

Isolation and characterization of 1,2,4-trichlorobenzene mineralizing *Bordetella* sp. and its bioremediation potential in soil

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Abstract

A soil which has been polluted with chlorinated benzenes for more than 25 years was used for isolation of adapted microorganisms able to mineralize 1,2,4-trichlorobenzene (1,2,4-TCB). A microbial community was enriched from this soil and acclimated in liquid culture under aerobic conditions using 1,2,4-TCB as a sole available carbon source. From this community, two strains were isolated and identified by comparative sequence analysis of their 16S-rRNA coding genes as members of the genus *Bordetella* with *Bordetella* sp. QJ2-5 as the highest homological strain and with *Bordetella petrii* as the closest related described species. The 16S-rDNA of the two isolated strains showed a similarity of 100%. These strains were able to mineralize 1,2,4-TCB within two weeks to approximately 50% in liquid culture experiments. One of these strains was reinoculated to an agricultural soil with low native 1,2,4-TCB degradation capacity to investigate its bioremediation potential. The reinoculated strain kept its biodegradation capability: ¹⁴C-labeled 1,2,4-TCB applied to this inoculated soil was mineralized to about 40% within one month of incubation. This indicates a possible application of the isolated *Bordetella* sp. for bioremediation of 1,2,4-TCB contaminated sites.

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1. Introduction

Chlorobenzenes (CBs) are important basic materials and additives in the production of pesticides, dyes, pharmaceuticals, disinfectants, rubbers, plastics, and electric goods (Beck, 1986; Rapp, 2001; Zhang et al., 2005). Their occurrence in the environment is widespread and they were found in the atmosphere (Popp et al., 2000), water (Monferrán et al., 2005), soil (Wang et al., 1995; Zolezzi et al., 2005), sediments (Lee et al., 2005), vegetables (Wang and Jones, 1994; Zhang et al., 2005), and biota (Vorkamp

et al., 2004). Since CBs can be accumulated in the food chain (Wang and Jones, 1994; Zhang et al., 2005), the elimination of these pollutants from the environment and from polluted sites is of great public interest. As chemical and photochemical degradation of CBs is very slow, biological degradation could be considered as a feasible process to eliminate these compounds from soil ecosystems.

Aerobic microbial degradation of mono-, di-, tri- and even tetra-chlorobenzenes is reported (Haigler et al., 1988; Potrawfke et al., 1998; Rapp and Timmis, 1999; Schroll et al., 2004; Monferrán et al., 2005; Rehfuss and Urban, 2005). Bacteria that are able to use these compounds as carbon and energy source have been isolated from polluted environments and are described as members of the genera *Alcaligenes*, *Pseudomonas*, *Burkholderia*,

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Xanthobacter and *Rhodococcus* and the species *Acidovorax avenae* and *Rhodococcus phenolicus* (Schraa et al., 1986; Spain and Nishino, 1987; van der Meer et al., 1987; Haigler et al., 1988; Sander et al., 1991; Brunsbach and Reineke, 1994; Spiess et al., 1995; Potrawfke et al., 1998; Rapp and Gabriel-Jürgens, 2003; Monferrán et al., 2005; Rehfuß and Urban, 2005).

In our study, we focused on 1,2,4-trichlorobenzene as this compound is one of the most widely used chlorobenzenes and also represents a possible intermediate product in the HCB degradation pathway (Brahushi et al., 2004). Some biodegradation studies of 1,2,4-TCB in pure cultures and environmental samples have been performed (van der Meer et al., 1987; Sander et al., 1991; Rapp and Timmis, 1999; Tchelet et al., 1999; Schroll et al., 2004). Biodegradation of 1,2,4-TCB in natural samples occurs in very low rates due to insufficient degradation capacity and slow adaptation of the indigenous microorganisms. In some cases, the biodegradation of 1,2,4-TCB in soil could be enhanced by inoculation with adapted bacteria like *Pseudomonas* sp. P51, *Burkholderia* sp. PS12 and *Burkholderia* sp. PS14 under laboratory conditions (van der Meer et al., 1987; Sander et al., 1991; Rapp and Timmis, 1999; Tchelet et al., 1999).

A former study (Schroll et al., 2004) showed an applicable method to considerably enhance the biodegradation of 1,2,4-TCB in a soil with low native degrading capacity by inoculating this soil with an adapted microbial community from a contaminated site via soil inoculum. In order to understand the observed decontamination process, the present study was carried out to isolate and identify the key organism of this degrading community and an isolated strain was inoculated to a soil with a low native degrading capacity to investigate its bioremediation potential.

