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Bioaugmentation of a Soil Bioreactor Designed for Pilot-Scale Anaerobic Bioremediation Studies

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The aim of this work was to answer the following questions: (i) Can we realize a long-term dechlorination with a pure anaerobic strain in soil and (ii) can we monitor the process on an adequate scale with a controlled simulator of in situ conditions (soil bioreactor of a 500-L scale). The soil bioreactor was fed continuously with 3-chlorobenzoate (3-CB) as a model chloroaromatic compound. Bioaugmentation was carried out by inoculating *Desulfomonile tiedjei* in localized areas in the bioreactor. Temporal and spatial distribution of the 3-CB dechlorination activity was investigated with specific biological activity tests for approximately 4 months following the inoculation. These tests involved the minimally invasive sampling of geometrically distinct points in the reactor and their off-site handling within reconstructed microcosms, allowing the assessment of dechlorinating and methanogenic soil activities. Using autoclaved and nonautoclaved agricultural soil, the results showed a heterogeneous distribution of the dechlorination activity in the bioreactor. The autoclaved soil expressed a high microbial activity as reflected by biogas production and 3-CB dechlorination. Furthermore, durable establishment of *D. tiedjei* in both autoclaved and nonautoclaved soil was shown, although in the latter portion of the reactor the microorganism was maintained only at the top surface. Polymerase chain reaction (PCR) detection of the 16S rDNA gene of *D. tiedjei* was directly correlated with 3-CB dechlorination activity. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR products demonstrated distinct bacterial community fingerprints in soil samples as a function of location and in response to process modifications. This investigation demonstrates for the first time the applicability and limitations of soil bioaugmentation with a pure anaerobic dechlorinating strain at a realistic pilot scale.

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Introduction

Bioremediation, the technology in which microorganisms are used to degrade toxic chemical compounds, is currently commanding interest for the efficient cleanup of polluted soils (*1*). Within the spectrum of bioremediation techniques, bioaugmentation can be defined as the use of competent consortia or characterized strains, with the abilities to degrade the target toxic molecules, as exogenous inocula for cleaning up polluted sites. Obligate anaerobes could potentially play a useful role as a source of inocula for use in contaminated sites (*2*−*4*), more specifically through reductive dehalogenation a process through which several anaerobes can remove halogens from highly halogenated organic compounds (*5, 6*). However, prior to introducing such microorganisms into the field, effective, reliable, rapid, and relatively inexpensive tools and techniques are required to assess survival and competitiveness of these introduced microbial strains. Several microcosms have been designed to determine whether the transferred organisms can express their specific activity in such environments (*7*−*12*). However, these studies have been carried out on a laboratory scale using bottles or small unidirectional columns and are generally confined to small volume samples. These tend to be homogeneous and uniform; thus, they are not representative of actual in situ conditions. Moreover, it appears that one of the challenges for soil bioremediation is the scaling up of these laboratory results to *in situ* or *on site* treatment (*13, 14*). Therefore, there is a need for intermediate- (pilot-) scale tools generating data useful for scaling up to an in situ soil bioremediation process. Recently, pilot-scale bioaugmentation for remediation of carbon tetrachloride-contaminated aquifer proved successful as shown by the inoculation of *Pseudomonas stutzeri* KC (*15*). The latter study showed both subsurface transport of strain KC and its assimilation into the aquifer community (*15*).

This work aimed to answer the following questions: (i) Can we realize a long-term dechlorination with a pure anaerobic strain in soil and (ii) can we monitor the process on an adequate scale with a controlled simulator of in situ conditions (soil bioreactor of a 500-L scale). The major advantage of such a system is that it offers the possibility to study bioremediation processes continuously on a three-dimensional level. Consequently, it makes it easy to follow both spatial and temporal distributions of the introduced microorganisms. In our present study, anaerobic bioaugmentation was realized by using a pure culture of *Desulfomonile tiedjei* as an anaerobic dechlorinating inoculum. This bacterium has been isolated as a strict anaerobic sulfate reducer capable of dechlorinating several chloroorganic compounds including 3-chlorobenzoate (3-CB), chlorophenols, and tetrachloroethene (*16*−*18*). The molecular monitoring of this bacterium in the bioreactor was performed with the polymerase chain reaction (PCR) amplification of the 16S rDNA gene. This methodology has been recently shown capable of specifically detecting this bacterium from a background of microorganisms in the agricultural soil used in this study (*12*).

Materials and Methods

Bioreactor. Figure 1 depicts schematically the model soil bioreactor. It is a parallelpiped constructed from transparent poly(vinyl chloride) (wall thickness: 10 mm) and measures $1.2 \times 0.7 \times 0.64$ m^3 ($L \times W \times H$) with a total volume of 0.538 m^3. The total bed volume was 500 L with a liquid volume of 275 L. An upflow−overflow liquid system was chosen as it

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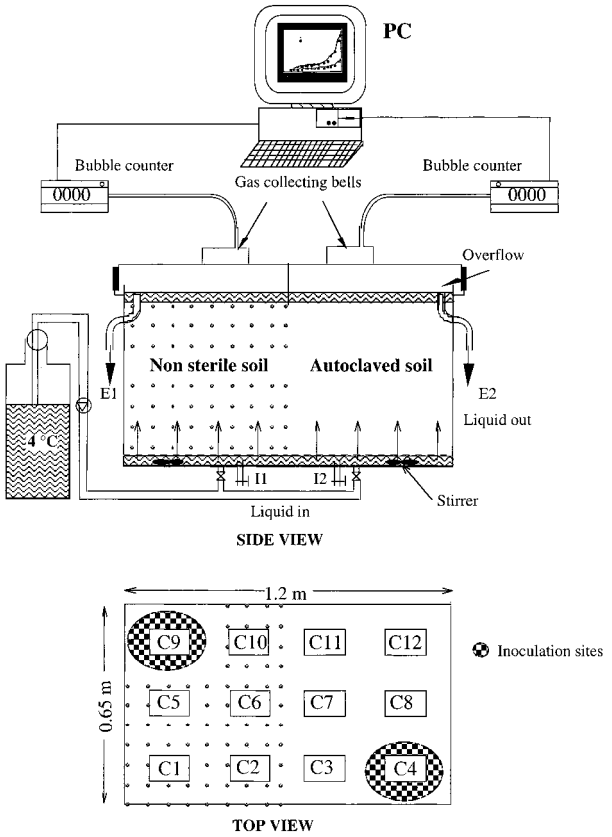

FIGURE 1. Schematic view of the soil bioreactor (see text for details). E1 and E2, effluent points. I1 and I2, influent points.

facilitates the recovery of the biogas produced by the soil microbial communities. Continuous biogas production was estimated with a homemade pair of electronic gas bubble counters connected to a personal computer. These counters were calibrated against a gas flow meter and frequently checked for their reliability. In the bottom of the reactor, a liquid zone measuring 15 cm in height was separated from the rest of the reactor by a grid which was completely covered with a "Bidim" fabric (LM Matériaux, Brussels, Belgium) permeable only to the liquid feed solution. Anaerobiosis was assured by submerged-state conditions and the top of the reactor was protected with an airtight plastic cover. This cover was divided into 12 numbered compartments. The reactor was installed inside a 35 °C constant temperature room. This temperature was chosen to achieve reasonably high methanogenesis.

To identify potential channels of preferential flow, sodium chloride (1000 ppm) was added as a mobile tracer. The breakthrough curve was obtained by measuring the chloride concentration at the effluent points. Furthermore, piezometers were introduced in the bioreactor at different points in terms of depth and width to allow experimental measurements of the distribution of the chloride concentration in different areas of the bioreactor.

