

Detection of *Dehalococcoides* spp. by Peptide Nucleic Acid Fluorescent in situ Hybridization

Anthony S. Danko^{a, c} Silvia J. Fontenete^b Daniel de Aquino Leite^{b, e} Patrícia O. Leitão^{a, c} Carina Almeida^{b, d} Charles E. Schaefer^f Simon Vainberg^f Robert J. Steffan^f Nuno F. Azevedo^b

^aCentro de Investigação em Geo-Ambiente e Recursos (CIGAR), Departamento de Engenharia de Minas, Faculdade de Engenharia, and ^bLaboratory for Process Engineering, Environment, and Energy and Biotechnology Engineering (LEPABE), Department of Chemical Engineering, Faculty of Engineering, University of Porto, Porto, ^cCentro de Recursos Naturais e Ambiente (CERENA), Instituto Superior Técnico, Lisboa, and ^dInstitute for Biotechnology and Bioengineering (IBB), Center of Biological Engineering, Universidade do Minho, Braga, Portugal; ^eLANASE, Departamento de Engenharia de Alimentos, Faculdade de Engenharia, Universidade Federal da Grande Dourados, Dourados, Brazil; ^fCB&I Federal Services, LLC., Lawrenceville, N.J., USA

Abstract

Chlorinated solvents including tetrachloroethene (perchloroethene and trichloroethene), are widely used industrial solvents. Improper use and disposal of these chemicals has led to a widespread contamination. Anaerobic treatment technologies that utilize *Dehalococcoides* spp. can be an effective tool to remediate these contaminated sites. Therefore, the aim of this study was to develop, optimize and validate peptide nucleic acid (PNA) probes for the detection of *Dehalococcoides* spp. in both pure and mixed cultures. PNA probes were designed by adapting previously published DNA probes targeting the region of the point mutations described for discriminating between the *Dehalococcoides* spp. strain CBDB1 and strain 195 lineages. Different fixation, hybridization and washing procedures were tested. The results indicated that the PNA probes hybridized specifically and with a high sensitivity to their corresponding lineages, and that the PNA probes developed during this work can be used in a duplex assay to distinguish between strain CBDB1 and strain 195 lineages, even in complex mixed cultures. This work demonstrates the effectiveness of using PNA fluorescence in situ hybridization to distinguish between two metabolically and genetically distinct *Dehalococcoides* strains, and they can have strong implications in the monitoring and differentiation of *Dehalococcoides* populations in laboratory cultures and at contaminated sites.

Introduction

Tetrachloroethene [perchloroethene (PCE)] and trichloroethene (TCE) have been widely used as industrial solvents and dry cleaning fluids, and are common ground- water pollutants that are highly toxic and potentially carcinogenic. Under anaerobic conditions, PCE and TCE can be transformed into non-chlorinated ethene by dechlorinating bacteria. This process can occur naturally or through the addition of electron donor compounds that stimulate the growth of indigenous microorganisms (bio- stimulation) and/or exogenous microorganisms (bioaugmentation) that are able to degrade these compounds [Scow and Hicks, 2005]. The complete reductive dechlorination of chlorinated ethenes is performed by microorganisms of the genus *Dehalococcoides*. In this reaction, the chlorinated compound serves as the acceptor for electrons with hydrogen as the electron donor. In this growth- linked process termed halorespiration, *Dehalococcoides* utilizes the chlorinated aliphatic compounds to obtain energy.

Dehalococcoides is notoriously difficult to isolate and grow, with doubling times of 1–2 days [He et al., 2003, 2005; Maymo-Gatell et al., 1997; Sung et al., 2006] and complex nutrient requirements. *Dehalococcoides* spp., therefore, are normally cultured as a consortium with other microorganisms, such as acetogens and methanogens. *Dehalococcoides* spp. have been found in a variety of locations, and their presence has been linked to the complete dechlorination of PCE at contaminated sites in North America and Europe [Hendrickson et al., 2002]. Although very few *Dehalococcoides* spp. have been isolated, some metabolic diversity has been identified between them. Originally, strain 195 was found to metabolize some chloroethenes [PCE, TCE, *cis*-dichloroethene (DCE) and 1,1-DCE], and the halogenated ethanes 1,2-dichloroethane and 1,2-dibromoethane [Maymo-Gatell et al., 1997, 1999]. Later research demonstrated the ability to metabolize higher chlorinated aromatics, such as hexachlorobenzene, pentachlorobenzene, 1,2,4,5-tetrachlorobenzene (TeCB) and 1,2,3,4-TeCB [Fennell et al., 2004]. Conversely, strain CBDB1 (of the Pinellas subgroup lineage) was found to metabolize these same chlorinated aromatics, and others such as 1,2,3,5- TeCB, 1,2,4-trichlorobenzene and 1,2,3-trichlorobenzene [Adrian et al., 2000]. In addition, recently, strain CBDB1 has been ascribed the ability to also metabolize the chloroethenes PCE and TCE [Marco-Urrea et al., 2011]. Other members of the Pinellas subgroup (of which CBDB1 is a member) are able to metabolize lower chlorinated ethenes such as *cis*-DCE, 1,1-DCE and vinyl chloride (VC) [He et al., 2003; Sung et al., 2006]. Table 1 (adapted from Eaddy [2008]) shows the differences between the two lineages. Thus, the ability to distinguish between different lineages of these bacteria at contaminated sites may provide insights into the potential fate of pollutants, the suitability of biostimulation remedial approaches and the need to perform bioaugmentation to reach remedial goals.

