamide. Density gradient centrifugation was carried out in an ultracentrifuge at 39 000 r.p.m. (130 000 *g*) and 20°C for 72 h (Lueders *et al.*, 2004a). After fractionation of the gradient, refractory indices were measured for each fraction. RNA in fractions was precipitated from the gradient medium with isopropanol (1 vol.) and 0.5% sodium acetate (pH 5.2), washed with 70% ethanol and eluted in 40 μl EB buffer (10 mM Tris-HCl, pH 8.5). Amplification of 16S rRNA was carried out with the Access one-tube reverse transcription polymerase chain reaction (RT-PCR) system (Promega) and primers Ba27f-FAM (6-carboxyfluorescein-labelled) and Ba907r (Bacteria) and Ar109f and Ar912rt-FAM (Archaea). After initial transcription at 48°C for 45 min, rcDNA was denatured at 94°C for 2 min followed by 23 cycles of denaturing (94°C, 30 s), primer annealing (52°C Bacteria, 55°C Archaea,

© 2008 The Authors

30 s) and elongation (68°C, 1 min) and a final 10 min extension at 68°C. 16S rcDNA amplicons were purified using MinElute columns (Qiagen, Hilden, Germany). PCR product (120 ng) was used for digestion with the restriction enzyme *Msp*I (37°C, *Bacteria*) or *Taq*I (65°C, *Archaea*) after (Lueders and Friedrich, 2002). Subsequent desalting with AutoSeq G50 columns (Amersham Pharmacia Biotech) and mixing of 1 μl of digest with 11 μl of Hi-Di formamide (Applied Biosystems) and 0.25 μl of Rox-labelled Map Marker 1000 ladder (Bio-Ventures, Murfreesboro, Tennessee) were performed. Then the samples were denatured for 4 min at 95°C, cooled on ice and analysed on an ABI 310 genetic analyser (Applied Biosystems). Electrophoresis parameters were selected as described in Kittelmann and Friedrich (2008). Analysis of T-RFLP electropherogrammes was performed with the Genescan 2.1 software (Applied Biosystems).

Construction of bacterial and archaeal 16S rRNA clone libraries

For cloning and sequence analysis of bacterial populations a high- and a low-density fraction were selected from the incubation with PCE together with a low-density fraction from the control. Amplicons were amplified with non-labelled primers Ba27f and Ba907r-TES (RT-PCR conditions as described above). Archaeal populations were amplified with primers Ar109f and Ar912rt from a light fraction of both the incubation with and without PCE. 16S rRNA genes were cloned using the pGEM-T Vector System II (Promega) and sequences were obtained from randomly selected clones by analysis at the core facility ADIS (Max Planck Institute for plant breeding research, Cologne). A total of 24 putative bacterial and 4 putative archaeal chimeric sequences were detected by Bellerophon (Huber *et al*., 2004) and Mallard (Ashelford *et al*., 2006) analysis, confirmed by fractional treeing (Ludwig *et al*., 1997) and excluded from corresponding libraries. Phylogenetic analysis was carried out with 136 bacterial sequences [TfC20H (58), TfC20L (42), TfP20L (36)] and 88 archaeal sequences [TfC20L (45), TfP20L (43)]. Sampling coverage of clones was checked on the level of major bacterial lineages by rarefaction analysis with the DOTUR software using the furthest neighbour sequence assignment (Schloss and Handelsman, 2005). Phylogenetic affiliation of clones was determined with the ARB software package (http://www.arb-home.de; version corrected January 2004, released January 2005; Ludwig *et al*., 2004) using the neighbour joining and fast parsimony methods for tree construction. For correlation of clones with their corresponding T-RFs the 'T-RF-cut' tool was used (Ricke *et al*., 2005). Sequence data have been deposited with GenBank under Accession No. EU362185–EU362408.