2. Materials and methods

2.1. Chemicals, culture medium and cell counting

Uniformly ^{14}C -ring-labeled 1,2,4-TCB was obtained from International Isotope (Munich, Germany). Non-labeled 1,2,4-TCB was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Scintillation cocktails were obtained from Packard (Dreieich, Germany). All other chemicals and solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Microbial enrichment steps, isolation procedures and degradation experiments were performed in mineral medium M3 (van der Meer et al., 1987) which contained 2.9 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.5 g KH_2PO_4 , 1 g NH_4NO_3 , 100 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 1 ml trace metal solution without EDTA (Zehnder et al., 1980) per liter of demineralized water.

Cell counting was performed by spreading serial dilutions of the liquid culture on Nutrient Broth (Sigma-Aldrich, Taufkirchen, Germany) agar plates and incubating them at 30 °C. Colony forming units (CFU) were determined after 48 h incubation.

2.2. Radioactivity measurement

For the determination of radioactivity in liquid samples, aliquots of the samples were mixed with Ultima Gold XR and measured in a liquid scintillation counter (Tricarb 1900 TR, Packard, Dreieich, Germany). In solid samples the radioactivity was measured by combusting aliquots of the samples (Oxidizer 306, Packard, Dreieich, Germany), trapping the evolved $^{14}\text{CO}_2$ in Carbosorb E (Packard, Dreieich, Germany), mixing it with scintillation cocktail (Permafluor E) and counting it in a liquid scintillation counter.

2.3. Biomineralization experiments

Soil suspension and liquid culture were placed in special flasks (Brahushi et al., 2004), spiked with 25 μl ^{14}C -1,2,4-TCB (see Section 2.4) and incubated on a shaker (120 rpm) in the dark at 20 ± 1 °C. Filters (0.20 μm , Sartorius, Göttingen, Germany) were installed at the air inlet and outlet of the flasks. Soil samples were placed in double walled 100 ml round flasks, spiked with ^{14}C -1,2,4-TCB (see Section 2.8) and incubated at 20 ± 1 °C in the dark. The flasks containing soil suspension, liquid culture or soil samples were connected to a closed laboratory trapping system (Schroll et al., 2004, modified) and aerated twice (soil suspension and liquid culture) or thrice (soil) per week for one hour at an air exchange rate of 11 h^{-1} to trap $^{14}\text{CO}_2$ and volatile ^{14}C -substances separately. The trapping system and sampling of the trapping solutions is described in Schroll et al. (2004). In order to keep the TCB concentration in the soil suspension and the liquid culture nearly constant, 25 μl ^{14}C -1,2,4-TCB were reapplied after each aeration.

2.4. Enrichment of microbial community

Soil material was collected from a site in Hungary, which was contaminated with CBs for more than 25 years (Schroll et al., 2004). Soil suspensions were prepared by adding 5 g of sieved (<2 mm) soil material to 50 ml mineral medium M3 in an incubation flask (Brahushi et al., 2004). 25 μl ^{14}C -labeled 1,2,4-TCB (750 μg , $8.33 \times 10^3 \text{ Bq}$, $11.11 \text{ Bq } \mu\text{g}^{-1}$) dissolved in acetone was applied to serve as the sole carbon and energy source via a Teflon–silicon septum on the side of the flask by a sterile syringe. Biomineralization experiments were conducted to monitor the 1,2,4-TCB degradation ability of the microbial community.

Every 2 weeks, an aliquot of the liquid culture (1 ml) was taken and transferred into 49 ml fresh, sterile medium M3. In total ten reinoculation steps of the enriched cultures were conducted to ensure the maintenance of the mineralization ability of the bacteria and to enrich the key organism for 1,2,4-TCB mineralization.

2.5. Isolation of pure strains from the enriched culture

From the 10th enriched culture, aliquots of the liquid culture were diluted in M3 mineral medium and serial dilutions

were spread on M3 agar plates (1.6% agar). The plates were incubated in desiccators at 20 ± 1 °C in the dark and in the presence of non-labeled 1,2,4-TCB vapour as the sole carbon source. Single colonies from each plate were transferred with a sterile inoculation loop on fresh medium plates to verify the purity of the isolates. After obtaining pure strains, each strain was controlled separately for its ability to mineralize 1,2,4-TCB by performing biomineralization tests.