Because of the importance of these microorganisms in degrading chlorinated pollutants, molecular tools based on the detection of 16S rRNA gene sequences have been developed and widely used for qualitative and quantitative detection of *Dehalococcoides* spp. [Holliger and Diekert, 1999; Maymo-Gatell et al., 1997; Rowe et al., 2008; Tas, 2009]. In addition, other non-PCR-based molecular methods, such as fluorescence in situ hybridization (FISH) and catalyzed reporter deposition FISH, have been used to distinguish between the different lineages of *Dehalococcoides* spp. [Dijk et al., 2008; Fazi et al., 2008; Yang and Zeyer, 2003]. The probes used for these FISH- based tools take advantage of the fact that the two different

lineages of *Dehalococcoides* spp. (strain 195 and Pinellas subgroup) can be distinguished from one another based on a conserved base difference in the 16S rRNA gene sequence.

FISH is based on the use of a fluorescently labeled nucleic acid probe (generally DNA) that is complementary to a sequence in the rRNA of the microorganism of interest [Almeida et al., 2009]. After exposure to the sample containing the microorganism, the probe diffuses through the cell wall and reaches the rRNA, where it forms a stable duplex if the complementary sequence is present. A subsequent washing step ensures that only the fully complementary probes remain within the cell and are detected by either flow cytometry or microscopy. Recently, peptide nucleic acid (PNA) has been replacing DNA as a probe in FISH. The backbone of these synthetic nucleic acid molecules is neutrally charged, which means that PNA has more affinity for duplexes with DNA and RNA than DNA itself [Armitage, 2003]. This higher affinity translates into shorter probes that can more easily diffuse through the microbial cell wall and hence increase the robustness of the FISH method. PNA-FISH has been successfully used for the detection of microorganisms in the food and health areas [Almeida et al., 2011; Azevedo et al., 2011; Guimaraes et al., 2007], but this improved methodology of detection has not yet been adapted to *Dehalococcoides* spp.

Because there are two different lineages of *Dehalococcoides* spp. that have different abilities to degrade pollutants, it is important to be able to identify which type of *Dehalococcoides* spp. is present in cultures or environmental samples to help predict the fate of pollutants or select the proper remedial treatments. Therefore, the aim of this work was to develop PNA-FISH probes and test them to determine whether this technique can be utilized to detect the two different lineages of *Dehalococcoides* spp. in both pure and mixed cultures.

Results and Discussion

Probe Development and Application to Pure Cultures

PNA probes were designed by adapting previously published DNA probes [Yang and Zeyer, 2003] targeting the 16S rRNA region of the point mutations described for detecting CBDB1 and 195 lineages of *Dehalococcoides* spp. Because the favorable size of PNA probes (related to their thermodynamic parameters) is between 13 and 16 bp, it was necessary to select a smaller sequence from the probe previously described. Consequently, the sequences 5-GTT CGC ACT GTT GC-3 and 5-GTT CAC ACT GTT GC-3 were selected and designated CBDB1 probe and 195 probe, respectively. The theoretical evaluation of the probes has shown high specificity and sensitivity values (table 2), since 55 of 56 *Dehalococcoides* sequences have been identified and only 1 non-target sequence has presented cross-hybridization with the probes. Additionally, melting temperatures and free energy (ΔG) values for the CBDB1 and 195 probes were very similar, which might indicate a good performance in a multiplex assay.

Initially, the development of a PNA-FISH procedure for the differentiation of *Dehalococcoides* spp. was found to be technically more challenging than PNA-FISH procedures developed earlier by our group to detect other microorganisms [Almeida et al., 2010; Guimaraes et al., 2007]. This may be related to the slow growth rates of the microbes in pure cultures and low cell yields of *Dehalococcoides* spp. [Duhamel and Edwards, 2006; Marco-Urra et al., 2011; Maymo-Gatell et al., 1997], which resulted in low concentrations of cells in the test cultures and, possibly, low ribosomal content of the cells.

The first issue was solved by concentrating the cells by centrifugation and counterstaining

the samples with 4',6'-diamidino-2-phenylindole (DAPI). Centrifugation allowed having a higher cell concentration in each field of view in the microscope. This also increased the signal-to-noise ratio by increasing the number of cells in the field of view relative to the occasional debris that might be present on the slide surface. This was particularly important while the method was not fully optimized and the fluorescent signal from the hybridization was faint. The number of *Dehalococcoides* spp. cells in each slide was assessed by counterstaining the sample with DAPI, a non-specific dye that intercalates in the double-stranded DNA present in all cells. By comparing the signal obtained in the red and green filters sensitive to the probes against the signal obtained in the blue filter with DAPI, we were able to ensure that the hybridization was equally successful for all cells present in the sample.