Screening for known reductive dehalogenases in trans*-DCE-producing incubation*

In order to evaluate the presence of genes with known dehalogenase motifs primers Dhar1000f and Dhu1350r (Rhee *et al*., 2003) were used to screen for dehalogenase genes in DNA extracts of the *trans*-DCE-producing incubation TfC20. DNA from *S. multivorans* (DSM 12446) served as positive control. PCR amplification was performed with a standard protocol (Kittelmann and Friedrich, 2008) applying an annealing temperature of 46°C and 35 cycles.

Acknowledgements

The study was supported by the Max Planck Society (MPG), the Deutsche Forschungsgemeinschaft (DFG) and the Fonds der chemischen Industrie. We thank Bianca Pommerenke and Peter Claus for excellent technical assistance, Torsten C. Schmidt (University of Duisburg), Lorenz Adrian (TU Berlin) and Frank E. Löffler (Georgia Tech) for helpful advice, and two anonymous reviewers for their useful comments.

References

Abelson, P.H. (1990) Inefficient remediation of groundwater pollution. *Science* **250:** 733.

Abraham, M.H., Chadha, H.S., Dixon, J.P., and Leo, A.J. (1994) Hydrogen bonding. 39. The partition of solutes between water and various alcohols. *J Phys Org Chem* **7:** 712–716.

Abrahamsson, K., Ekdahl, A., Collen, J., and Pedersen, M. (1995) Marine algae – a source of trichloroethylene and perchloroethylene. *Limnol Oceanogr* **40:** 1321–1326.

Agency for Toxic Substances and Disease Registry (1996) *Toxicological profile for 1,2-dichloroethene* [WWW document]. URL http://www.atsdr.cdc.gov/toxprofiles/tp87.pdf.

Ansbaek, J., and Blackburn, T.H. (1980) Method for the analysis of acetate turnover in a coastal marine sediment. *Microbial Ecol* **5:** 253–264.

Ashelford, K.E., Chuzhanova, N.A., Fry, J.C., Jones, A.J., and Weightman, A.J. (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl Environ Microbiol* **72:** 5734–5741.

Bak, F., Scheff, G., and Jansen, K.H. (1991) A rapid and sensitive ion chromatographic technique for the determination of sulfate and sulfate reduction rates in fresh-water lake sediments. *FEMS Microbiol Ecol* **85:** 23–30.

Becker, J.G., Berardesco, G., Rittmann, B.E., and Stahl, D.A. (2005) The role of syntrophic associations in sustaining anaerobic mineralization of chlorinated organic compounds. *Environ Health Persp* **113:** 310–316.

Brand, W.A. (1996) High precision isotope ratio monitoring techniques in mass spectrometry. *J Mass Spectrom* **31:** 225–235.

Chang, Y.C., Hatsu, M., Jung, K., Yoo, Y.S., and Takamizawa, K. (2000) Isolation and characterization of a tetrachloroethylene dechlorinating bacterium, *Clostridium bifermentans* DPH-1. *J Biosci Bioeng* **89:** 489–491.

Christof, O., Seifert, R., and Michaelis, W. (2002) Volatile halogenated organic compounds in European estuaries. *Biogeochem* **59:** 143–160.

Conrad, R., Schutz, H., and Babbel, M. (1987) Temperature limitation of hydrogen turnover and methanogenesis in anoxic paddy soil. *FEMS Microbiol Ecol* **45:** 281–289.

Conrad, R., Klose, M., and Claus, P. (2000) Phosphate inhibits acetotrophic methanogenesis on rice roots. *Appl Environ Microbiol* **66:** 828–831.

© 2008 The Authors

De Rooij, C., Boutonnet, J.C., Garny, V., Lecloux, A., Papp, R., Thompson, R.S., and Van Wijk, D. (1998) Euro Chlor risk assessment for the marine environment Osparcom Region: North Sea – Tetrachloroethylene. *Environ Monit Assess* **52:** 489–508.

