ELSEVIER

FEMS Microbiology Ecology 47 (2004) 381–386

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Inhibition of anaerobic microbial *o*-xylene degradation by toluene in sulfidogenic sediment columns and pure cultures

Rainer U. Meckenstock [a,*], Rolf J. Warthmann [a,1], Wolfgang Schäfer [b]

[a] *Lehrstuhl für Mikrobielle Ökologie, Universität Konstanz, Universitätsstr. 10, D-78457 Konstanz, Germany*

[b] *Universität Heidelberg, Interdisziplinäres Zentrum für Wissenschaftliches Rechnen, Im Neuenheimer Feld 368, D-69120 Heidelberg, Germany*

Received 6 October 2003; received in revised form 15 December 2003; accepted 18 December 2003

First published online 16 January 2004

Abstract

It is frequently observed in aromatic hydrocarbons such as benzene, toluene, ethylbenzene, xylene (BTEX)-contaminated aquifers that toluene degrades faster than xylenes and benzene. In sediment column experiments which were run with a mixture of BTEX compounds toluene degradation started after a lag period of several weeks. When we omitted toluene from the culture medium *o*-xylene degradation started. Xylene degradation could be inhibited by adding toluene back to the medium and could be recovered when toluene was omitted again. This was observed repeatedly when toluene concentrations higher than 20 μM were added. Two sulphate-reducing bacterial species, isolated from the column material, were used to investigate the degradation behaviour in detail. Strain TRM1 degraded exclusively toluene, strain OX39 degraded preferentially *o*-xylene and toluene only after an adaptation period of more than 90 days when added as the sole substrate. Growth and *o*-xylene degradation of strain OX39 were inhibited by toluene concentrations as low as 40 μM, whereas, in contrast, toluene degradation by strain TRM1 was not inhibited by *o*-xylene concentrations up to 0.5 mM. Both the column data and the batch experiments indicated that two organisms were responsible for the toluene/xylene degradation in the sediment column. One strain degraded only toluene and was not effected by xylene and the second degraded xylene and was inhibited by toluene. Our findings offer an explanation that the observed differential degradation of BTEX compounds in contaminated aquifers could originate from a partial metabolic inhibition of xylene-degrading organisms by toluene.

© 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Biodegradation; Natural attenuation; Aromatic hydrocarbon; Mixture of aromatic hydrocarbons such as benzene, toluene, ethylbenzene, xylene; Polycyclic aromatic hydrocarbon; Metabolic inhibition; Sulfate-reducing bacterium

1. Introduction

Aromatic hydrocarbons such as benzene, toluene, ethylbenzene, xylene (BTEX), and polycyclic aromatic hydrocarbons (PAH) are frequent contaminants in the environment. They can either originate from biological activities such as microbial production of toluene in anoxic lake sediments [1–3] or from anthropogenic contaminations. High concentrations of aromatic hydrocarbons, which are of concern for ground water quality mostly originate from spills of mineral oil products or industrial production such as former gas work plants. The majority of contaminants disappear within the anoxic zones of aquifers implying that anaerobic microbial degradation is the driving force for contaminant removal [4]. Toluene appears to be the most easily degradable aromatic hydrocarbon under anoxic conditions and toluene-degrading bacteria could be cultivated for all the environmentally important electron-accepting processes such as denitrification, sulphate reduction, iron(III) reduction, fermentation, and methanogenesis [5–12]. For anaerobic *o*,*m*,*p*-xylene degradation, a few examples have been reported under sulphate-reducing, methanogenic, and denitrifying conditions [8,13–16]. So far, it is not known why toluene-degrading cultures appear to be easier to cultivate than xylene-degrading organisms.

In contaminated aquifers, organisms face mixtures of

* Corresponding author. Present address: Institut für Grundwasserökologie, GSF, Forschungszentrum für Umwelt und Gesundheit, Ingolstädter Landstraße 1, D-85764 Neuherberg, Germany.
Tel.: +49 (89) 3187-2561; Fax: +49 (89) 3187-3361.
E-mail address: rainer.meckenstock@gsf.de (R.U. Meckenstock).