2.6. DNA extraction, PCR amplification, and sequencing of 16S-rRNA coding genes

High molecular weight DNA from two isolated strains, E3 and F2, was extracted with a commercially available kit (NucleoSpin Tissue, Macherey-Nagel, Düren, Germany). The 16S-rRNA coding genes were amplified from the bacteria total genomic DNA by using PCR with the conserved flanking primer pair 616-F (5'-AGAGTTTYMTGGCT-CAG-3') and 630-R (5'-CAKAAAGGAGGTGATCC-3') (Juretschko et al., 1998). PCR mixture contained 1 U Taq polymerase (Fermentas, St. Leon-Rot, Germany), 5 µl 10× buffer, 5 µl 10× dNTP, 5 µl MgCl₂ (25 mmol), 1 µl forward primer and 1 µl reverse primer (50 pmol), 25 ng of DNA template and sterile water to 50 µl final reaction volume. PCR was started with an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 45 s, and 72 °C for 1.5 min and the last elongation step was done at 72 °C for 10 min (Juretschko et al., 1998). Almost full length PCR amplicons of the 16S-rRNA coding genes were analyzed by horizontal agarose gel electrophoresis and purified with the commercially available QIAEX II extraction kit (Qiagen, Hilden, Germany). The purified PCR products were sequenced directly using the Big-Dye-Terminator Sequencing Kit (Applied Biosystems, Darmstadt, Germany) and the internal conserved primers 609-Forward (5'-GGATTAGATACCCBDGTA-3', corresponding position in *E. coli*: 785–802) and 612-Reverse (5'-GTAAGGTTYTNCGCGT-3', corresponding position in *E. coli*: 969–984). Both strands were sequenced with an ABI-Prism-377 automated Sequencer (Applied Biosystems, Darmstadt, Germany).

The BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) was used for gene homology search with the standard program default. Phylogenetic analyses of the obtained 16S-rDNA sequences were performed with the software package ARB (Ludwig et al., 2004, <http://www.arb-home.de/>). All tools for automatic alignment and tree calculation were included in ARB. Phylogenetic trees were calculated by applying distance matrix (Felsenstein, 1993), Maximum Parsimony (ARB, PHYLIP) and Maximum Likelihood (fast DNAML) methods.

2.7. Biomineralization of 1,2,4-TCB in liquid culture by the isolated strains E3 and F2

Isolated strains E3 and F2 were inoculated into 50 ml sterile M3 mineral medium to investigate their 1,2,4-TCB

mineralization ability. Mineral medium without inoculation of 1,2,4-TCB degrading bacteria served as a control. Application, incubation and sampling were identical to the procedures described above. At the end of the experiment, aliquots (1 ml) of the liquid culture were filtered through a 0.20 µm filter (Sartorius, Göttingen, Germany). Aliquots of the liquid phase (100 µl) were taken for radioactivity analysis before and after filtration.

2.8. Biomineralization of 1,2,4-TCB in soil by isolated strain F2

An agricultural soil (Mollic Gleysol, clay 24%, silt 60%, sand 16%, Corg. 1.7%, pH 7.1) with low native capacity of 1,2,4-TCB mineralization was used for the mineralization experiment with the isolated strain F2. Soil material was collected from a soil depth of 0–20 cm, mixed homogeneously, sieved (<2 mm) and stored at 4 °C. Prior to starting the laboratory experiments, soil samples were equilibrated for one week at 20 ± 1 °C at a soil moisture of 23% gravimetric water content.

After application of ¹⁴C-labeled 1,2,4-TCB (5 µg g⁻¹ soil (dry weight), 2.4 × 10⁴ Bq, 30.85 Bq µg⁻¹, application procedure see Schroll et al., 2004), 2.5 ml liquid culture inoculum of *Bordetella* strain F2 (1.40 × 10⁸ CFU in total) was added to the soil. Then the spiked soil sample was transferred to the incubation flask and pressed to a soil density of 1.3 g cm⁻³; additional water was applied to achieve a soil water potential of -15 kPa for optimal mineralization of the chemical (Schroll et al., 2006). At the end of the experiment, the soil samples were extracted with hexane/acetone (3/1, v/v) by accelerated solvent extraction (ASE 200, Dionex, Idstein, Germany) at a temperature of 90 °C and a pressure of 10 MPa. The residual radioactivity in soil after extraction was determined by combustion.

3. Results

3.1. Enrichment and isolation of 1,2,4-TCB-mineralizing bacteria

1,2,4-TCB mineralization in soil suspension (microbial community) was about 47% after two weeks and 58% after 3 weeks of incubation (Fig. 1).