The second issue that reduced detection sensitivity was related to the low ribosomal content of the cells, which has been reported previously [Fazi et al., 2008; Fletcher et al., 2011]. To minimize this issue, we coupled the PNA probes with Alexa Fluor dyes. These dyes are well known for being more photostable and brighter than other commonly used fluorescent dyes operating at similar wavelengths [Berlier et al., 2003; Panchuk-Voloshina et al., 1999]. Because growth phase also can affect the rRNA content of the cells, we also evaluated the effect of the growth phase of the microorganisms on hybridization. Samples from the two different pure culture strains of *Dehalococcoides* were taken on different days and for different growth phases (fresh and old cultures). After optimization, the PNA-FISH method was able to detect cells in pure culture in all growth stages (fig. 1). Negative controls without probes for the two different pure cultures of *Dehalococcoides* did not show fluorescence (online suppl. fig. S2; for all online suppl. material, see www.karger.com/doi/10.1159/000362790).

Other possible causes of low intensity of the fluorescence signal for the two strains tested could have been an insufficient penetration of the probe through the bacterial cell wall due to the thick cell wall of the Gram-positive *Dehalococcoides* spp. [Perry-O'Keefe et al., 2001; Stender, 2003]. By performing the experiment with PNA instead of DNA, we guaranteed that the diffusion of our probe would be easier, as PNA probes are electrically uncharged and smaller than DNA probes [Cerqueira et al., 2008; Pellestor and Paulasova, 2004].

Development of a Multiplex Assay

The probes hybridized specifically to their corresponding strains meaning that the 1 base mismatch between the sequences was sufficient to discriminate between both strains using this technology. An important aspect was that the optimum temperature of hybridization for the two probes differed in 4°C. This was unexpected as there was only 1 base difference, but can be explained by the fact that the base pair for the 195 strain was formed between adenine and thymine (which creates two hydrogen bonds), whereas the CBDB1 base pair was between guanine and cytosine (which creates three hydrogen bonds). Nevertheless, the two probes will also work in a multiplex experiment if a hybridization average temperature of 57°C is selected (fig. 2). However, higher hybridization temperatures (59 and 61 °C) have proven to favor the binding of the CBDB1 probe.

While the method is adequate to discriminate between these 2 strains of *Dehalococcoides* spp., the probes were also designed to minimize the detection of other microorganisms present in environmental samples (99.99% specificity to *Dehalococcoides*, as indicated in table 2). A sequence search of the Ribosomal Database Project (RDP) confirmed that the probes detect very low numbers of other microorganisms. One exception, however, was the

complementarity observed between the 195 probe and the *Escherichia coli* strain W [Archer et al., 2011]. Overall, because 1 strain is not representative of the whole species and because the morphology of *Dehalococcoides* spp. And *E. coli* are very different, the risk for a misidentification is rather low. This sequence complementarity was also observed for the DNA-FISH probes reported earlier by Yang and Zeyer [2003].

Application of Probes to Mixed Cultures

To further confirm the specificity and performance of the probe, we tested the PNA-FISH multiplex method on mixed cultures containing other microorganisms (e.g. acetogens and methanogens). Results showed that the probe only detected coccoid cells in these cultures. For the Hawaii-05TM culture, both lineages of *Dehalococcoides* were observed (fig. 2), whereas SDC-9TM only contained the 195 lineage. Interestingly, 16S rRNA gene sequencing performed with SDC-9 suggested that the culture contains at least 4 different strains of *Dehalococcoides* [Trotsky et al., 2010], and the PNA-FISH testing performed here suggests that those strains may all be from the 195 lineage. For certain samples, autofluorescence by other microorganisms could be observed, but the signal-to-noise ratio of the probe was still sufficient to discriminate between populations (fig. 3). Both cultures exhibit metabolic behavior specific for their lineage. For example, SDC-9 and Hawaii-05 have the ability to metabolize a variety of chlorinated ethenes, including *cis*-DCE and VC, but neither culture is able to dechlorinate chlorobenzenes. As previously mentioned, the strain 195 and Pinellas subgroup lineages have metabolic similarities but also have some distinguishing features. Strain CBDB1 has the ability to metabolize a wide variety of chlorinated aromatics but is limited in its ability with chloroethenes. Conversely, other members of the Pinellas subgroup (FL2, GT and BAV1) have the ability to metabolize many of the lower chlorinated organics (table 1). Strain 195 is the only member of its lineage and demonstrates many of the same metabolic activities of the Pinellas subgroup, including the ability to metabolize chlorinated aromatics and ethenes.

This work demonstrates the effectiveness of using PNA-FISH to distinguish between 2 metabolically and genetically distinct *Dehalococcoides* strains. In previous works, PNA-FISH has been shown to surpass standard DNA-FISH in terms of robustness, and is now starting to be commercially available to detect microorganisms in clinical samples [Cerqueira et al., 2013]. Hence, the method can have strong implications for the monitoring and discrimination of *Dehalococcoides* populations in laboratory cultures and at contaminated sites. Nevertheless, future work should be performed to compare the method developed here with FISH and catalyzed reporter deposition FISH to determine which method is most effective in determining the concentration of *Dehalococcoides* spp. and also the application of this method to field samples.