De Wever, H., Cole, J.R., Fettig, M.R., Hogan, D.A., and Tiedje, J.M. (2000) Reductive dehalogenation of trichloroacetic acid by *Trichlorobacter thiogenes* gen. nov., sp. nov. *Appl Environ Microbiol* **66:** 2297–2301.

Dollhopf, S.L., Hashsham, S.A., and Tiedje, J.M. (2001) Interpreting 16S rDNA T-RFLP data: application of self-organizing maps and principal component analysis to describe community dynamics and convergence. *Microbial Ecol* **42:** 495–505.

Dumont, M.G., and Murrell, J.C. (2005) Stable isotope probing – linking microbial identity to function. *Nat Rev Microbiol* **3:** 499–504.

El Fantroussi, S., Naveau, H., and Agathos, S.N. (1998) Anaerobic dechlorinating bacteria. *Biotechnol Progr* **14:** 167–188.

Fathepure, B.Z., and Boyd, S.A. (1988) Dependence of tetrachloroethylene dechlorination on methanogenic substrate consumption by *Methanosarcina* sp. strain DCM. *Appl Environ Microbiol* **54:** 2976–2980.

Fathepure, B.Z., Nengu, J.P., and Boyd, S.A. (1987) Anaerobic bacteria that dechlorinate perchloroethene. *Appl Environ Microbiol* **53:** 2671–2674.

Friedrich, M.W. (2006) Stable-isotope probing of DNA: insights into the function of uncultivated microorganisms from isotopically labeled metagenomes. *Curr Opin Biotechnol* **17:** 59–66.

Gantzer, C.J., and Wackett, L.P. (1991) Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environ Sci Technol* **25:** 715–722.

Gribble, G.W. (1994) The abundant natural sources and uses of chlorinated chemicals. *Am J Public Health* **84:** 1183.

Gribble, G.W. (2003) The diversity of naturally produced organohalogens. *Chemosphere* **52:** 289–297.

Griffin, B.M., Tiedje, J.M., and Löffler, F.E. (2004) Anaerobic microbial reductive dechlorination of tetrachloroethene to predominately *trans*-1,2-dichloroethene. *Environ Sci Technol* **38:** 4300–4303.

Häggblom, M.M., Ahn, Y.B., Fennell, D.E., Kerkhof, L.J., and Rhee, S.K. (2003) Anaerobic dehalogenation of organohalide contaminants in the marine environment. *Adv Appl Microbiol* **53:** 61–84.

He, J.Z., Sung, Y., Dollhopf, M.E., Fathepure, B.Z., Tiedje, J.M., and Löffler, F.E. (2002) Acetate versus hydrogen as direct electron donors to stimulate the microbial reductive dechlorination process at chloroethene-contaminated sites. *Environ Sci Technol* **36:** 3945–3952.

Holliger, C., and Schraa, G. (1994) Physiological meaning and potential for application of reductive dechlorination by anaerobic bacteria. *FEMS Microbiol Rev* **15:** 297–305.

Holliger, C., Schraa, G., Stams, A.J.M., and Zehnder, A.J.B. (1993) A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. *Appl Environ Microbiol* **59:** 2991–2997.

Holliger, C., Wohlfarth, G., and Diekert, G. (1998) Reductive dechlorination in the energy metabolism of anaerobic bacteria. *FEMS Microbiol Rev* **22:** 383–398.

Huber, T., Faulkner, G., and Hugenholtz, P. (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20:** 2317–2319.

Jablonski, P.E., and Ferry, J.G. (1992) Reductive dechlorination of trichloroethylene by the Co-Reduced Co dehydrogenase enzyme complex from *Methanosarcina thermophila*. *FEMS Microbiol Lett* **96:** 55–59.

Jørgensen, B.B. (1978) Comparison of methods for the quantification of bacterial sulfate reduction in coastal marine sediments 1. Measurement with radiotracer techniques. *Geomicrobiol J* **1:** 11–27.