Mineralization of 1,2,4-TCB in the following M3 enrichment cultures was always about 45% after 2 weeks of incubation. The isolation of single strains by M3 selective agar plates was started from the 10th enrichment culture. 150 strains were isolated on M3 agar plates. The isolated strains E3 and F2 showed highest mineralization rates in liquid culture and were further characterized by comparative sequence analysis of their 16 S-rRNA.

3.2. Characterization of 1,2,4-TCB-mineralizing bacteria

The isolated strains E3 and F2 grew on 1,2,4-TCB as only carbon and energy source. The bacteria colonies were

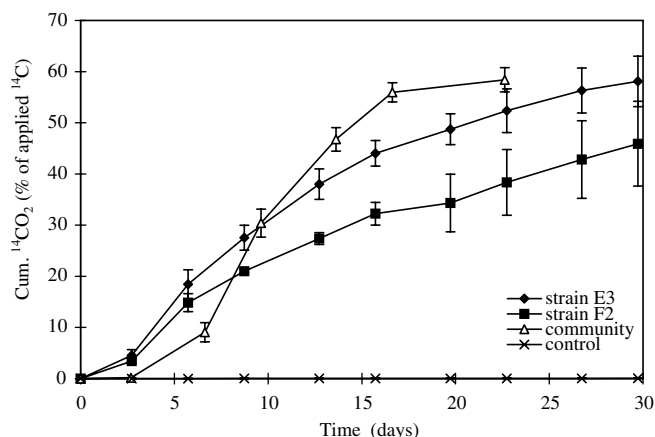


Fig. 1. Mineralization of ¹⁴C-1,2,4-TCB in mineral medium M3 by microbial community and by *Bordetella* strains, E3 and F2.

typically circular, convex, smooth, shining, entire and about 1 mm in diameter after they grew on nutrient broth agar at 30 °C for 48 h. Under the microscope, they showed a rod shape and had a dimension of about 1.5–2.5 μm in length and about 0.5 μm in width.

The nucleotide sequences of the 16S-rDNA from the isolated strains, E3 and F2, were determined (comprising 1527 nucleotides) and deposited in the Gene Bank databases (<http://www.ncbi.nlm.nih.gov>) under accession numbers DQ453688 and DQ453689, respectively. A phylogenetic

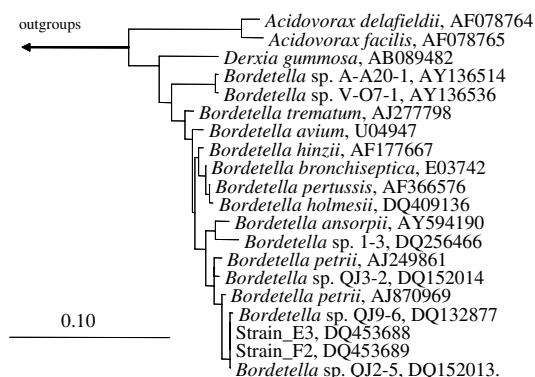


Fig. 2. Phylogenetic tree based on 16S-rRNA gene sequence analysis, illustrating the relationship of the isolates able to mineralize 1,2,4-TCB to the closely related bacteria. The scale bar corresponds to 0.10 estimated nucleotide substitution per sequence position. Dendrogram was calculated according to a Maximum Likelihood approach.

tree was constructed based on the obtained 16S-rRNA coding gene sequences of the isolates and the nearest relatives (Fig. 2). The 16S-rDNA of strains E3 and F2 showed a similarity of 100%. Both strains had the highest similarity of 100% to *Bordetella* species QJ2-5. They had a close match to the described species, *Bordetella petrii* AJ870969, with a homology of 99.4%.

3.3. 1,2,4-TCB mineralization by isolated strains E3 and F2 in liquid culture

The isolated strains, E3 and F2, showed nearly as high mineralization capacities as the enriched mixed culture (Fig. 1), with 58% and 46% mineralization of 1,2,4-TCB, respectively, within 30 days of incubation. Highest mineralization rates were observed 6 days after incubation. In the corresponding control samples, mineralization of 1,2,4-TCB was negligible (0.06% of applied radioactivity).