[1] Present address: Eidgenössische Technische Hochschule Zürich (ETH), Geologisches Institut, Sonneggstr. 5, CH-8092 Zürich, Switzerland.

0168-6496/04/$22.00 © 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/S0168-6496(03)00303-9

xenobiotic compounds in varying concentrations and with stimulating or inhibitory effects to their growth or activity. In mixtures of aromatic hydrocarbons (BTEX), toluene always seems to be the preferentially degraded compound [17,18]. Moreover, it is frequently observed that toluene plumes are much shorter than the corresponding plumes of xylenes or benzene in the same aquifer. Thus, changes of the ratio of the toluene to benzene concentrations along the ground water flow path have been even used as an indicator of biodegradation [19]. Further attempts to elucidate the differential degradation of BTEX compounds have been performed in a field experiment where mixtures of aromatic hydrocarbons and nitrate as electron acceptor were injected into the Borden aquifer [20]. In these studies degradation of xylenes was inhibited by the addition of toluene. As a further example of inhibitory effects by complex substrate mixtures, toluene degradation was inhibited by the presence of alkanes, gasoline, or trichlorethylene [18,21].

Here, we report on anaerobic microcosm studies investigating inhibitory effects of toluene on *o*-xylene degradation with sulphate as electron acceptor. With the help of pure sulphate-reducing bacterial cultures originating from the same sediment columns, inhibition processes in the microcosms were investigated in detail. The results suggested that the partial inhibition of the pure cultures by BTEX compounds takes place in the same way as in the microcosms leading to a preferential degradation of toluene.

2. Materials and methods

2.1. Sediment columns

Microcosm experiments were performed with quaternary river sediment material taken from a BTEX-contaminated aquifer near Stuttgart, Germany, at 8 m beneath soil surface. Sediment-filled glass columns (5×45 cm) were operated bottom to top with carbonate-buffered (30 mM) fresh-water medium, pH 7.2 [22], supplemented with 3 mM $FeCl_2$ as reducing agent and 1 mM Na_2SO_4 as electron acceptor. The basal medium consisted of 1.0 g l^{-1} NaCl, 0.4 g l^{-1} $MgCl_2{\cdot}6H_2O$, 0.2 g l^{-1} KH_2PO_4, 0.25 g l^{-1} NH_4Cl, 0.5 g l^{-1} KCl, 0.15 g l^{-1} $CaCl_2{\cdot}2H_2O$, 7 vitamin solution, and trace element solution SL10 [22]. The medium flow speed through the column was approximately 2 m per day. The medium was pumped with peristaltic pumps (Minipuls 3, Gilson) through a mixing chamber where a mixture of hydrocarbons was continuously added with a SP 220i infusion pump (World Precision Instruments, Berlin, Germany). The standard hydrocarbon mixture was naphthalene (20 μM), benzene (150 μM), toluene (200 μM) and *o*,*m*,*p*-xylene (60 μM each) at the column inlet. However, the hydrocarbon concentrations in the feeding solution could be varied for special experiments. When increased hydrocarbon concentrations were used, the sulphate concentration was adjusted to allow a complete oxidation of the hydrocarbons to CO_2. Three separate sediment columns were operated for three to four months at 16°C in the dark. All tubings were of Viton quality (Kummer, Freiburg, Germany) to avoid oxidation of the medium and unspecific loss of hydrocarbons.

2.2. Cultivation of bacterial strains

The sulphate-reducing strains TRM1 and OX39 were enriched and isolated from the sediment columns as described elsewhere [9,16]. The organisms were cultivated in 100 ml serum bottles half-filled with the above-mentioned fresh-water medium, flushed with N_2/CO_2 (80/20), and closed with Viton rubber stoppers (Maag Technik, Dübendorf, Switzerland). The large head space was applied to allow repetitive sampling without producing under pressure in the bottles which could change the pH or the proportion of volatile hydrocarbons. Na_2SO_4 (10 mM) was added as electron acceptor, 1 mM Na_2S as reducing agent, and 3 mM $FeCl_2$ in order to scavenge produced sulfide.