Jørgensen, B.B. (1982) Mineralization of organic-matter in the Sea Bed – the role of sulfate reduction. *Nature* **296:** 643–645.

Kengen, S.W.M., Breidenbach, C.G., Felske, A., Stams, A.J.M., Schraa, G., and de Vos, W.M. (1999) Reductive dechlorination of tetrachloroethene to *cis*-1,2-dichloroethene by a thermophilic anaerobic enrichment culture. *Appl Environ Microbiol* **65:** 2312–2316.

Kittelmann, S., and Friedrich, M.W. (2008) Identification of novel PCE-respiring microorganisms in anoxic river sediment by RNA-based stable isotope probing. *Environ Microbiol* **10:** 31–46.

Köpke, B., Wilms, R., Engelen, B., Cypionka, H., and Sass, H. (2005) Microbial diversity in coastal subsurface sediments: a cultivation approach using various electron acceptors and substrate gradients. *Appl Environ Microbiol* **71:** 7819–7830.

Krumholz, L.R., Sharp, R., and Fishbain, S.S. (1996) A freshwater anaerobe coupling acetate oxidation to tetrachloroethylene dehalogenation. *Appl Environ Microbiol* **62:** 4108–4113.

Llobet-Brossa, E., Rabus, R., Böttcher, M.E., Könneke, M., Finke, N., Schramm, A., *et al.* (2002) Community structure and activity of sulfate-reducing bacteria in an intertidal surface sediment: a multi-method approach. *Aquat Microb Ecol* **29:** 211–226.

Löffler, F.E., and Sanford, R.A. (2005) Analysis of trace hydrogen metabolism. *Environ Microbiol* **397:** 222–237.

Löffler, F.E., Sanford, R.A., and Tiedje, J.M. (1996) Initial characterization of a reductive dehalogenase from *Desulfitobacterium chlororespirans* Co23. *Appl Environ Microbiol* **62:** 3809–3813.

Löffler, F.E., Champine, J.E., Ritalahti, K.M., Sprague, S.J., and Tiedje, J.M. (1997) Complete reductive dechlorination of 1,2-dichloropropane by anaerobic bacteria. *Appl Environ Microbiol* **63:** 2870–2875.

Löffler, F.E., Sun, Q., Li, J.R., and Tiedje, J.M. (2000) 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. *Appl Environ Microbiol* **66:** 1369–1374.

Ludwig, W., Bauer, S.H., Bauer, M., Held, I., Kirchhof, G., Schulze, R., *et al.* (1997) Detection and *in situ* identification of representatives of a widely distributed new bacterial phylum. *FEMS Microbiol Lett* **153:** 181–190.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, *et al.* (2004) ARB: a software environment for sequence data. *Nucl Acids Res* **32:** 1363–1371.

© 2008 The Authors

Lueders, T., and Friedrich, M.W. (2002) Effects of amendment with ferrihydrite and gypsum on the structure and activity of methanogenic populations in rice field soil. *Appl Environ Microbiol* **68:** 2484–2494.

Lueders, T., Manefield, M., and Friedrich, M.W. (2004a) Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ Microbiol* **6:** 73–78.

Lueders, T., Pommerenke, B., and Friedrich, M.W. (2004b) Stable-isotope probing of microorganisms thriving at thermodynamic limits: syntrophic propionate oxidation in flooded soil. *Appl Environ Microbiol* **70:** 5778–5786.

Manefield, M., Whiteley, A.S., Griffiths, R.I., and Bailey, M.J. (2002) RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Appl Environ Microbiol* **68:** 5367–5373.

Mazur, C.S., and Jones, W.J. (2001) Hydrogen concentrations in sulfate-reducing estuarine sediments during PCE dehalogenation. *Environ Sci Technol* **35:** 4783–4788.