Experimental Procedures

Pure cultures of *Dehalococcoides* strain CBDB1 and *Dehalococcoides mccartyi* strain 195 were grown at 30°C in glass serum bottles [Adrian et al., 2000; Löffler et al., 2013; Marco-Urrea et al., 2011]. Acetate (5 mM) was used as the carbon source for the pure cultures.

Mixed cultures SDC-9 and Hawaii-05 were chosen due to their application as commercial bioaugmentation cultures that have been used at a variety of sites because of their ability to

utilize chloroethenes [Schaefer et al., 2009, 2010a, b; Vainberg et al., 2009]. SDC-9 was enriched from a chlorinated solvent-contaminated site in southern California with PCE and sodium lactate [Vainberg et al., 2009]. In 2005, Hawaii-05 was enriched from aquifer material from the Hickman Air Force Base with TCE and sodium lactate [Vainberg et al., 2009]. Both cultures contain *tceA* and *vcrA* dehalogenase genes. They were grown at 28°C in glass serum bottles with lactate (5 mM) as the carbon source [Vainberg et al., 2009].

The initial PCE concentration was approximately 100 μ M for CBDB1, SDC-9 and Hawaii-05, and 200 μ M for strain 195. Aliquots of cells were removed from the serum bottles after 50% (CBDB1) and 100% (195, SDC-9 and Hawaii-05) of the initial PCE concentration was consumed. VC (99.5%) and ethene (99.95%) were obtained from Fluka. PCE (99%), TCE (99.5%), sodium lactate (60% solution) and sodium acetate (99%) were obtained from Sigma-Aldrich. *cis*-DCE (97%) and *trans*-DCE (99%) were obtained from Acros.

Concentrations of PCE and daughter products were analyzed using headspace samples (0.1 or 0.5 ml) injected onto a Shimadzu GC-2014 gas chromatograph with flame ionization detection and a 1% SP-1000 on a 60/80 Carbo- pack B column (Supelco). The column temperature program, and injector and detector temperatures have been described previously [Freedman and Gossett, 1989]. The production of PCE degradation daughter products (TCE, DCEs, VC and ethene) was used to confirm that the pure and mixed cultures were actively growing.

Probe Design

Specific PNA probes were designed by considering the position of a point mutation that has been used previously to distinguish between the strain 195 and Pinellas sub- groups of *Dehalococcoides* [Berlier et al., 2003] and the predicted melting temperature (T_m) value of the possible PNA oligomers. The theoretical T_m was calculated based on thermodynamic parameters described previously by SantaLucia and Hicks [2004]. Once the probe sequence was selected, a search was made for the available 16S rRNA gene sequences (RDP-II), version 10 (<http://rdp.cme.msu.edu/>) to confirm the theoretical specificity and sensitivity of the probe against other microorganisms. For this analysis, only high quality sequences with more than 1,200 bp were selected. The specificity and sensitivity values were determined as previously reported [Almeida et al., 2010]. The sequences were synthesized by PANAGENE (South Korea), purified by reverse-phase HPLC, and the N-terminus of the CBDB1 and 195 oligomers was connected to Alexa Fluor 594 and Alexa Fluor 488, respectively, via a double AEEA linker.

Hybridization Procedure

The PNA hybridization method used was developed based on the procedure reported [Azevedo et al., 2011; Perry-O'Keefe et al., 2001], but different fixation, hybridization and washing procedures were tested to optimize the method. Briefly, cell suspensions of the test cultures were prepared by harvesting cultures that were exponentially grown (online suppl. fig. S1). Thirty milliliters of each diluted sample were then collected by centrifugation [30 min at 3,200 *g* (Eppendorf centrifuge 5810, Eppendorf AG, Hamburg, Germany)]. In order to fix the cells, the pellet was resuspended in 1 ml of 4% paraformaldehyde (Sigma Aldrich) for 1 h at room temperature, followed by centrifugation. The fixed cells were then resuspended in 1 ml of 50% ethanol (vol/vol) for at least 30 min at -20°C. Then, 20 μ l were placed into microscopy well slides (10 mm; Thermo Scientific, Braunschweig, Germany) and allowed to air dry.

The tests slides were then covered with 20 µl of probe (400 nM) diluted in a hybridization solution that contained 10% (wt/vol) dextran sulfate, 10 mM NaCl, 30% (vol/vol) formamide, 0.1% (wt/vol) sodium pyrophosphate, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) Ficoll, 5 mM disodium EDTA, 0.1% (vol/vol) Triton X-100 and 50 mM Tris-HCl (all from Sigma). Control slides were covered with hybridization solution without the probe and, afterwards, both control and tests slides were covered with coverslips and then placed in moist chambers and incubated for 90 min at 57°C for the 195 lineage and 61°C for CBDB1 lineage.

To perform the multiplex assay, 20 µl of fixed SDC-9 and Hawaii-05 cells were placed onto microscopy slides and allowed to air dry. Hybridization was performed using a solution with 20 µl of hybridization buffer (previously described) with 100 nM of each respective probe covering each smear individually. Samples were sealed with coverslips and incubated for 90 min at 57°C. Subsequent steps were performed as described below.