Experimental Procedure. *Desulfomonile tiedjei* DCB-1 (ATCC 49306) was obtained from the American Type Culture Collection, Bethesda, MD. The bacterium was maintained by periodic transfer in a defined medium (*16*) with 2 mM 3-CB as the electron acceptor and 20 mM pyruvate as the electron donor. Throughout this study, an agricultural soil originating from Michamps, Belgium was used. It consists of 60% silt, 30% clay, and 10% sand. The soil contained 2% organic matter; its cation exchange capacity (CEC) was 8−10 mequiv/100 g, and its pH in water was 6 (*12*). The bioreactor was filled with soil at a 1:1 ratio of a nonautoclaved portion which was introduced in vertical contact with a previously autoclaved portion of the soil (Figure 1). The portion destined to be autoclaved was first air-dried and then steam-sterilized in three steps for 2 h at 121 °C. The autoclaving steps were separated by at least 24 h. Air-dried soil or soil with a low water content (less than about 60% of moisture-holding capacity) is known to allow better sterilization (*19*). Just before the two parts of the soil (autoclaved and nonautoclaved portion) were added into the reactor, the vessel was vertically separated in the middle with a plastic barrier, which was removed immediately after the loading of each portion into the appropriate half of the reactor, ensuring complete vertical contact between autoclaved and nonautoclaved soil. *D. tiedjei* inoculation was performed in the nonautoclaved part (Figure 1, compartment 9) as well as in the autoclaved part (Figure 1, compartment 4). Strictly localized inoculation was ensured by installing two soil-containing cylinders made of poly(vinyl chloride) (65 cm in height and 15 cm in diameter) in parts 4 and 9 of the reactor (Figure 1) before the bioreactor was filled with soil. The soil in each cylinder (±6 kg) was mixed separately with fresh cultures of *D. tiedjei* to give approximately 10^5 cells/g of soil. This mixture was transferred into the cylinders. The reactor was fed daily with a mineral solution (containing, in mg/L: KH_2PO_4, 500; $Na_2HPO_4 \cdot 2H_2O$, 485; $MgCl_2 \cdot 6H_2O$, 80; $CaCl_2 \cdot 2H_2O$, 10; NH_4Cl, 500; NaCl, 400), a trace metal solution (containing, in mg/L: $ZnSO_4 \cdot 7H_2O$, 10; $MnSO_4 \cdot H_2O$, 2.6; H_3BO_3, 30; $CoSO_4 \cdot H_2O$, 24; $NiSO_4 \cdot 6H_2O$, 2.2; $Na_2MoO_4 \cdot 2H_2O$, 3.0; Na_2SeO_3, 1.0; $CuSO_4 \cdot 5H_2O$, 3.7), and vitamins (in mg/L: biotin, 0.05; pyridoxine, 0.05; riboflavin, 0.05; panthothenic acid, 0.05; *p*-aminobenzoic acid, 0.05; folic acid, 0.05; thiamine hydrochloride, 0.05; cyanocobalamine, 0.05; nicotinamide, 0.50; 1,4-naphthoquinone, 0.20). After 10 days from the start of feeding, the cylinders were removed to allow spatial distribution of the introduced bacterium. The reactor was operated at a flow rate of 2 L/h with a hydraulic retention time of 7 days. The cosubstrates used in the feed were acetate plus formate at various concentrations shown in Figure 3A. The feed concentrations of 3-CB used are also shown in Figure 3B. Dechlorinating activity was monitored by two means: (i) The concentration of 3-CB and its dechlorinated product (benzoate) were measured in the culture fluid at the influent sampling points I1 and I2 as well as at the effluent points E1 and E2 (Figure 1). (ii) Spatial distribution of the dechlorinating activity was studied by assaying samples of soil from different areas. Soil samples were then put inside batch bottles under standard conditions, allowing the comparison of dechlorinating activity over the course of the experiment (see below). A specially designed nondisturbing pinch-type soil sampler was used. It is a long metal rod (90 cm) equipped with a sampling compartment which has a lozenge shape. Before the sampler was introduced, the sampling compartment was closed. Once introduced into the soil, the sampling compartment was opened 5 cm above the depth of sampling. After the sampling (approximately 50 g of soil), the compartment was closed and the sampler was slowly pulled out.

Dechlorination Activity Tests. Twenty grams of soil taken from different compartments of the reactor were placed in a 120-mL glass flask and 50 mL of the basal medium containing 0.1% yeast extract, mineral solution, and vitamins (*16*) was added. To this, a supplemental cosubstrate consisting of 5 mM acetate plus 6 mM formate was added. The mixture was flushed under 100% N_2 and the pH was adjusted to 7.3 with $NaHCO_3$. Transfers to flasks were conducted under anaerobic conditions. The flasks were sealed under an atmosphere of 20% CO_2 plus 80% N_2. Sodium dithionite was added as reducing agent (final concentration 500 μM). 3-CB (1−1.5 mM) was added from a concentrated solution. To follow the time course of the 3-CB dechlorinating activity in different parts of the reactor, soil samples were always taken

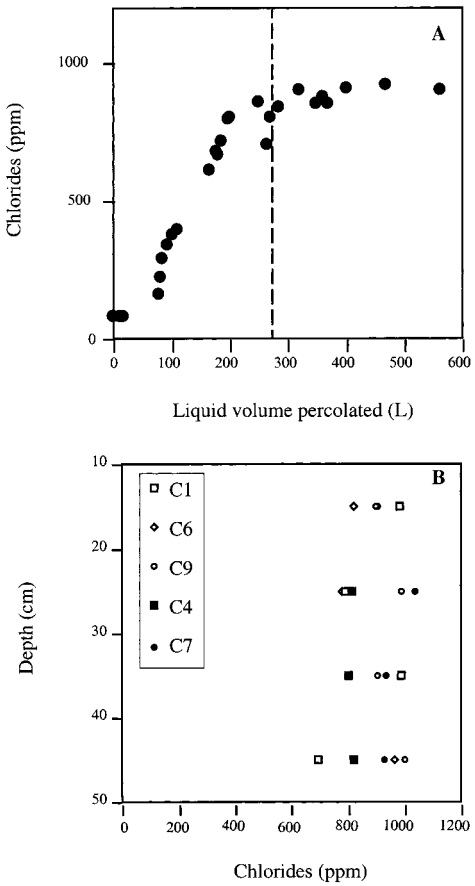

FIGURE 2. The movement of the liquid flow in the bioreactor. (A) Breakthrough curve. Dashed line corresponds to the volume of the liquid percolated which coincides with the total liquid volume of the bioreactor (275 L). (B) Measurements of chloride concentration in different parts of the bioreactor.

at the same depth (30 cm from the top). In addition, soil samples were also taken at the interface between the soil and the liquid zone submerging the soil, at day 93. The bottles, shaken at 125 rpm, were incubated at 35 °C for 10 days. All experiments were performed in duplicate. The dechlorination capacity was deduced from the drop of 3-CB levels with time. The same bottles were used to assess the methanogenic capacity of the soil samples (*20*).

Soil DNA Extraction and PCR Detection. Total DNA from soil samples was extracted using previously described protocol (*20*). In this study we used oligonucleotide primers (Dt1 and Dt2) targeting the 16S rDNA gene which were previously designed as specific for detecting *D. tiedjei* introduced in nonautoclaved soil (*12*). One to two microliters of extracted DNA were amplified by PCR with a 9600 thermal cycler (Perkin-Elmer, Norwalk, CT). Throughout this study DNA polymerase commercially available as the High Fidelity PCR system (Boehringer, Mannheim, Germany) was used. PCR experiments were carried out in 50 μL final volume with 5 μL of Ampli *Taq* 10× reaction buffer, 2.5 pmol of primers, 0.2 mM dNTP, and 0.6 units of polymerase. Two to four hundred ng/mL of an ultrapure and molecular biology grade bovine serum albumin (BSA) (Boehringer) and 100−150 ng/mL of T4 gene protein 32 (Boehringer) was added to the PCR mixture. Samples were amplified with 30 cycles of the following conditions: 92 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. A final extension was carried out at 72 °C for 10 min. Confirmation of PCR products was obtained by restriction mapping. The choice of enzymes was made by comparing restriction maps of the closely related species using the program of the Genetic Computer Group, Inc.