Miller, G.S., Milliken, C.E., Sowers, K.R., and May, H.D. (2005) Reductive dechlorination of tetrachloroethene to *trans*-dichloroethene and *cis*-dichloroethene by PCB-dechlorinating bacterium DF-1. *Environ Sci Technol* **39:** 2631–2635.

Neumann, A., ScholzMuramatsu, H., and Diekert, G. (1994) Tetrachloroethene metabolism of *Dehalospirillum multivorans*. *Arch Microbiol* **162:** 295–301.

Oremland, R.S., and Taylor, B.F. (1978) Sulfate-reduction and methanogenesis in marine sediments. *Geochim Cosmochim Ac* **42:** 209–214.

Peng, J., and Wan, A. (1997) Measurement of Henry's constants of high-volatility organic compounds using a headspace autosampler. *Environ Sci Technol* **31:** 2998–3003.

Rhee, S.K., Fennell, D.E., Haggblom, M.M., and Kerkhof, L.J. (2003) Detection by PCR of reductive dehalogenase motifs in a sulfidogenic 2-bromophenol-degrading consortium enriched from estuarine sediment. *FEMS Microbiol Ecol* **43:** 317–324.

Ricke, P., Kolb, S., and Braker, G. (2005) Application of a newly developed ARB software-integrated tool for *in silico* terminal restriction fragment length polymorphism analysis reveals the dominance of a novel *pmoA* cluster in a forest soil. *Appl Environ Microbiol* **71:** 1671–1673.

Sansone, F.J., and Martens, C.S. (1982) Volatile fatty-acid cycling in organic-rich marine sediments. *Geochim Cosmochim Ac* **46:** 1575–1589.

Schloss, P.D., and Handelsman, J. (2005) Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* **71:** 1501–1506.

Schwarzenbach, R.P., Gschwendt, P.M., and Imboden, D.M. (2003) *Environmental Organic Geochemistry*, 2nd edn. New York, USA: Wiley Interscience.

Sharma, P.K., and McCarty, P.L. (1996) Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenates tetrachloroethene to *cis*-1,2-dichloroethene. *Appl Environ Microbiol* **62:** 761–765.

Shelton, D.R., and Tiedje, J.M. (1984) Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. *Appl Environ Microbiol* **48:** 840–848.

Smidt, H., and de Vos, W.M. (2004) Anaerobic microbial dehalogenation. *Annu Rev Microbiol* **58:** 43–73.

Sung, Y., Ritalahti, K.M., Sanford, R.A., Urbance, J.W., Flynn, S.J., Tiedje, J.M., and Löffler, F.E. (2003) Characterization of two tetrachloroethene-reducing, acetate-oxidizing anaerobic bacteria and their description as *Desulfuromonas michiganensis* sp. nov. *Appl Environ Microbiol* **69:** 2964–2974.

US Environmental Protection Agency (2006) *Superfund information system, National Priorities List (NPL) Sites.* [WWW document]. URL http://cfpub.epa.gov/supercpad/cursites/srchsites.cfm.

Watts, J.E.M., Fagervold, S.K., May, H.D., and Sowers, K.R. (2005) A PCR-based specific assay reveals a population of bacteria within the *Chloroflexi* associated with the reductive dehalogenation of polychlorinated biphenyls. *Microbiology* **151:** 2039–2046.

Wilms, R., Sass, H., Köpke, B., Cypionka, H., and Engelen, B. (2007) Methane and sulfate profiles within the subsurface of a tidal flat are reflected by the distribution of sulfate-reducing bacteria and methanogenic archaea. *FEMS Microbiol Ecol* **59:** 611–621.

Wu, Q.Z., Sowers, K.R., and May, H.D. (2000) Establishment of a polychlorinated biphenyl-dechlorinating microbial consortium, specific for doubly flanked chlorines, in a defined, sediment-free medium. *Appl Environ Microbiol* **66:** 49–53.

© 2008 The Authors