The amount of ¹⁴C-volatile substances and the chemical residues in the liquid phase are shown in Table 1. In the control liquid culture, 45% of the applied radioactivity was trapped as volatile substances, whereas in the liquid cultures with inoculums of the microbial community and strains E3 and F2, volatile substances were much lower corresponding to a much higher 1,2,4-TCB-mineralization. In the control samples, there was no significant difference between the ¹⁴C-concentration in the mineral medium before and after filtration step at the end of the experiment. But in the liquid media inoculated with the microbial community and strains E3 and F2, the ¹⁴C-concentration was much higher before filtration than after filtration, indicating that a large amount of radioactivity was retained on the filter together with the microbial cells.

3.4. Mineralization of 1,2,4-TCB in soil by isolated strain F2

Strain F2 was inoculated to soil (Mollic Gleysol) to study the potential of this strain to increase the 1,2,4-TCB mineralization in a soil with a low indigenous 1,2,4-TCB mineralization capability. In the inoculated soil, the mineralization rates increased without any lag phase. After 30 days about 39% of 1,2,4-TCB was converted to CO₂. In the soil without inoculation, a very low mineralization of 2% of applied radioactivity was measured. These results indicate that inoculation of the soil with *Bordetella* strain F2 strongly enhanced the mineralization of 1,2,4-TCB.

Table 1

Distribution and mass balance of ¹⁴C-1,2,4-TCB in the liquid cultures with inoculation of the microbial community and the strains E3 and F2 and control samples (in % of applied radioactivity, n = 3, ±SD)

Treatments	¹⁴ CO ₂	¹⁴ C Volatile substances	¹⁴ C In medium before filtration	¹⁴ C Recovery	¹⁴ C In medium after filtration
Control ^a	0.06 ± 0.01	45.38 ± 7.61	22.03 ± 3.76	67.47 ± 8.55	19.60 ± 3.35
Community ^b	58.40 ± 3.06	0.73 ± 0.11	14.38 ± 2.96	73.51 ± 4.30	2.36 ± 0.34
Strain E3 ^a	58.10 ± 4.92	2.38 ± 0.30	40.56 ± 2.34	101.04 ± 7.23	12.93 ± 0.65
Strain F2 ^a	45.91 ± 8.27	3.01 ± 1.98	31.54 ± 2.10	80.46 ± 13.00	12.63 ± 1.01

^a Incubation time: 30 days.

^b Incubation time: 22 days.

Table 2
Distribution and mass balance of ^{14}C -1,2,4-TCB in soil samples with inoculation of strain F2 and control samples (in % of applied radioactivity, $n = 3$, \pm SD, incubation time: 30 days)

Treatments	$^{14}\text{CO}_2$	^{14}C Volatile substances	^{14}C Extractable	^{14}C Nonextractable	^{14}C Recovery
Control	2.23 \pm 0.16	35.26 \pm 5.08	19.76 \pm 4.74	5.28 \pm 0.23	63.52 \pm 1.50
Strain F2	39.13 \pm 1.45	9.81 \pm 0.65	8.74 \pm 0.29	18.21 \pm 0.27	75.89 \pm 2.61

The ^{14}C residues in the soils varied significantly between the two treatments (Table 2). In the soil without inoculum extractable residues were about 20% and the nonextractable residues were about 5% of the applied radioactivity. Whereas in the soil with inoculum of strain F2, extractable residues were much lower (8.7%) and nonextractable residues were much higher (18.2%).

A considerably higher amount of volatile substances was trapped in the control soil in comparison with the soil inoculated with strain F2. The results show that the amount of nonextractable residues increased parallel with the increase of mineralization and the decrease of evaporation of volatile substances.

4. Discussion

At sites where microbial communities have been exposed to pollutants for a long period, the degradation ability of microorganisms appears to be closely dependent on their long-term adaptation to contaminated habitats (van der Meer et al., 1998). For our enrichment and isolation experiments, we therefore used a soil with a long-term exposure to chlorobenzenes which contained an adapted microbial community capable of 1,2,4-TCB degradation as was demonstrated in a previous study (Schroll et al., 2004). After transfer of this adapted native community from soil to liquid culture, the microbes maintained their 1,2,4-TCB degradation ability and thus we could successfully isolate degrading strains from the enriched liquid cultures which belong to the genus *Bordetella*.