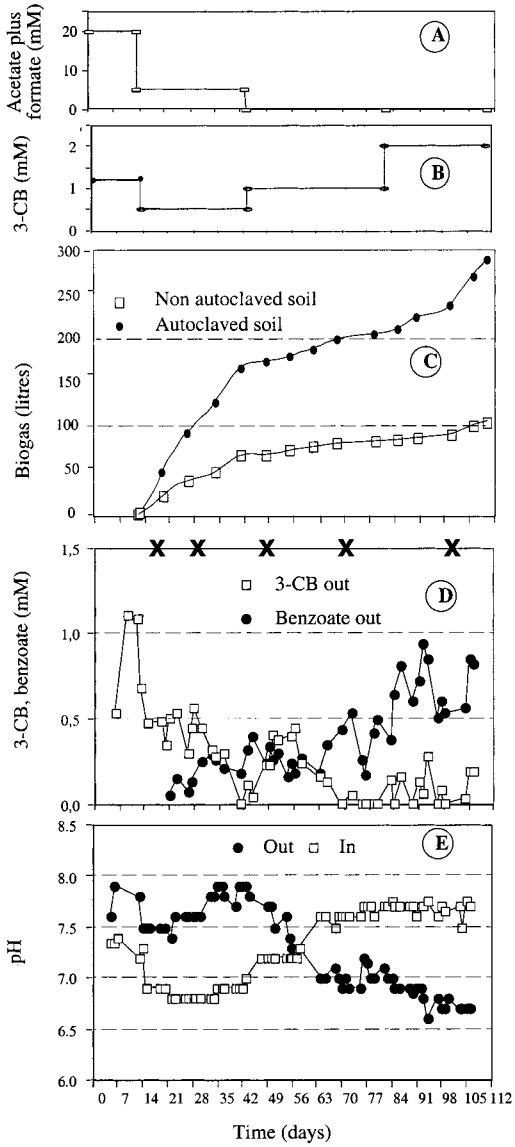

FIGURE 3. Evolution of the biological activity in the soil bioreactor inoculated with *D. tiedjei* in combination with chemical feeding modifications. (A) Cosubstrate concentration in the feeding solution over time. (B) Evolution of 3-CB concentration in the feeding solution. (C) Cumulative biogas production recovered from the autoclaved soil part and from the nonautoclaved soil part. (D) 3-CB transformation and benzoate evolution in effluent samples recovered from effluent sampling point E2. (E) pH evolution in liquid samples recovered from influent point I2 (pH In) as well as from effluent point E2 (pH Out). Points I2 and E2 are indicated in Figure 1. (X) Soil sampling times.

(Wisconsin package, version 8.1-UNIX, 1995). *Bsi*HKAI which has two sites and generated three fragments (648, 208, and 134 bp) was used.

Denaturing Gradient Gel Electrophoresis (DGGE). The V3 region of 16S rRNA genes from soil microbial communities was amplified by PCR with a set of primers consisting of the PRBA338f primer (5′ACTCCTACGGGAGGCAGCAG3′) which complements a region conserved among members of the domain *Bacteria* and of the primer PRUN518r (5′ATTACCGCGGCTGCTGG3′) based on the universally conserved region. The primer PRBA338f incorporates a 40-base GC clamp (5′CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGCACGGGGGG) (*21*).

DGGE was performed using the Bio-Rad D Gene System (Hercules, CA). PCR samples were loaded onto 8% (wt/vol)

TABLE 1. Comparison between Measured and Expected Values for Methane Production in the Soil Bioreactor

days	biogas produced (L)	average %CH_4	CH_4 produced (L)	acetate[a] consumed (mmol)	formate[a] consumed (mmol)	aromatic[b] compound consumed (mmol)	experimental[c] CH_4 (exp) production (mmol)	theoretical[d] CH_4 (th) production (mmol)	ratio CH_4 (exp)/ CH_4 (th)
19−41	154	80 ± 5	123	4125	4125	0	5125	5156	0.99
49−69	27	90 ± 4	24.3	0	0	392	1012	1443	0.68
81−93	42	90 ± 4	37.8	0	0	470	1575	1762	0.89

[a] The calculation was based on the theoretical assumption that there was a complete transformation of acetate (5 mM) and formate (5 mM) to methane and carbon dioxide. The total liquid volume of the reactor is 275 L with a hydraulic retention time of 7 days. [b] The average concentrations of the aromatic compound consumed were deduced from the experimental values reported in Figure 3. These are 0 mM between days 19 and 41, 0.5 mM between days 49 and 69, and 1 mM between days 81 and 93. [c] Experimental values of CH_4 production were calculated as follows: CH_4 (mmol) = [(volume of biogas produced × %CH_4)/24] × 1000. [d] Theoretical values for CH_4 production were calculated on the basis of the reactions reported in Table 1 in combination with concentrations reported in columns (*a*) and (*b*): CH_4 (mmole) = $VL_{\text{total liquid volume percolated}}$ × ([acetate concentration[a]] + [formate concentration[a]]/4 + [aromatic compound concentration[b]] × 3.75).

polyacrylamide gels in 1xTAE (20 mM Tris, 10 mM acetate, and 0.5 mM EDTA pH 7.4). The polyacrylamide gels were made with a denaturing gradient ranging from 40−55% (where 100% of the denaturant contains 7 M urea and 40% formamide). The electrophoresis was run overnight at 60 °C and 35 V. After the electrophoresis, the gel was soaked for 30 min in SYBR GreenI nucleic acid gel stain (1:10000 dilution; FMC BioProducts, Rockland, ME). The stained gel was immediately photographed on an UV transillumination table with a video camera module (Vilbert Lourmat, Marne-la-Valle, France).

The comparison of different patterns has been done using the GelCompar software 4.1 (Applied Maths, Kortrijk, Belgium). The band-based cluster analysis was calculated using two similarity coefficients: (i) the coefficient of Jaccard (Sj) using band positions. For each pattern Sj divides the number of corresponding bands by the total number of bands in both patterns. (ii) The area-sensitive coefficient (SD) which is very similar to the coefficient of Jaccard but modified to penalize differences in band intensities. This coefficient gives more weight to matching bands.

Analytical Methods. Samples for quantification of 3-CB and benzoate were analyzed by reverse-phase high-performance liquid chromatography (HPLC) as previously described (*12*). The gas composition was determined by gas chromatography (*22*). Mineral chloride concentration was determined as previously described by Boucquey et al. (*22*).

Results

Identification of Channels of Preferential Flow. To determine whether there is preferential flow of water in the bioreactor, we used high concentration chloride (1000 ppm) as a chemical tracer. Assuming that there is essentially a plug flow of liquid, if there is no preferential channeling, it can be expected that the chloride concentration, measured at outlet points, in a continuous feeding system will become constant when the percolated volume equals the total liquid volume of the bioreactor (275 L). This assumption was proven experimentally by data reported in Figure 2A. Furthermore, the analysis of liquid samples taken from different parts in the reactor showed no significant difference in chloride concentrations (Figure 2B). On the basis of these results, we can state that there are no preferential channels in the bioreactor.

Reactor Studies. The reactor was fed with acetate + formate as a substrate for methanogenesis (20 mM) together with 3-CB at an initial concentration of 1 mM over the first 10 days (Figure 3A,B). As no methane was observed in this period, this concentration was judged as too high for the indigenous bacterial population, and it was subsequently decreased to 0.5 mM. Immediately thereafter, methane production started, followed by 3-CB dechlorination after about 1 week (Figure 3C). The biological activity of the soil reactor was monitored by following the biogas production collected from the nonautoclaved as well as from the autoclaved soil parts throughout the experiment. The results showed a clear difference between the autoclaved and the nonautoclaved half of the reactor (Figure 3C). The cumulative biogas production was considerably higher in the part containing the previously autoclaved soil. Concerning the biogas analysis, samples were taken from different compartments and the average methane production is depicted in Table 1. The results reported in Figure 3D show that the 3-CB dechlorination activity was expressed after approximately 3 weeks from the start of the experiment. 3-CB was measured in effluent point E2 (Figure 3D). The data show a progressive decrease in 3-CB concentration which was converted to benzoate. When 3-CB concentration reached zero (day 41), we decided to stop the cosubstrate feeding and the initial concentration of 3-CB was doubled in the influent to 1 mM. After this modification, a tendency toward a transient accumulation of 3-CB was observed for approximately 2 weeks followed by a decrease in 3-CB concentration. However, between days 42 and 70 the biogas production decreased significantly (Figure 3C). On the basis of a mass balance and taking into account the mean hydraulic retention time (HRT = 7 d), one sees that 3-CB dechlorination was diminished during that interval, but never completely arrested (Figure 3D). At day 80, the concentration of 3-CB was again doubled in the feed to reach 2 mM. After this second modification, no accumulation of 3-CB was noted in the samples taken from the sampling point E2 (Figure 3D), while at the same time, a commensurately higher rate of methanogenesis was noted (Figure 3C). In the effluent outlets E1 (data not shown) and E2 (Figure 3D) there was no complete disappearance of benzoate, even though an increase in the cumulative biogas production was observed in the two reactor halves (autoclaved and nonautoclaved soil) as depicted in Figure 3C. Experimental measurements of 3-CB and benzoate transformations (Figure 4) in combination with mass-balance calculations for methane production (Table 1) performed on the basis of theoretical reactions reported in Table 2 show clearly that (a) there is no adsorption of 3-CB nor of benzoate on soil particles and (b) that the part of 3-CB transformed was mineralized to CH_4 (Table 1). Figure 3E displays the variation (time course) of the pH in the influent and effluent samples. The curves reveal a decrease in pH coinciding with the beginning of benzoate transformation in the reactor (day 36). However, this decrease in pH was apparently attenuated by the mineralization of benzoate as shown in Figure 3E from day 80 onward.