Following hybridization, coverslips were removed and slides were submerged in a prewarmed washing solution (the temperature was similar to the one used for hybridization) containing 5 mM Tris base (Sigma), 15 mM NaCl (Sigma) and 1% (vol/vol) Triton X-100 (pH 10, Sigma) for 15 min and then removed and allowed to air dry. Finally, 1 µl of DAPI was added and the slides were covered with 1 drop of nonfluorescent immersion oil. Slides were stored at 4°C in the dark for a maximum of 24 h before microscopy.

Microscopy Visualization

Visualization was performed using an Olympus BX51 epifluorescence microscope (Olympus Portugal SA, Porto, Portugal) equipped with a filter sensitive to the Alexa Fluor 488 signaling molecule attached to the 195 PNA probe (absorbance, 495 nm, emission, 519 nm) and a filter sensitive to the Alexa Fluor 594 signaling molecule attached to the CBDB1 PNA probe (excitation, 530–550 nm; barrier, 570 nm; emission long-pass filter, 591 nm). To ensure that the signal obtained was not related to autofluorescence, all samples were visualized with other available filters. For every experiment, a negative control was performed simultaneously. For the negative control, all the steps described above were performed, but no probe was added during the hybridization procedure. All images were acquired using the Olympus CellB (Olympus Portugal) software with a magnification of $\times 1,000$.

Acknowledgments

The authors gratefully acknowledge the University of Porto– Mobile Program and the Portuguese Science and Technology Foundation (FCT) for the research project (DNA mimics – reference PIC/IC/82815/2007), a PhD grant (SFRH/BD/44876/2011) and two research positions (Ciência 2008 Program). The authors wish to thank Lorenz Adrian and Ivonne Nijenhuis (Helmholtz Center for Environmental Research) and Steven Zinder (Cornell University) for providing *Dehalococcoides* strains 195 and CBDB1. In addition, the assistance of Julian Renpenning (Helmholtz Center for Environmental Research) and Heather Fullerton (Cornell University) is greatly appreciated.

References

- Adrian L, Szewzyk U, Wecke J, Gorisch H: Bacterial dehalorespiration with chlorinated benzenes. *Nature* 2000;408:580–583.
- Almeida C, Azevedo NF, Fernandes RM, Keevil CW, Vieira MJ: Fluorescence in situ hybridization method using a peptide nucleic acid probe for identification of *Salmonella* spp. in a broad spectrum of samples. *Appl Environ Microbiol* 2010;76:4476–4485.
- Almeida C, Azevedo NF, Iversen C, Fanning S, Keevil CW, Vieira MJ: Development and application of a novel peptide nucleic acid probe for the specific detection of *Cronobacter* genomospecies (*Enterobacter sakazakii*) in powdered infant formula. *Appl Environ Microbiol* 2009;75:2925–2930.
- Almeida C, Azevedo NF, Santos S, Keevil CW, Vieira MJ: Discriminating multi-species populations in biofilms with peptide nucleic acid fluorescence in situ hybridization (PNA- FISH). *PLoS One* 2011;6:e14786.
- Archer CT, Kim JF, Jeong H, Park JH, Vickers CE, Lee SY, Nielsen LK: The genome sequence of *E. coli* w (ATCC 9637): comparative genome analysis and an improved genome-scale reconstruction of *E. coli*. *BMC Genomics* 2011;12:9.
- Armitage BA: The impact of nucleic acid secondary structure on PNA hybridization. *Drug Discov Today* 2003;8:222–228.
- Azevedo NF, Jardim T, Almeida C, Cerqueira L, Almeida AJ, Rodrigues F, Keevil CW, Vieira MJ: Application of flow cytometry for the identification of *Staphylococcus epidermidis* by peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) in blood samples. *Antonie Van Leeuwenhoek* 2011;100:463–470.
- Berlier JE, Rothe A, Buller G, Bradford J, Gray DR, Filanoski BJ, Telford WG, Yue S, Liu J, Cheung CY, Chang W, Hirsch JD, Beechem JM, Haugland RP: Quantitative comparison of long-wavelength Alexa Fluor dyes to Cy dyes: fluorescence of the dyes and their bio- conjugates. *J Histochem Cytochem* 2003;51: 1699–1712.
- Cerqueira L, Azevedo NF, Almeida C, Jardim T, Keevil CW, Vieira MJ: DNA mimics for the rapid identification of microorganisms by fluorescence in situ hybridization (FISH). *Int J Mol Sci* 2008;9:1944–1960.
- Cerqueira L, Fernandes RM, Ferreira RM, Oleastro M, Carneiro F, Brandão C, Pimentel-Nunes P, Dinis-Ribeiro M, Figueiredo C, Keevil CW, Vieira MJ, Azevedo NF: Validation of a fluorescence in situ hybridization method using peptide nucleic acid probes for detection of *Helicobacter pylori* clarithromycin resistance in gastric biopsy specimens. *J Clin Microbiol* 2013;51:1887–1893.
- Dijk JA, Breugelmans P, Philips J, Haest PJ, Smolders E, Springael D: Catalyzed reporter deposition-fluorescent in situ hybridization (CARD-FISH) detection of *Dehalococcoides*. *J Microbiol Methods* 2008;73:142–147.
- Duhamel M, Edwards EA: Microbial composition of chlorinated ethene-degrading cultures dominated by *Dehalococcoides*. *FEMS Microbiol Ecol* 2006;58:538–549.
- Eaddy A: Scale-Up and Characterization of an Enrichment Culture for Bioaugmentation of the P-Area Chlorinated Ethene Plume at the Savannah River Site; MS thesis; in Environmental Engineering and Earth Sciences. Clemson, Clemson University, 2008.
- Fazi S, Aulenta F, Majone M, Rossetti S: Improved quantification of *Dehalococcoides* species by fluorescence in situ hybridization and catalyzed reporter deposition. *Syst Appl Microbiol* 2008;31:62–67.