Hydrocarbons (5 μl toluene or 10 μl *o*-xylene) were added with a syringe through the stoppers. Strain OX39 was cultivated in the presence of Amberlite XAD7 (Fluca, Buchs, Switzerland) functioning as a substrate buffer and providing the cultures with a low *o*-xylene concentration of 50–100 μM [16].

The adsorber resin XAD7 was carefully washed five times with ethanol (99.8%) and five times with distilled water to remove organic compounds from the resin. Traces of ethanol were removed by lyophylisation and drying for 2–3 days at 90°C. XAD7 (0.3 g) was autoclaved in a 100 ml serum bottle and the medium was added. Then, the hydrocarbons were injected through the stopper and allowed to adsorb to the XAD resin for one week before the cultures were inoculated. During the cultivation, the hydrocarbons were in equilibrium with the resin and showed almost constant concentrations in the aqueous phase.

Batch inhibition experiments with strains TRM1 and OX39 were performed under the same respective cultivation conditions as used for the enrichment procedure only varying the hydrocarbon concentrations. Different amounts of aromatic hydrocarbons were added to the cultures and substrate utilisation was assessed by following sulfide production, measured by the method of [23]. Sulfide was measured instead of the substrates itself because the hydrocarbons were adsorbed in the bottles with XAD7 and stayed constant at very low concentrations although degradation of e.g. xylene occurred. For the inhibition experiments with strain OX39, the same amount of xylene was added to every bottle, whereas the amount of toluene added to the XAD-containing bottle was varied between

0 and 120 μM finally measured toluene concentration at day 55 in the aqueous phase (the larger portion of the hydrocarbons being adsorbed to the XAD phase). Inhibition experiments with strain TRM1 were performed without XAD addition as this strain could stand higher hydrocarbon concentrations as compared to strain OX39. Here, the same amount of toluene was added to the bottles and the xylene concentrations were varied between 0 and 110 μM as measured on day 55. Until this time, the respective inhibitory substrate concentrations (e.g. toluene in the xylene degradation experiment and vice versa) remained unchanged whereas the substrates already underwent significant degradation.

2.3. Analytics

Hydrocarbon concentrations were determined by high-performance liquid chromatography (HPLC) on a Beckman System-Gold equipped with a C_{18}-reversed phase column and an ultraviolet (UV) detector (206 nm). Eluent was acetonitrile/ammonium phosphate buffer, pH 3.5 (70/30). Samples for HPLC analysis (250 μl) were added to 1 ml ethanol (analytical grade, 99.8%), mixed and precipitates were removed by centrifugation (5 min, $15\,000 \times g$).

Sulfate was measured with an HPLC ion chromatograph (Sykam, Gilching, Germany) equipped with a LCA A03 ion-exchange column (Sykam). Elution condition was isocratic with an aqueous eluent containing 5 mM Na_2CO_3, 0.4 mM hydroxybenzonitrile, and 10% (v/v) acetonitrile.

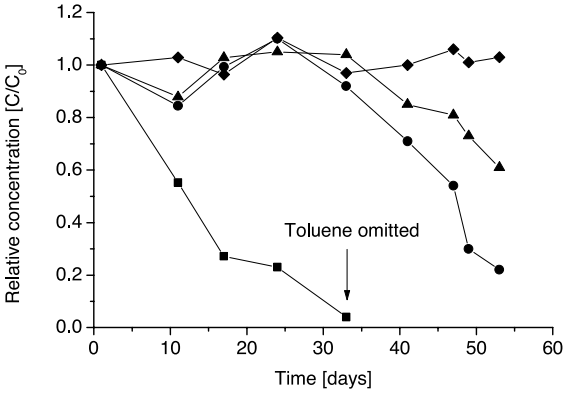

Fig. 1. Relative concentrations (C/C_0) of a mixture of aromatic hydrocarbons during anoxic sediment column experiments with material from a contaminated aquifer. C and C_0 depict the outlet and inlet concentrations, respectively. Experiment with a mixture of toluene (■), *o*-xylene (●), *m,p*-xylene (▲), and benzene (♦) as substrates. Toluene was omitted from the substrate mixture on day 33.