Distribution of 3-CB Dechlorination Activity in the Bioreactor Over Time. To assess the distribution of the dechlorination activity in the bioreactor, different soil samples were taken from the bioreactor using the soil sampler and were tested in microcosms as described in the Materials and

TABLE 2. Reactions and Gibbs Free Energy Changes ($\Delta G^{\circ\prime}$) for Various Substrates Involved in Methane Production in the Bioreactor

reactions	$\Delta G^{\circ\prime}$ (kJ/reaction)	refs
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	−32	*41*
$4HCOO^- + 4H^+ \rightarrow 3CO_2 + CH_4 + 2H_2O$	−134	*41*
$4C_6H_5COO^- + 28H_2O \rightarrow 12CH_3COO^- + 12H^+ + 4HCO_3^- + 12H_2$	+359	*42*
$12H_2 + 3HCO_3^- + 3H^+ \rightarrow 3CH_4 + 9H_2O$	−407	*42*
$4C_6H_5COO^- + 19H_2O \rightarrow 12CH_3COO^- + HCO_3^- + 3CH_4$	−48	*42*

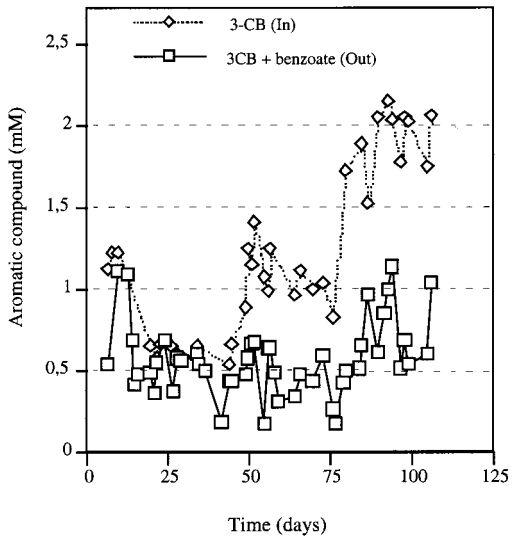

FIGURE 4. Measurements of 3-CB and benzoate concentrations at the effluent "Out" and influent "In" points.

Methods section. Throughout the experiment, five soil sampling tests were performed at days 13, 24, 43, 62, and 93 (Figure 3). Activity measurements of methanogenesis and benzoate formation/utilization (data not shown) and dechlorination were obtained from these soil samples. Figure 5 depicts the comparison of 3-CB dechlorination activity between samples taken from the nonautoclaved (Figure 5A) and autoclaved (Figure 5B) soil at different days. All samples were taken at 30 cm of depth. Figure 5A shows the results of samples taken from different points of the nonautoclaved soil. Not much 3-CB dechlorination activity was observed in the noninoculated points (C1 and C6). In the inoculated site (C9), significant dechlorination activity was observed on samples taken at days 13 and 24 with 50 and 80% transformation of 3-CB, respectively. However, in this location, the dechlorination activity decreased drastically in the soil samples taken on days 43, 62, and 93. It is important to note that the three latter tests were performed on samples obtained after the reactor feeding with the cosubstrate was stopped and the 3-CB inlet concentration was doubled. Concerning the autoclaved soil samples, considerable 3-CB dechlorination activity was observed with all samples from day 43 onward. This activity was observed in the area inoculated with *D. tiedjei* (C4) as well as in the noninoculated areas (C7 and C12). These results are particularly indicative of the striking difference in the dechlorination activity between nonautoclaved (Figure 5A) and autoclaved (Figure 5B) soil.

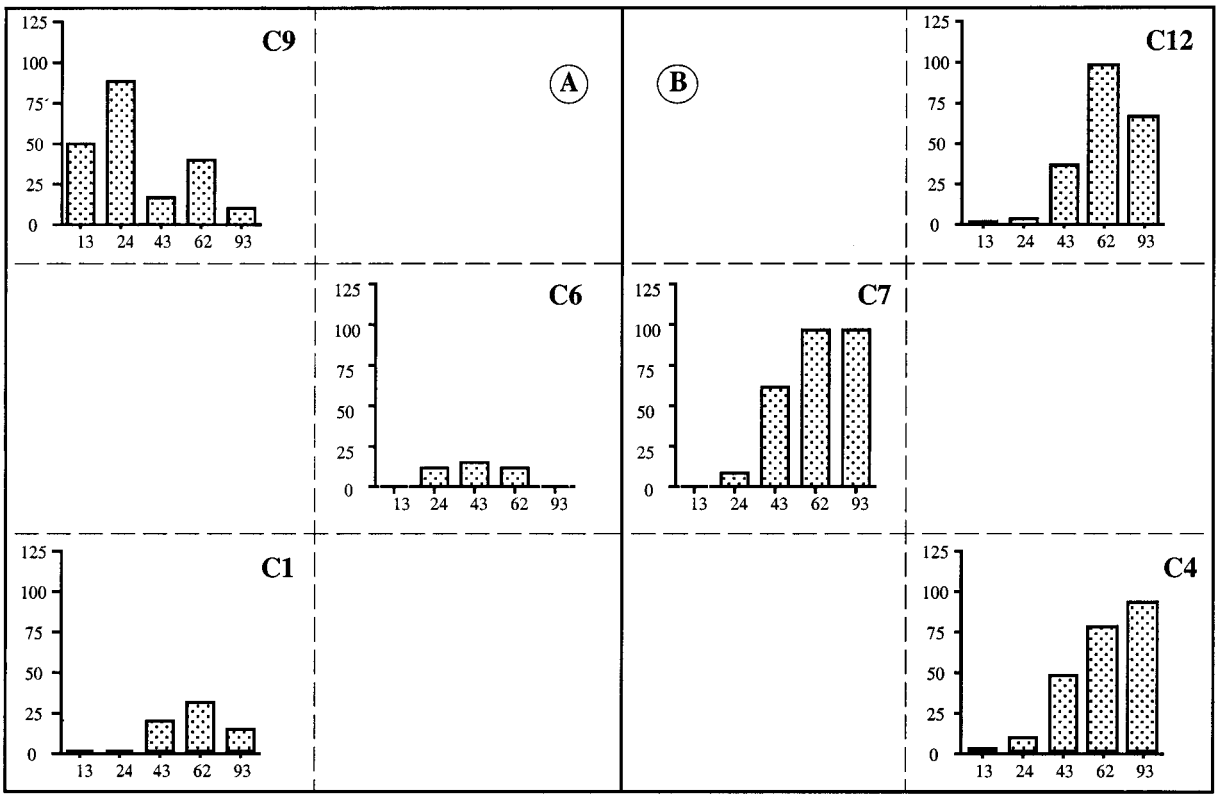

FIGURE 5. Dechlorination activity expressed as a percentage of 3-CB transformed in nonautoclaved (A) and autoclaved (B) parts of the soil bioreactor. Days of sampling are indicated on the abscissa. Soil samples were taken from different areas at a depth of 30 cm and incubated in batch bottles for 10 days before 3-CB analysis. Sample notation corresponds to that of Figure 1.