- Fennell DE, Nijenhuis I, Wilson SF, Zinder SH, Haggblom MM: Dehalococcoides ethenogenes strain 195 reductively dechlorinates diverse chlorinated aromatic pollutants. Environ Sci Technol 2004;38:2075–2081.
- Fletcher KE, Costanza J, Cruz-Garcia C, Ramaswamy NS, Pennell KD, Löffler FE: Effects of elevated temperature on Dehalococcoides dechlorination performance and DNA and RNA biomarker abundance. Environ Sci Technol 2011;45:712–718.
- Freedman DL, Gossett JM: Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl Environ Microbiol 1989;55:2144–2151.
- Guimaraes N, Azevedo NF, Figueiredo C, Keevil CW, Vieira MJ: Development and application of a novel peptide nucleic acid probe for the specific detection of Helicobacter pylori in gastric biopsy specimens. J Clin Microbiol 2007;45:3089–3094.
- He J, Ritalahti KM, Yang KL, Koenigsberg SS, Löffler FE: Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nature 2003;424:62–65.
- He J, Sung Y, Krajmalnik-Brown R, Ritalahti KM, Löffler FE: Isolation and characterization of Dehalococcoides sp. strain FL2, a trichloroethene (TCE)- and 1,2-dichloroethene-respiring anaerobe. Environ Microbiol 2005;7: 1442–1450.
- Hendrickson ER, Payne JA, Young RM, Starr MG, Perry MP, Fahnestock S, Ellis DE, Ebersole RC: Molecular analysis of Dehalococcoides 16S ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe. Appl Environ Microbiol 2002;68:485–495.
- Holliger C, Wohlfarth G, Diekert G: Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol Rev 1999;22:383–398.
- Löffler FE, Yan J, Ritalahti KM, Adrian L, Edwards EA, Konstantinidis KT, Muller JA, Fullerton H, Zinder SH, Spormann AM: Dehalococcoides mccartyi gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, Dehalococcoidia classis nov., order Dehalococcoidales ord. nov. and family Dehalococcoidaceae fam. nov., within the phylum Chloroflexi. Int J Syst Evol Microbiol 2013;63:625–635.
- Marco-Urrea E, Nijenhuis I, Adrian L: Transformation and carbon isotope fractionation of tetra- and trichloroethene to trans-dichloroethene by Dehalococcoides sp. strain CBDB1. Environ Sci Technol 2011;45:1555–1562.
- Maymo-Gatell X, Anguish T, Zinder SH: Reductive dechlorination of chlorinated ethenes and 1,2-dichloroethane by ‘Dehalococcoides ethenogenes’ 195. Appl Environ Microbiol 1999;65:3108–3113.
- Maymo-Gatell X, Chien Y, Gossett JM, Zinder SH: Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science 1997;276:1568–1571.
- Panchuk-Voloshina N, Haugland RP, Bishop-Stewart J, Bhalgat MK, Millard PJ, Mao F, Leung WY: Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. J Histochem Cytochem 1999;47:1179–1188.
- Pellestor F, Paulasova P: The peptide nucleic acids, efficient tools for molecular diagnosis (review). Int J Mol Med 2004;13:521–525.
- Perry-O’Keefe H, Rigby S, Oliveira K, Sorensen D, Stender H, Coull J, Hyldig-Nielsen JJ: Identification of indicator microorganisms using a standardized PNA-FISH method. J Microbiol Methods 2001;47:281–292.