The genus *Bordetella* comprises eight species as shown in Fig. 2. Most of them were isolated from humans and animals (Gerlach et al., 2001; von Wintzingerode et al., 2001; Fingerhann et al., 2006). Recently there have been some reports about the identification of *Bordetella* sp. in environmental samples with *Bordetella petrii* as the only described sp. Among the isolated strains, reported in the literature, *Bordetella petrii* DSM12804 could degrade selenate (von Wintzingerode et al., 2001) and another *Bordetella petrii* strain could degrade naphthalene (Bianchi et al., 2005) in pure culture experiments. But the strains *Bordetella* sp. A-A20-1 and *Bordetella* sp. V-O7-1 could degrade PAH only in a mixed culture (Eriksson et al., 2003). There are two other *Bordetella* spp. published, *Bordetella* sp. HPC772 and *Bordetella* sp. M1-6 (Katoh et al., 2004), but we did not include these two strains into the phylogenetic tree (Fig. 2) because the first one had only partial 16S-rDNA sequences (580 bp) and the second one was misclassified and did not belong to *Bordetella* sp.

according to our sequence analysis (Accession No.: AB039335). The strains we isolated had the closest match to *Bordetella petrii* AJ870969 (Fig. 2). To our knowledge, these are the first isolated *Bordetella* strains able to degrade 1,2,4-TCB.

The ability to degrade 1,2,4-TCB has been previously described for *Pseudomonas* sp. P51 (van der Meer et al., 1987), *Ralstonia* (formerly *Burkholderia*) sp. PS12 and *Ralstonia* (formerly *Burkholderia*) sp. PS14 (Sander et al., 1991; Rapp and Timmis, 1999) and *Rhodococcus* sp. MS11 (Rapp and Gabriel-Jürgens, 2003), but there are no previous reports on the biodegradation of 1,2,4-TCB by *Bordetella* sp. Furthermore, our results show that the isolated *Bordetella* sp. could directly degrade 1,2,4-TCB to a non-toxic metabolite (CO_2) and was thus able to detoxicate 1,2,4-TCB completely.

The isolated *Bordetella* strains and the microbial community showed a similar 1,2,4-TCB mineralization kinetic in liquid culture and in soil samples: an exponential phase of degradation at the beginning and a high rate of total mineralization, which is characteristic for “metabolic degradation” (Torstensson, 1980). In contrast, the mineralization in the control samples was very low with a mineralization dynamic which was defined as a “co-metabolic degradation” by Torstensson (1980). Our results show that the mineralization ability of the isolated bacteria was conserved in soil.

In the control soil, only low amounts of nonextractable residues were formed whereas in the soil inoculated with *Bordetella* sp. the nonextractable residues were much higher. The formation of nonextractable residues is often explained by binding of a xenobiotic to the soil matrix, especially to the soil organic matter (Gevao et al., 2000). Therefore, it should be expected that the formation rates of nonextractable residues should be the same in the soil with and without inoculum since the soil samples are identical. However, the inoculated soil showed a higher portion of nonextractable residues, which cannot be explained by different abiotic soil properties. In addition, the mineralization of 1,2,4-TCB was much higher in the inoculated soil than in the control soil. From the strong correlation between increasing formation of nonextractable residues and increasing mineralization, it can be concluded that the nonextractable residues could also be built by the formation of ^{14}C -biomass. This explanation is supported by the results from the liquid culture experiments: a high amount of radioactivity was related to the bacterial cells at the end of the experiment. Thus, it can be assumed that parallel to the 1,2,4-TCB mineralization, ^{14}C -biomass was

formed as well. In pre-experiments with ^{14}C -glucose (data not presented) in liquid culture experiments, we could show that parallel to glucose mineralization a large amount of nonextractable ^{14}C -residues was formed, which are most likely to consist of ^{14}C -biomass, which agreed with the results reported in a previous study (Rapp and Timmis, 1999).

The evaporation of volatile compounds in the liquid cultures and inoculated soil samples was much lower than for the control samples without inoculums. Due to the high mineralization capacity of the applied microbes and the probable formation of ^{14}C -labeled microbial biomass volatilization of ^{14}C -substances was reduced; these processes also resulted in a higher ^{14}C -mass balance.

5. Conclusions

Two strains, capable to mineralize 1,2,4-TCB, were isolated from a chlorobenzene contaminated soil and were identified as *Bordetella* sp. by comparative sequence analysis of their 16S-rRNA coding genes. When applying the isolated *Bordetella* strains to an artificially contaminated soil, the degradation of 1,2,4-TCB could be tremendously increased. Therefore, these microorganisms are possibly applicable for very effective remediation techniques. But further studies have to show whether application of microorganisms with a high degradation capacity can be a suitable technique on field scale and it has to be proven what could be the most efficient approach to produce, multiply and apply these microorganisms to contaminated sites. Also the sustainability of this technique for *in situ*-decontamination processes has to be tested.

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