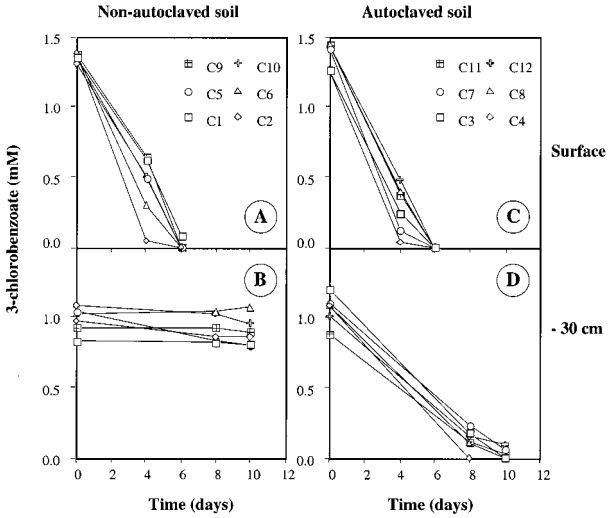

FIGURE 6. Time course data of 3-CB disappearance in soil samples taken from different compartments and at different depths in the reactor on day 93 (Sample notation corresponds to that of Figure 1). (A and B) Samples taken in the nonautoclaved part at the top surface and at 30 cm of depth, respectively. (C and D) Samples taken in the autoclaved part at the top surface and at 30 cm of depth, respectively.

Samples taken from the autoclaved soil expressed a dechlorination activity increasing over the course of the experiment.

Spatial Distribution of 3-CB Dechlorination Activity in the Bioreactor. Figure 6 depicts the time course data of 3-CB dechlorination activity on soil samples taken from different compartments in the bioreactor at day 93. In this experiment, the soil samples were taken from the top surface as well as from 30 cm in depth both in the nonautoclaved (Figure 6A and 6B) and in the autoclaved (Figure 6C and 6D) parts. There is a strong difference in dechlorination activity between samples taken from 30 cm in depth in the nonautoclaved soil (Figure 6B) and those taken from the other parts of the reactor (Figure 6A,C,D). In both autoclaved and nonautoclaved soil, samples taken from the top showed similar, and high, dechlorination activities (Figure 6A,C). However, there is a striking difference in the dechlorination activity of samples taken from 30 cm in depth between nonautoclaved (Figure 6B) and autoclaved sections (Figure 6D): while the former exhibited practically no dechlorination activity, the latter showed good activity, although clearly lower than that observed at the top of either section (Figure 6A,C). As reflected by all these data, there is a heterogeneous distribution of the 3-CB dechlorination activity in the soil.

PCR Detection of the 16S rDNA Genes. This molecular detection methodology was applied both to the group of archaea methanogens presumed to be present in the soil as well as to the dechlorinator eubacterium, *D. tiedjei*, that was introduced for bioaugmentation. In the case of the methanogenic communities, specific primers previously described as being capable of amplifying 1.1 kb of the archaean 16S rDNA genes (*23*) were used. In the nonautoclaved soil, the PCR signal was detected from the beginning of the experiment, whereas in the autoclaved portion there was no detection of archaen 16S rDNA before day 24 (results not shown). To explore the origin of the dechlorination activity seen in the reactor and to possibly correlate it with the presence of *D. tiedjei*, we also monitored the 16S rDNA of this bacterium. PCR amplification of this genomic fragment was performed using primers previously designed for specific detection of *D. tiedjei* in nonsterile soil slurry microcosms (*12*). Figure 7 shows typical gels of PCR signals detected in reactor soil samples taken at day 93 from the nonautoclaved

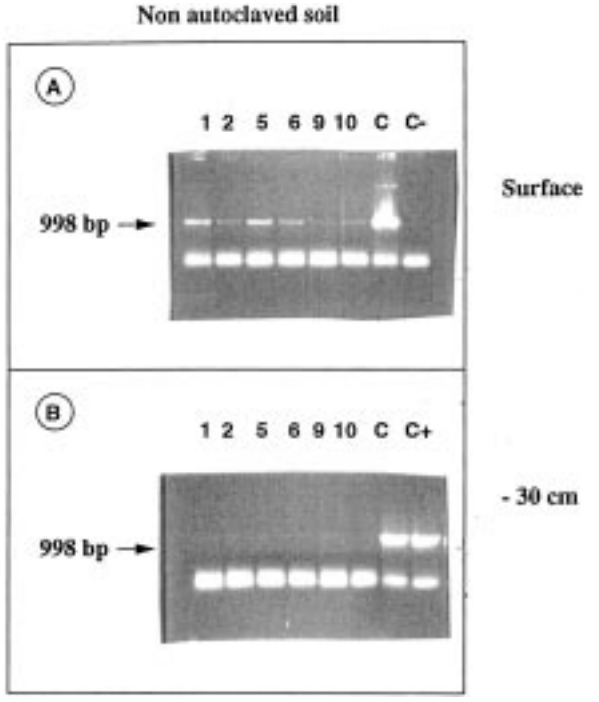

FIGURE 7. PCR detection of the 16S rDNA gene of *D. tiedjei* in soil samples taken from different areas and depths in the bioreactor. (A and B) Detection in soil samples taken from the nonautoclaved soil at the top surface and at 30 cm of depth, respectively. The numbers correspond to the different compartments indicated in Figure 1. C+, C−, and C correspond to control with *D. tiedjei* DNA, control without addition of DNA, and control with *D. tiedjei* DNA plus DNA extracted from soil, respectively. The lower bands correspond to the primers used in the PCR.

TABLE 3. Correlation between 3-Chlorobenzoate (3-CB) Dechlorination Activity and PCR Detection of the 16S rDNA Gene of *D. tiedjei* Using Soil Samples Taken from Different Areas in the Reactor at 30 cm of Depth over Time[a]

	nonautoclaved			autoclaved		
	C1	C6	C9	C4	C7	C12
13 days 3-CB transformation	−	−	+	−	−	−
13 days PCR detection	−	−	+	−	−	−
24 days 3-CB transformation	−	−	+	−	−	−
24 days PCR detection	−	−	+	(+/−)[b]	−	−
43 days 3-CB transformation	−	−	−	+	+	+
43 days PCR detection	−	−	+	+	+	+
62 days 3-CB transformation	−	−	+	+	+	+
62 days PCR detection	−	−	+	+	+	+
93 days 3-CB transformation	−	−	−	+	+	+
93 days PCR detection	−	−	−	+	+	+

[a] The samples were incubated for 10 days in the presence of 2 mM 3-CB. C1−C12 indicate the different compartments in the reactor. [b] Signal not detected by normal one-round PCR but by a nested PCR procedure using primers previously described (*12*).

soil. Whereas positive signals were detected in all soil samples at the soil surface (Figure 7A), no signal was detected in samples taken from 30 cm of depth (Figure 7B). Table 3 groups together the results obtained with PCR amplification of the 16S rDNA gene of *D. tiedjei* in different samples taken from the reactor over time. The results are presented in combination with measurements of 3-CB dechlorination activity. We observed a correlation between 3-CB dechlorination activity and the PCR detection of 16S rDNA from *D. tiedjei* with samples taken at days 13 and 24. During these initial sampling times, this genomic fragment was detected only in samples taken from the inoculated part of the nonautoclaved soil

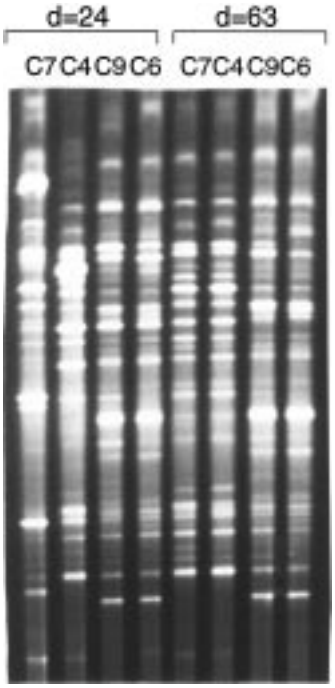

FIGURE 8. DGGE analyses of 16S rDNA fragments of soil samples taken from different compartments of the bioreactor. C4, C6, C7, and C9: compartment 4, 6, 7, and 9 as reported in Figure 1. 24 and 63 correspond to dates of sampling.

(C9). Samples taken from autoclaved soil at day 43 showed a correlation between 3-CB dechlorination activity and PCR detection. However, in compartment C9, although a remarkable decrease in dechlorination activity was observed at day 43, a positive signal was still detected by PCR. One may assume that upon cutting the cosubstrate feed and doubling the 3-CB input, the bacterium was still present in this area but its dechlorination activity was reduced to a low level. To test the authenticity of PCR detection, several amplified fragments detected from different parts of the soil reactor at different times were subjected either to sequencing or to restriction mapping using a specific enzyme. The patterns were identical with those expected by restriction mapping of the PCR product obtained with pure DNA of *D. tiedjei* (data not shown).