- Rowe AR, Lazar BJ, Morris RM, Richardson RE: Characterization of the community structure of a dechlorinating mixed culture and comparisons of gene expression in planktonic and biofloc-associated 'Dehalococcoides' and Methanospirillum species. *Appl Environ Microbiol* 2008;74:6709–6719.
- SantaLucia J Jr, Hicks D: The thermodynamics of DNA structural motifs. *Annu Rev Biophys Biomol Struct* 2004;33:415–440.
- Schaefer CE, Condee CW, Vainberg S, Steffan RJ: Bioaugmentation for chlorinated ethenes using *Dehalococcoides* sp.: comparison between batch and column experiments. *Chemosphere* 2009;75:141–148.
- Schaefer CE, Lippincott DR, Steffan RJ: Field-scale evaluation of bioaugmentation dosage for treating chlorinated ethenes. *Ground Water Monit Remediat* 2010a;30:113–124.
- Schaefer CE, Towne RM, Vainberg S, McCray JE, Steffan RJ: Bioaugmentation for treatment of dense non-aqueous liquid in fractured sand-stone blocks. *Environ Sci Technol* 2010b;44: 4958–4964.
- Scow KM, Hicks KA: Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. *Curr Opin Biotechnol* 2005;16:246–253.
- Stender H: PNA FISH: an intelligent stain for rapid diagnosis of infectious diseases. *Expert Rev Mol Diagn* 2003;3:649–655.
- Sung Y, Ritalahti KM, Apkarian RP, Löffler FE: Quantitative PCR confirms purity of strain GT, a novel trichloroethene-to-ethene-respiring *Dehalococcoides* isolate. *Appl Environ Microbiol* 2006;72:1980–1987.
- Tas N: *Dehalococcoides* spp. in River Sediments: Insights in Functional Diversity and Dechlorination Activity; PhD thesis, Wageningen University, Wageningen, 2009, p 206.
- Trotsky J, Wymore RA, Lamar MR, Sorenson KD: A low-cost, passive approach for bacterial growth and distribution for large-scale implementation of bioaugmentation. in ESTCP Project ER-200513 – Final Report. 2010, http://www.clu-in.org/download/contaminantfocus/dnapl/Treatment_Technologies/ER-200513-FR.pdf.
- Vainberg S, Condee CW, Steffan RJ: Large-scale production of bacterial consortia for remediation of chlorinated solvent-contaminated groundwater. *J Ind Microbiol Biotechnol* 2009;36:1189–1197.
- Yang Y, Zeyer J: Specific detection of *Dehalococcoides* species by fluorescence in situ hybridization with 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* 2003; 69:2879–2883.

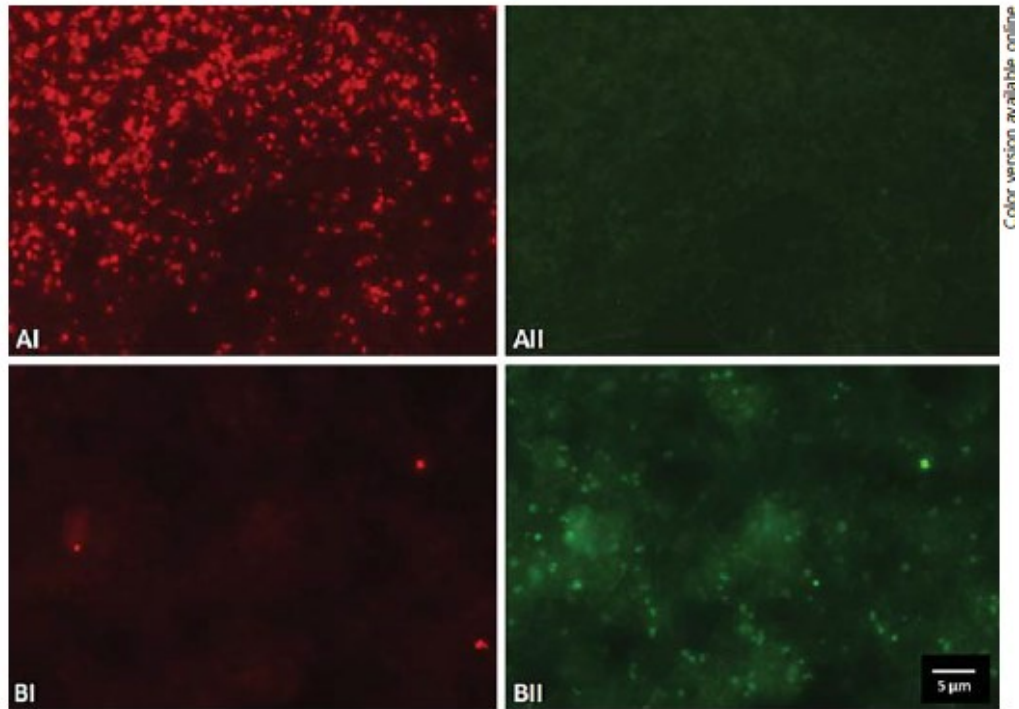


Fig. 1. Identification of *Dehalococcoides* spp. (strain 195 and CBDB1) by epifluorescence microscopy. **A** Detection of *Dehalococcoides* CBDB1 in pure culture using the CBDB1 PNA probe labeled with Alexa Flu- or 594 (**AI**) and observation of the same field of view in the green channel that is not sensitive to the fluorochrome used (**AII**). **B** Detection of *Dehalococcoides* 195 in pure culture using the 195 PNA probe labeled with Alexa Fluor 488 (**BII**) and observation of the same field of view in the red channel (**BI**). The experiments were performed in parallel, and images were obtained with equal exposure times