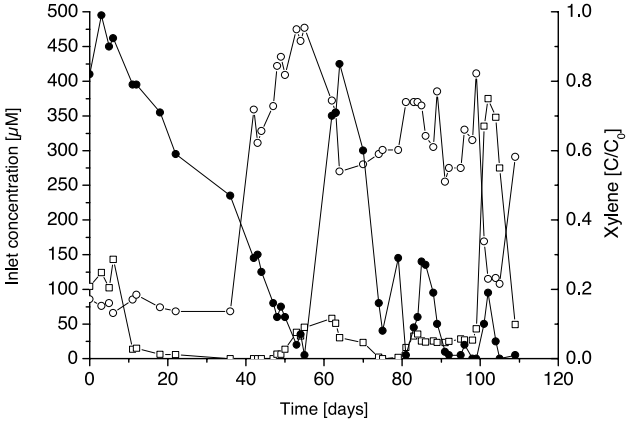

Fig. 2. Inhibition of anaerobic *o*-xylene degradation by toluene in a restarted anoxic sediment column with changing concentrations of the only two substrates. A: Measured toluene (□) and *o*-xylene (○) inlet concentrations are shown together with the relative outlet concentrations (C/C_0) of *o*-xylene (●).

3. Results

3.1. Sediment column experiments

The 65 cm glass columns were filled with natural sediment from a contaminated site and operated with sulphate as electron acceptor and a mixture of benzene, toluene, *o,m,p*-xylene, and naphthalene (data not shown) as carbon and electron source. The hydrocarbon concentrations were monitored over time at the inlet and the outlet of the column. After the equilibrium concentrations in the sediment column were established, the outlet concentration of only toluene decreased continuously until toluene was almost completely degraded during the column passage at day 33 (Fig. 1). An electron balance was calculated based on the disappearance of sulphate and toluene and revealed that 74% of the electrons from toluene were used for sulphate reduction indicating a complete microbial oxidation to CO_2. At day 33, toluene was omitted from the hydrocarbon mixture which served as sole carbon and energy source and a continuously increasing degradation of *o*-xylene established (Fig. 1). This was reproducibly observed in three independent experiments with different sediment columns (data not shown).

As this experiment indicated that toluene itself or toluene degradation products inhibited *o*-xylene degradation, a sediment column which had already shown total removal of toluene and *o*-xylene was run with the two substrates, and the concentrations were monitored at the inlet and outlet (Fig. 2). When 100 μM toluene was present in the medium at the first day of the experiment no *o*-xylene removal could be observed but toluene was completely degraded. This continued until toluene was omitted from the mixture after 7 days. As observed before, the *o*-xylene concentration decreased steadily until it was almost completely removed in the column at day 55 although the *o*-xylene concentration was significantly increased to about

400 μM. Then, 50 μM toluene was added back to the medium. *o*-Xylene degradation decreased immediately when the toluene concentration increased and recovered only when toluene was again omitted from the medium. The absence of toluene again resulted in a complete *o*-xylene removal about 10 days later. When only 25 μM toluene was added to the medium at day 77, *o*-xylene degradation was again inhibited. The outlet concentration increased to 70% of the inlet concentration. However, the xylene-degrading activity recovered within 5 days although the toluene inlet concentration was not reduced. A last increase to almost 400 μM toluene could again produce a minor inhibition of *o*-xylene degradation but also in this case the *o*-xylene degradation recovered within 2 days. During the whole experiment, toluene was almost completely degraded in the column. Toluene appeared at the outlet only at day 77 (0.9 μM) when it was added back to the medium at an inlet concentration of 25 μM and at days 98 and 99 (31.4 and 1.4 μM respective outlet concentrations) when the toluene concentration at the inlet was increased to approximately 400 μM. A second, similar experiment was performed with another column and produced similar results (data not shown).