Characterization of Soil Microbial Communities with Denaturing Gradient Gel Electrophoresis (DGGE). Figure 8 shows DGGE patterns of PCR products obtained after amplification of 16S rDNA genes from bioreactor soil samples. The equal-sized 16S rDNA fragments were separated according to their sequences to study the microbial populations in different locations of the soil bioreactor. Each band in the pattern can be assigned to one bacterial species in the original sample. The same figure depicts the comparison of soil bacterial communities between autoclaved and nonautoclaved soil before and after the cosubstrate feed is suspended. There are clear differences between samples from autoclaved and nonautoclaved soil as can be observed from the absence and presence of several bands in the corresponding DGGE gel (Figure 8). In addition, a reassortment of bacterial communities in different areas of the reactor after the cosubstrate feeding is cut is clearly seen from the DGGE patterns reported in Figure 8.

The degrees of similarity among the communities were quantified by numerically analyzing the DGGE band patterns. Figure 9 shows the similarity dendrograms, based on two band-based coefficients. The similarity dendrograms indicate significant differences in the microbial community structures. There is less than 60% similarity between the community

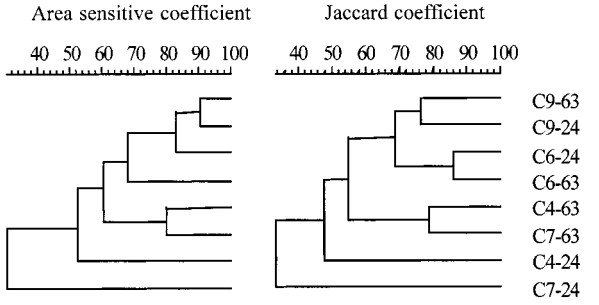

FIGURE 9. Dendrograms of community relatedness of different soil samples taken from the soil bioreactor. S: sterilized (autoclaved). C4, C6, C7, and C9: compartment 4, 6, 7, and 9 as reported in Figure 1. 24 and 63 correspond to dates of sampling. The percentage of similarity was calculated on the basis of two band-based coefficients, the Jaccard and area-sensitive coefficients, using the GelCompar software.

fingerprints of autoclaved and nonautoclaved soil. Furthermore, it seems that the cosubstrate plays a key role in the compositions of soil microbial communities as a clear difference in the percentage of similarity can be seen with all soil compartments (Figure 9). The results obtained with DGGE suggest that a reassortment of microbial communities has occurred both in autoclaved and nonautoclaved soil as the cosubstrate feed has been modified during the experiment.

Discussion

Soil Bioreactor. Among major areas suggested for high-priority research in soil bioremediation, there is a need to improve the design of bioremediation processes to demonstrate that the potential results obtained in the laboratory can actually be realized in the field site (*24, 13*). In a preliminary approach, these concerns can be overcome by scaling up the experiments from simple laboratory systems to pilot operations. A useful discussion of the challenges inherent in the study of biodegradation in soil at appropriate scales together with methodological considerations (mass balances, hydraulics, etc.) and system setup is given by Schmidt and Scow (*14*). In this spirit, we have designed a relatively simple model soil bioreactor to serve as a useful pilot-scale tool for studying anaerobic bioremediation processes. We have demonstrated the establishment of anaerobic microbial activity in the bioreactor as seen by the development of the methanogenic community. Furthermore, bioaugmentation using *D. tiedjei* as the source of inoculum proved possible, even though remarkable differences in dehalogenation activities were observed between autoclaved and nonautoclaved soil. The bioreactor was also designed to allow easy sample collection and handling from both soil and liquid phases. This is important as it permits the establishment of biodegradation mass balances, leading to the prediction of the behavior and fate of added toxic compounds and microbes. On the other hand, an accurate assessment of the distribution of the bioremediation activity in different areas of the reactor was demonstrated with activity tests on soil samples taken from different compartments (Figures 5 and 6). The bioreactor was also designed to facilitate the collection of the gas that was produced (Figure 3C). This is a useful tool since it offers the possibility to monitor the biological activity of the bioreactor over time and the mineralization of the toxic target molecule when the latter is added as the sole source of carbon.

3-CB Transformation. The difference in biological activity between autoclaved and nonautoclaved soil, as it is reflected by the high rate of gas production, can be explained by the fact that the dechlorination of 3-CB occurs better in auto-

claved soil as a result of a good distribution of *D. tiedjei* (see also Table 3 for a correlation between dechlorination activity and PCR detection). Consequently, much more substrate for methanogenesis is provided in the autoclaved part. Furthermore, the sterilization has reduced the number of microorganisms in the autoclaved soil in contrast to the nonautoclaved soil where microoganisms other than methanogens can use the organic substrates for their growth and metabolism. Additional aspects of autoclaved vs nonautoclaved soil behavior (microbiological and physical/chemical) which may have an impact on the establishment of dechlorination activity are discussed below.

Concerning the difference between the top and the bottom of the soil matrix, especially in nonautoclaved soil (Figure 6), it is important to note that there was a remarkable difference in the soil texture between the top, which is in reality the interface between the soil and the liquid zone, and the bottom where the soil is compacted. The situation at the top resembles strongly the batch soil slurry microcosms in which we recently showed durable establishment of de novo dechlorination activity (*12*). This difference in soil texture could influence the movement of microorganisms and the mass transfer of the toxic compounds and nutrients. In addition, the microbial density could also be modified since the number of microrganisms is higher in compacted soil (bottom) than in slurry (top). The high dechlorination capacity seen in samples taken from the top of nonautoclaved sections (Figure 6A) appears to be the result of cross contamination through surface water coming from the top of autoclaved sections (Figure 6C).

The tendency to transiently accumulate 3-CB and the concurrent slow of methanogenesis that were seen upon elimination of cosubstrate feeding and doubling of 3-CB concentration in the feed (Figure 3C,D) are to be expected since the indigenous populations must become adapted to the utilization of a sole source of reducing equivalents, namely, benzoate. The latter is considerably less readily convertible into methane than acetate + formate (the cosubstrate). However the dechlorination activity never stopped completely even in compartment C9 in the nonautoclaved soil (Figure 5, C9): this modification in the feed apparently caused the local microbial community arrangement (*D. tiedjei* in association with local flora) to be impaired to the point that *D. tiedjei* was unable to use at least 50% of the 3-CB supplied over the 10 days of the incubation test, as required for scoring a sample as "dechlorination positive" (+) in Table 3). On the other hand, it is important to note that, in the nonautoclaved soil, the dechlorination activity was affected at the bottom (−30 cm) but never at the top surface (see Figure 6). We can hypothesize that different reassortments of microbial communities have occurred, as can be seen in part from DGGE evidence (see below). In the autoclaved soil there was no difference in the dechlorination activity between the top and bottom, which may indicate that a stable syntrophic association has occurred between the dechlorinator and the indigenous microorganisms throughout the soil core, which moreover, was not disturbed by the modification (shutting off the cosubstrate and increasing the 3-CB concentration) in the feed: 3-CB dechlorination continued while benzoate served as the source of reducing equivalents.

In this study we observed the formation of benzoate as an intermediate metabolite of 3-CB biotransformation. Taking into consideration the cumulative biogas production, we observed that the mineralization of benzoate required approximately 40 days. This is in contrast to the results obtained by Ahring et al. (*25*) who reported that no benzoate accumulation was observed in upflow anaerobic granular sludge blanket reactors (UASB) inoculated with *D. tiedjei*. We can speculate that the granules used by Ahring et al. (*25*) may have had immediate benzoate-mineralization ability, whereas the soil microbial community investigated in this study may have needed a lag period to be adapted for benzoate mineralization.

Effect of Sterilization by Autoclaving on Microbiological, Chemical, and Physical Properties of Soils. The difference in the results found in this study between autoclaved and nonautoclaved soil is of interest to be explored further. In samples taken from the autoclaved soil at 30 cm of depth, we observed dechlorination activity in all areas. In contrast, for nonautoclaved soil no significant dechlorination activity was observed in samples taken at the same depth. This may have been due to the modification of the soil composition induced by the autoclave treatment.