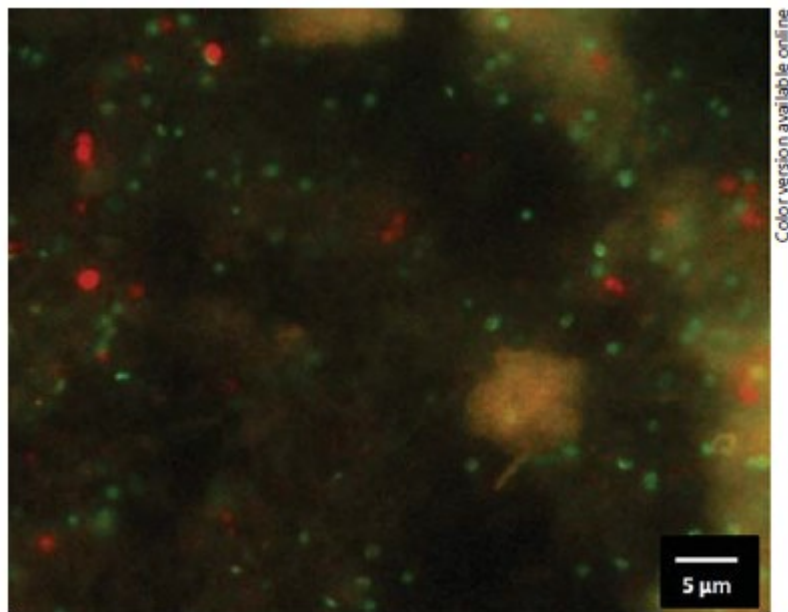


Fig. 2. Epifluorescence microscopy image of a multiplex experiment performed on the Hawaii-05 showing the two lineages.

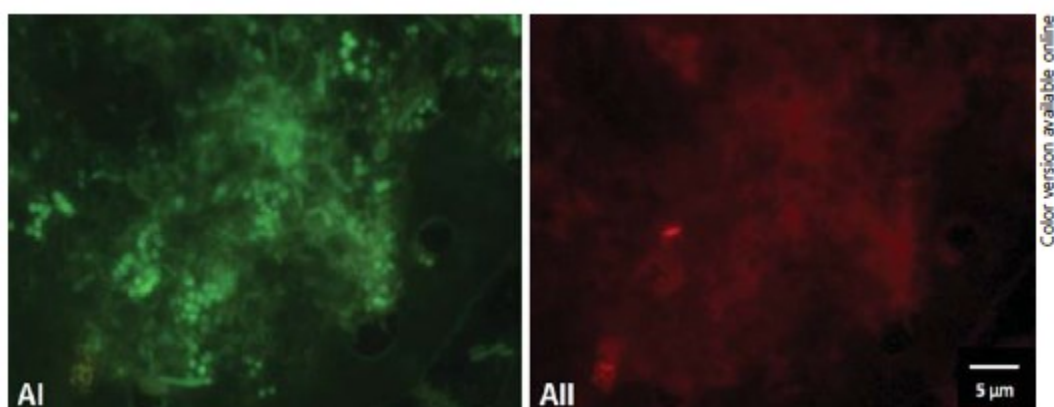


Fig. 3. Epifluorescence microscopy image of a multiplex experiment performed on the SDC-9 showing the detection of the CBDB1 lineage against a background of other cells (**AI**). This autofluorescence was also detected in the red filter that was not sensitive to the fluorochrome attached to the probe (**AII**).

Table 1. Comparison of different *Dehalococcoides* pure cultures and their ability to utilize different electron acceptors (adapted from Eaddy [2008])

Compound	Strain 195 (Cornell)	BAV1 (Pinellas)	GT (Pinellas)	FL2 (Pinellas)	CBDB1 (Pinellas)
PCE	+	●	–	●	+
TCE	+	●	+	+	+
<i>cis</i> -DCE	+	+	+	+	–
<i>trans</i> -DCE	●	+	–	+	–
1,1-DCE	+	+	+	–	–
VC	●	+	+	●	–
CB	–	?	?	?	–
1,2-DCB	–	?	?	?	?
1,3-DCB	–	?	?	?	?
1,4-DCB	–	?	?	?	?
1,2,3-TCB	●	?	?	?	+
1,2,4-TCB	●	?	?	?	+
1,3,5-TCB	●	?	?	?	–
1,2,3,4-TeCB	+	?	?	?	+
1,2,3,5-TeCB	●	?	?	?	+
1,2,4,5-TeCB	+	?	?	?	+
PeCB	+	?	?	?	+
HCB	+	?	?	?	+

+ = Used as a terminal electron acceptor (TEA). ● = Used cometabolically. – = Not used as a TEA. CB = Chlorobenzene; DCB = dichlorobenzene; HCB = hexachlorobenzene; PeCB = pentachlorobenzene; TCB = trichlorobenzene.

Table 2. Theoretical evaluation of the PNA probes

Probe	Sequence (5'-3')	Target position (<i>E. coli</i> numbering)	kcal/mol ΔG	°C T _m	Sensitivity, %	Specificity, %
CBDB1	GTTCGCACTGTTGC	1,257–1,270	–17.92	71.30*	98.21	99.99
195	GTTCACACTGTTGC	1,257–1,270	–16.36	68.13*		
* Considering a probe concentration of 1 × 10 ^{–4} M.						