3.2. Batch culture experiments

Two sulphate-reducing pure cultures were isolated from the sediment columns as reported elsewhere [9,16]. Strain TRM1 could only utilise toluene as a carbon source and none of the different xylene isomers. When incubated with different mixtures of toluene and *o*-xylene, the sulfide production from toluene degradation by strain TRM1 was not affected by the *o*-xylene concentration in the medium although *o*-xylene was added up to 110 μM final concentration in the medium (Fig. 3).

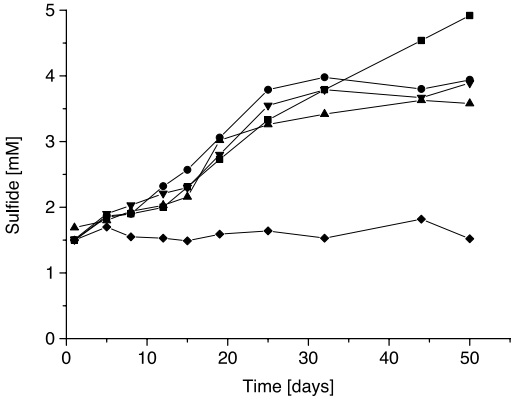

Fig. 3. Batch cultures of strain TRM1 showing sulfide production from toluene degradation in the presence of *o*-xylene. The means of two sulfide production curves by cultures supplied with the same toluene and *o*-xylene concentrations are shown for a typical experiment out of three replicates. The initial sulfide concentration of 1–1.5 mM originates from sodium sulfide added to the medium as the reducing agent. Toluene concentrations in the beginning of the experiment were set to 300 μM in all vials (except the control with xylene only) and were below the detection limit of 0.5 μM on day 55. Concentrations of *o*-xylene on day 55 were (■) no *o*-xylene added, (●) 10 μM *o*-xylene, (▲) 25 μM *o*-xylene, (▼) 115 μM *o*-xylene, and (♦) 55 μM for the vials where only *o*-xylene and no toluene was added. The xylene concentrations were measured on day 55 of the experiment.

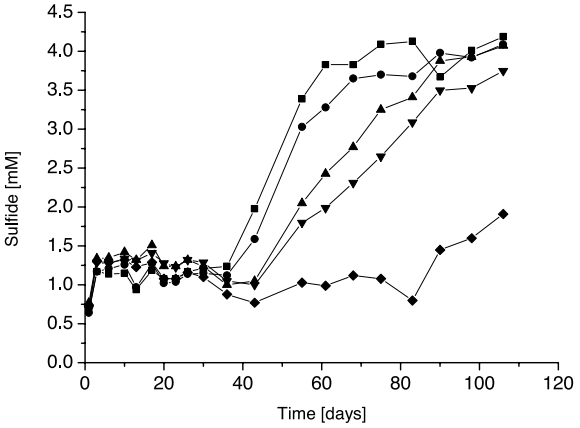

Fig. 4. Sulfide production from *o*-xylene degradation by strain OX39 in the presence of (■) 36 μM *o*-xylene and no toluene, (●) 10 μM *o*-xylene and no toluene, (▲) 30 μM *o*-xylene and 45 μM toluene, (▼) 40 μM *o*-xylene and 110 μM toluene, and (♦) 105 μM toluene only. Xylene and toluene concentrations were measured on day 55 of the experiment. The xylene concentrations in the medium are effected by adsorption to the XAD7 phase and varied in addition due to different extents of degradation that already occurred.

Within the first 50 days of observation, sulfide was produced by strain OX39 degrading *o*-xylene (Fig. 4) and *m*-xylene (data not shown) but not toluene (Fig. 4) or *p*-xylene (data not shown). After incubation of strain OX39 for more than 90 days, sulfide production started slowly in the incubations with only toluene. When strain OX39 was incubated with mixtures of *o*-xylene and toluene, sulfide development from *o*-xylene utilisation was slowed down if the toluene concentration exceeded 40 μM final concentration in the medium (Fig. 4).