It has been shown previously that autoclaving could alter the microbial, chemical, and physical composition of soil. Using three different soils, Wolf et al. (*26*) have shown that autoclaving the soils once for 2 h reduced bacterial numbers but did not eliminate the microbial population, whereas autoclaving three times eliminated the complete microbial population. Although we cannot presume that indigenous microbial biomass was completely eliminated, we however, can assume that the number of microorganisms was significantly decreased by autoclaving and that a reassortment of bacteria, probably due to the increase in the number of heat resistant microorganisms such as spore-forming bacteria, has occurred in the autoclaved part. This reassortment of bacteria after autoclaving (supportive evidence for which comes from the striking difference in DGGE patterns between autoclaved and nonautoclaved soil samples, Figure 8) seems to be favorable for both the survival and migration of *D. tiedjei* in this part of the soil reactor and the effect of microbial competition was also significantly reduced. As stated above, such a reassortment would allow stable syntrophism between *D. tiedjei* and the indigenous microorganisms that have survived the autoclaving and/or have proliferated in this open, nonaxenic system. In this connection, it has been previously suggested that in natural soil microorganisms may be susceptible to competition, toxins, and predators (*27, 28*). For instance, some *Pseudomonas* strains capable of mineralizing 2,4-dichlorophenol failed to function when inoculated into natural soil whereas the same strains expressed a good biodegradation activity in sterile soil (*27*).

Concerning the chemical alterations, it has been shown that autoclaving treatments once, twice, or three times increased the Mn^{2+} levels in the soils by an average of approximately 500% (*26*). This increased production of Mn^{2+} after soil autoclaving was also demonstrated by other authors (*29, 30*). It has also been shown that soil autoclaving released ammonium and amino acids (*31*). In addition to these chemical modifications, it has been shown that the sterilization by autoclaving has an effect on the physical characteristics of the soils. Indeed, Jenneman et al. (*32*) have shown an increase in the porosity of the autoclaved cores along with decreases in the amount of silica, aluminum, and potassium, which are elements often associated with clays. This may imply that autoclaving causes the destruction or alteration of clay particles. Scanning electron micrographs performed by these authors (*32*) showed a typical clay morphology after autoclaving. The clay particles were clumped, with smooth and rounded edges (*32*). This reduction of the surface area was also demonstrated by Wolf et al. (*26*). More importantly, Jenneman et al. (*32*) showed that the autoclaving resulted in an increase of the chloride content and a reduction in the content of aluminum and potassium. This mineralogic alteration probably resulted in an increase in the negatively charged ions on the surfaces of soil particles and in a reduction in surface area available for cell adhesion. We observed somewhat finer and more separated grains in the autoclaved soil, which appeared less compact than the

TABLE 4. Introduction and Detection of *D. tiedjei* in Different Natural Habitats

habitat	reactor	method of detection	refs
granules	UASB (200 mL)	immunofluorescence	*25*
bacterial biofilm	fixed bed (50cm × 2.5cm)	microscopic visualization	*39*
sediments	flasks (150 mL)	membrane fatty acids	*40*
soil	pilot (500 L)	PCR 16S rDNA	this study

untreated soil. On the basis of this visual evidence and the above literature data, we can speculate that the rapid establishment of the 3-CB activity in autoclaved soil of the bioreactor studied here was also due to the soil modifications brought about by autoclaving.

Detection of *D. tiedjei*. In nonsterile environments, like soil, the measurement of physiological activities alone is inadequate to distinguish between intrinsic bioremediation, also termed natural attenuation, and bioaugmentation, which involves active exogenous inocula. This insufficiency to prove that the specific activity (such as the 3-CB dechlorination activity studied here) is due to the fact that the introduced microorganism becomes more complicated when natural nonautoclaved soil is investigated in experiments involving long time incubations. Indeed, the lengthy exposure of the microbial populations living in the soil to the target molecules can bring about the adaptation by natural genetic processes (*33*) either by inducement of novel enzymes or by genetic transfer of degradative genes that can occur between introduced microorganisms and the indigenous microbial population (*34*, *35*). However, we must admit that factors other than natural genetic processes can account for biodegradation after a lengthy lag period. Factors such as the exhaustion of preferential substrates and the enrichment of the requisite competent population could be the cause as well.

The molecular monitoring of *D. tiedjei* proved successful, with a good correlation between PCR detection of the 16S rDNA gene and 3-CB dechlorination activity (Table 3). A striking illustration of this correlation is shown in the two inoculated compartments, C9 (nonautoclaved part) and C4 (autoclaved part), as can be gathered by juxtaposing Figure 5 and Table 3. Whereas in C9 there is already a measurable presence of both dechlorination activity and detection of *D. tiedjei* using one-round PCR in the initial period of the bioreactor run after inoculation (days 13 and 24), in C4 more time was apparently needed for these indicators to be observed. This difference may be due to the following: As previously shown (*36*, *37*) *D. tiedjei* is more easily established in the presence of a methanogenic consortium than in pure culture. We can reasonably assume that in autoclaved soil a syntrophic association between the dechlorinator and the indigenous methanogenic consortium was needed for the dechlorination activity to start, but such an association may not have been established in the early days after inoculation. This is consistent with the detection of the 16S rDNA gene of archaea methanogens in which a positive signal of one-round PCR for autoclaved soil was observed only in samples taken after day 24 (data not shown). In addition, to better interpret the data of Table 3 it is to be noted that the threshold of detection using one-round PCR is 10^3 cells/g of soil. The sensitivity can be increased by using a nested PCR procedure (*12*), which, however, is more time-consuming and not practicable for routine monitoring on a large scale. The use of nested PCR gave a signal of *D. tiedjei* presence in compartment C4 for the first time on day 24 (Table 3).

The authenticity of the detected fragment upon PCR amplification of DNA extracted from our soil samples was confirmed using restriction mapping. The results are significant in verifying fragment authenticity, and thus, suggesting strongly the presence of *D. tiedjei* in our samples, since the restriction enzyme used was chosen in the highly conserved regions of the *D. tiedjei* 16S rDNA gene. Moreover, sequencing several PCR products confirmed the authenticity of the amplified fragments.

DGGE Analysis. DGGE proved to be a very useful means for probing the microbial diversity in the soil bioreactor in this study. Detection of bacteria by DGGE analysis of PCR-amplified 16S rDNA fragments contributes to a better understanding of the organization of microbial communities in environmental sites (*21*, *38*), especially by the fact that it is fast and reproducible compared to other methodologies such as BIOLOG. Furthermore, it is a cultivation-independent approach which takes into consideration also the nonculturable microorganisms. In this study we have shown for the first time the value of using the numerical analysis of DGGE patterns in a dynamic system, such as a soil bioreactor, using appropriate software (Figure 9). This gives better insight into the shift of bacterial communities after the modification of environmental conditions. The most striking result obtained by DGGE is the demonstration that bacterial community fingerprints are distinct between samples from autoclaved and nonautoclaved soil as well as between samples taken after the cutting of the cosubstrate feed (Figure 8).

The successful inoculation of *D. tiedjei* in soil on a large, realistic scale constitutes a new positive argument for using pure strains isolated in the laboratory to help clean up contamined sites. Furthermore, our results enlarge the demonstration of the capacity of this bacterium to survive in different anaerobic environments. Indeed, in addition to granular sludge, sediments, and enriched biofilm, we have now shown for the first time that an agricultural soil can also be used as a natural matrix for this bacterium (Table 4). A useful extension of this work would be to determine the effect that varying temperatures will have on bioaugmentation. Future experiments with more common contaminants such as mono- or polychlorinated benzenes or polychlorinated biphenyls may be instructive. Also, inexpensive cosubstrates should be evaluated.

The results obtained in the top surface of the nonautoclaved soil, which represents the real-life situation, lead us to suggest that the most practical application of our study could be the land-farming treatment in which polluted soils can be treated on site by using engineered bioremediation where the soil could be manipulated at the surface to allow an effective inoculation. If, on the other hand, an on-site or ex-situ treatment of soil is envisaged, then a slurry reactor setup might be indicated (*14*). Finally, an additional implication of this work may be that soils where habitually used biocides (e.g., pesticides) have reduced the endogenous microbial communities (analogy to partial sterility) are better candidates for bioaugmentation through inoculation of a pure anaerobic dechlorinator strain. Because of relatively poor inoculation efficiencies, especially for nonsterile soil as seen in this work, full-scale implementation of anaerobic bioaugmentation could be achieved by a combination of different strategies: applying successive repeated inoculations at higher dechlorinator bacterial densities ($>10^5$ cells/g of soil), ensuring better anaerobiosis with inundation, a cheap cosubstrate (methanol or molasses), and a tapestry-like soil cover after plowing (land farming) for better distribution,

and finally, enhancing the capacity of the soil to establish the dechlorinators by prior treatments with biocides (sterilization).

In conclusion, this investigation demonstrated for the first time the applicability and limitations of soil bioaugmentation with a pure anaerobic dechlorinating strain at a realistic pilot scale. Using autoclaved and nonautoclaved soil, the results showed a heterogeneous distribution of the microbial activity in the bioreactor. The autoclaved soil expressed high microbial activity and a high dechlorination capacity. PCR detection of the 16S rDNA gene of *D. tiedjei* was directly correlated to 3-CB dechlorination activity, and evidence for microbial community arrangements in the reactor portions in response to feed modification was clearly demonstrated by DGGE. This study confirms that an integrated approach combining bioprocess engineering, molecular methodologies, and microbiological techniques may lead to a better overall understanding of the possibilities and limitations of bioremediation processes.

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Literature Cited

(1) MacDonald, J. A.; Rittmann, B. E. *Environ. Sci. Technol.* **1993**, *27*, 1974−1979.
(2) Lowe, S. E.; Jain, M. K.; Zeikus, J. G. *Microbiol. Rev.* **1993**, *57*, 451−509.
(3) Morris, J. G. *Appl. Biochem. Biotechnol.* **1994**, *48*, 75−106.
(4) El Fantroussi, S.; Naveau, H.; Agathos, S. N. *Biotechnol. Prog.* **1998**, *14*, 167−188.
(5) Mohn, W. W.; Tiedje, J. M. *Microbiol. Rev.* **1992**, *56*, 482−507.
(6) Dolfing, J.; Beurskens, J. E. M. *Adv. Microbial Ecol.* **1995**, *14*, 143−206.
(7) Oldenhuis, R.; Kujik, L.; Lammers, A.; Janssen, D. B.; Witholt, B. *Appl. Microbiol. Biotechnol.* **1989**, *30*, 211−217.
(8) Brunsbach, F. R.; Reineke, W. *Appl. Microbiol. Biotechnol.* **1995**, *43*, 529−533.
(9) Angle, J. S.; Levin, M. A.; Gagliardi, J. V.; McIntosh, M. S. *Appl. Environ. Microbiol.* **1995**, *61*, 2835−3839.
(10) Klasson, K. T.; Barton, J. W.; Evans, B. S.; Reeves, M. E. *Biotechnol. Prog.* **1996**, *12*, 310−315.
(11) Lestan, D.; Lestan, M.; Chapelle, J. A.; Lamar, R. T. *J. Ind. Microbiol.* **1996**, *16*, 286−294.
(12) El Fantroussi, S.; Mahillon, J.; Naveau, H.; Agathos, S. N. *Appl. Environ. Microbiol.* **1997**, *63*, 806−811.
(13) National Research Council. In *In situ Bioremediation, When Does It Work*; National Academy Press: Washington, DC, 1993; pp 63−90.
(14) Schmidt, S. K.; Scow, K. M. In *Manual of Environmental Microbiology*; Hurst, C. J., Knudsen, G. R., McInerney, M. J., Stetzenbach, L. D., Walter, M. V., Eds.; American Society for Microbiology, Washington, DC, 1997; pp 822−829.
(15) Dybas, M. J.; Barcelona, M.; Bezborodnikov, S.; Davies, S.; Forney, L.; Heuer, H.; Kawka, O.; Mayotte, T.; SepulvedaTorres, L.; Smalla, K.; Sneathen, M.; Tiedje, J.; Voice, T.; Wiggert, D. C.; Witt, M. E.; Criddle, C. S. *Environ. Sci. Technol.* **1998**, *32*, 3598−3611.
(16) DeWeerd, K. A.; Mandelco, L.; Tanner, R. S.; Woese, C. R.; Suflita, J. M. *Arch. Microbiol.* **1990**, *154*, 23−30.
(17) Mohn, W. W.; Kennedy, K. J. *Appl. Environ. Microbiol.* **1992**, *58*, 1367−1370.
(18) Townsend, G. T.; Suflita, J. M. *Appl. Environ. Microbiol.* **1996**, *62*, 2850−2853.
(19) Trevors, J. T. *J. Microbiol. Methods* **1996**, *26*, 53−59.
(20) El Fantroussi, S.; Mahillon, J.; Naveau, H.; Agathos, S. N. *Biodegradation* **1997**, *8*, 125−133.
(21) Ovreas, L.; Forney, L.; Daae, F. L.; Torsvik, V. *Appl. Environ. Microbiol.* **1997**, *63*, 3367−3373.
(22) Boucquey, J. B.; Renard, P.; Amerlynck, P.; Modesto Filho, P.; Agathos, S. N.; Naveau, H.; Nyns, E.-J. *Biotechnol. Bioeng.* **1995**, *47*, 298−307.
(23) Hales, B. A.; Edwards, C.; Ritchie, D. A.; Hall, G.; Pickup, R. W.; Saunders, J. R. *Appl. Environ. Microbiol.* **1996**, *62*, 668−675.
(24) Alexander, M. *Environ. Sci. Technol.* **1991**, *25*, 1972−1973.
(25) Ahring, B. K.; Christiansen, N.; Mathrani, I. H.; Hendriksen, V.; Macario, A. J. L.; Conway de Macario, E. *Appl. Environ. Microbiol.* **1992**, *58*, 3677−3682.
(26) Wolf, D. C.; Dao, T. H.; Scott, H. D.; Lavy, T. L. *J. Environ. Qual.* **1989**, *18*, 39−44.
(27) Goldstein, R. M.; Mallory, L. M.; Alexander. M. *Appl. Environ. Microbiol.* **1985**, *50*, 977−983.
(28) Pritchard, R. P. *Curr. Opin. Biotechnol.* **1992**, *3*, 232−243.
(29) Bohn, H. L. *Soil Sci. Soc. Am. Proc.* **1970**, *34*, 195−197.
(30) Martin, J. P.; Farmer, W. J.; Ervin, J. O. *Soil Sci. Soc. Am. Proc.* **1973**, *37*, 56−60.
(31) Alef, K.; Nannipieri, P.; *Methods in Applied Soil Microbiology and Biochemistry*; Academic Press: San Diego, CA, 1995.
(32) Jenneman, G. E.; McInerney, M. J.; Crocker, M. E.; Knapp, R. M. *Appl. Environ. Microbiol.* **1986**, *51*, 39−43.
(33) Van der Meer, J. R.; de Vos, W. M.; Harayama, S.; Zehnder, A. J. B. *Microbiol. Rev.* **1992**, *56*, 677−694.
(34) Mergeay, M.; Springael, D.; Top, E., In *Bacterial Genetics in Natural Environments*; Fry, J. C., Day, M. J., Eds; Chapman and Hall: London, 1990; pp 152−171.
(35) Zhou, J. Z.; Tiedje, J. M. *Mol. Ecol.* **1995**, *4*, 613−618.
(36) Dolfing, J.; Tiedje, J. M. *FEMS Microbiol. Ecol.* **1986**, *38*, 293−298.
(37) Dolfing, J. In *Anaerobic biodegradation of Xenobiotic Compounds*; Jacobsen, B. N., Zeyer, J., Jensen, B., Westermann, P., Ahring, B., Eds.; Report No. 25 in the Water Pollution Report Series of the Environmental Research Programme of the Commission of the European Communities, Directorate-General for Science, Research and Development; Brussels, 1990; pp 47−64.
(38) Ferris, M. J.; Muyzer, G.; Ward, D. M. *Appl. Environ. Microbiol.* **1996**, *62*, 340−346.
(39) Fathepure, B. Z.; Tiedje, J. M. *Environ. Sci. Technol.* **1994**, *28*, 746−752.
(40) Ringelberg, D. B.; Townsend, G. T.; DeWeerd, K. A.; Suflita, J. M.; White, D. C. *FEMS Microbiol. Ecol.* **1994**, *14*, 9−18.
(41) Blaut, M. *Antonie van Leeuwenhoek* **1994**, *66*, 187−208.
(42) Kamagata, Y.; Kitagawa, N.; Tasaki, M.; Nakamura, K.; Mikami, E. *J. Ferment. Bioeng.* **1992**, *73*, 213−218.

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