4. Discussion

Preferential degradation of toluene over xylene can be observed frequently in BTEX-contaminated aquifers indicating a general rule or mechanism behind this observation. One reason which could possibly influence the preferential degradation in situ was identified in this work as the partial inhibition of the xylene-degrading activity by toluene. This could be shown in batch culture experiments with two different sulphate-reducing bacterial cultures isolated from the columns. Whereas the toluene-degrading strain TRM1 could stand mixtures of toluene and xylene in equal amounts without interference with respect to toluene degradation it appeared that the xylene-degrading strain OX39 was inhibited by toluene concentrations as small as 40 μM final concentration in the medium. If the two organisms represented the major degrading populations in the sediment column this could explain that toluene is preferentially degraded in the columns.

The sediment column experiments with different toluene and xylene concentrations indicated as well that a specific xylene-degrading and a separate toluene-degrading population are responsible for the observed degradation and not only one type of organism that can degrade both. When toluene was omitted from the carbon source, *o*-xylene degradation started slowly. If the same organism was responsible for the degradation of toluene and *o*-xylene, then the regulation between toluene and xylene degradation would not last for several weeks if the biomass was already present. As soon as the induction of enzyme expression for *o*-xylene degradation would have started, resulting in an increasing *o*-xylene degradation, the whole population should have shifted to xylene degradation. However, this was not observed during the initial phase when the activity was slowly increasing (Fig. 2). In contrast, after *o*-xylene degradation was established and inhibited by the first toluene pulse, *o*-xylene degradation recovered much faster when toluene was omitted, which indicates that the xylene-degrading biomass was already present in this case.

It remains an open question how toluene inhibited xylene degradation in our experiments although some possibilities can be excluded. The inhibition could have been either due to a general toxicity on organism level or to specific enzyme inhibition or metabolic regulation. On the organism level, one possibility is that the overall hydrocarbon concentration was too high for the xylene-degrading organism because e.g. the membranes could have been disrupted when the toluene concentration increased. However, the inhibition of *o*-xylene degradation took place with concentrations as low as 25 μM toluene in the sediment columns. This almost completely excludes the hypothesis that the inhibition is due to a non-specific toxicity of elevated hydrocarbon concentrations. This is also obvious from the batch experiments where strain OX39 could degrade *o*-xylene at a much higher total hydrocarbon concentration of 30 μM xylene plus 45 μM toluene. Lethal toxic effects on the organisms are also not very likely because the xylene-degrading activity recovered immediately after toluene was omitted from the medium in the sediment columns. On the enzyme level there could be inhibition of the xylene-degrading enzymes either by toluene itself or by one of the metabolites produced, or on the regulation of enzyme expression. These processes cannot be distinguished so far. Metabolites of toluene degradation such as benzylsuccinate could be responsible for such effects, as these metabolites are sometimes found in concentrations of more than 1 μM in the culture media [24]. Toluene degradation could interfere with the very similar *o*-xylene degradation pathway because for the two degradation pathways the addition of fumarate to the methyl group is observed as the initial reaction [15,25,26]. The following degradation steps of toluene and xylene are probably also very similar.

It was observed that with time the *o*-xylene-degrading activity in the sediment columns reacted progressively less to the toluene treatment which was obvious by the last toluene addition of 400 μM causing only minor inhibition effects on *o*-xylene degradation. This observation could be explained by a general feature of degradation experiments that we observed frequently with column microcosms. With increasing operation time of column experiments the organisms tended to concentrate at the column inlet [27]. Consequently, the degradation activity was located in the first few centimetres after the column inlet and the rest of the column was no more supplied with substrate. If such a scenario took place in our sediment columns the toluene-degrading organisms would have concentrated at the column inlet after a reasonable operation time. With every toluene pulse this effect would enhance and finally generate areas in the outlet region of the column which were not exposed to toluene during the pulse because it was already degraded in the first parts of the column. In these zones, *o*-xylene degradation could occur and would have been protected from the toluene addition. Although this aspect was not investigated in detail in the presented experiments, this explanation is consistent with all observations of the three different experimental approaches.

5. Conclusion

Our findings of the preferential degradation of toluene have major implications for microbiological degradation tests which are often performed to investigate the degradation capacity of contaminated aquifers with respect to bioremediation. Based on our results, every compound of interest has to be tested individually for biodegradation. If complex mixtures of aromatic hydrocarbons are used in sediment column experiments or microcosms inhibitory effects might occur. On the other hand, if a potential inhibition between different substrates is taken into account, the feeding of mixed substrates might represent a more realistic image of environmental conditions.

Acknowledgements

The authors are grateful to Prof. Bernhard Schink, Konstanz for substantial support of this work.

References

[1] Jüttner, F. and Henatsch, J.J. (1986) Nature 323, 797–798.
[2] Jüttner, F. (1988) Naturwissenschaften 75, 151–153.
[3] Fischer-Romero, C., Tindall, B.J. and Jüttner, F. (1996) Int. J. Syst. Bacteriol. 46, 183–188.
[4] Christensen, T.H., Kjeldsen, P., Bjerg, P.L., Jensen, D.L., Christensen, J.B., Baun, A., Albrechtsen, H.J. and Heron, C. (2001) Appl. Geochem. 16, 659–718.
[5] Dolfing, J., Zeyer, J., Binder-Eicher, P. and Schwarzenbach, R.P. (1990) Arch. Microbiol. 154, 336–341.

[6] Lovley, D.R. and Lonergan, D.J. (1990) Appl. Environ. Microbiol. 56, 1858–1864.
[7] Rabus, R., Nordhaus, R., Ludwig, W. and Widdel, F. (1993) Appl. Environ. Microbiol. 59, 1444–1451.
[8] Edwards, E.A. and Grbic-Galic, D. (1994) Appl. Environ. Microbiol. 60, 313–322.
[9] Meckenstock, R.U. (1999) FEMS Microbiol. Lett. 177, 67–73.
[10] Heider, J., Spormann, A.M., Beller, H.R. and Widdel, F. (1999) FEMS Microbiol. Rev. 22, 459–473.
[11] Spormann, A.M. and Widdel, F. (2000) Biodegradation 11, 85–105.
[12] Widdel, F. and Rabus, R. (2001) Curr. Opin. Biotechnol. 12, 259–276.
[13] Evans, P.J., Mang, D.T. and Young, L.Y. (1991) Appl. Environ. Microbiol. 57, 450–454.
[14] Häner, A., Höhener, P. and Zeyer, J. (1995) Appl. Environ. Microbiol. 61, 3185–3188.
[15] Harms, G., Zengler, K., Rabus, R., Aeckersberg, F., Minz, D., Rossello-Mora, R. and Widdel, F. (1999) Appl. Environ. Microbiol. 65, 999–1004.
[16] Morasch, B., Annweiler, E., Warthmann, R.J. and Meckenstock, R.U. (2001) J. Microbiol. Methods 44, 183–191.
[17] Haag, F., Reinhard, M. and McCarty, P.L. (1991) Environ. Toxicol. Chem. 10, 1379–1389.
[18] Phelps, C.D. and Young, L.Y. (1999) Biodegradation 10, 15–25.
[19] Thierrin, J., Davis, G.B., Barber, C., Patterson, B.M., Pribac, F., Power, T.R. and Lambert, M. (1993) Hydrol. Sci. 38, 309–322.
[20] Barbaro, H.R., Barker, J.F., Lemon, L.A. and Mayfield, C.I. (1992) J. Contam. Hydrol. 11, 245–272.
[21] Johnston, J.J., Borden, R.C. and Barlaz, M.A. (1996) J. Contam. Hydrol. 23, 263–283.
[22] Widdel, F. and Bak, F. (1992) In: The Prokaryotes (Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H., Eds.), Vol. 4, pp. 3352–3378. Springer, New York.
[23] Cline, J.D. (1969) Limnol. Oceanogr. 14, 454–458.
[24] Beller, H.R. (2000) Biodegradation 11, 125–139.
[25] Krieger, C.J., Beller, H.R., Reinhard, M. and Spormann, A.M. (1999) J. Bacteriol. 181, 6403–6410.
[26] Beller, H.R. and Spormann, A.M. (1997) J. Bacteriol. 179, 670–676.
[27] Tchelet, R., Meckenstock, R., Steinle, P. and van der Meer, J.R. (1999) Biodegradation 10, 113–125.