Microbial colonization of the subsurface at the test site and degradation of chlorobenzenes by autochthonous bacteria of the quarternary aquifer

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Introduction

The groundwater in the region of Bitterfeld has been contaminated to a high degree with industrial chemicals - intermediates, final- and by-products - of very different chemical structure, properties and risk potentials due to activities of the chemical industry in this region for more than hundred years.

Considering the huge dimension of the groundwater pollution (about 200 million m^3 distributed over an area of more than 25 km^2) only the development of special passive remediation techniques using in situ- reactors ("reactive barriers") for plume treatment according to the "funnel and gate" principle might offer suitable solutions for such an environmental problem. Bioremediation processes using the degradative potential of the autochthonous microbial community of the contaminated aquifer can be regarded as promising variants of this procedure.

The hydrogeological situation at the selected test site is characterized by the presence of two aquifers separated by a lignite seam (in a depth between about 22 and 28 m) which acts as a local aquitard. The upper, quarternary aquifer is polluted predominantly by chlorobenzenes, mainly monochlorobenzene (MCB). Chloroaliphatics were found only in traces (range of some µg/l), unsubstituted benzene was detected in relatively low concentrations (Table 1). The lower, tertiary aquifer ranging from about 28 m down to the regionally distributed clay aquitard which marks the border of groundwater bearing strata in about 50 m depth is contaminated only with small concentrations (some µg/l) of di- and trichloroethenes and traces of different chloroaromatics at the test site.

Based on the different degree of contamination by chloro-organics and the technical-economic feasibility of an in situ-remediation process according to the "funnel and gate"-principle the investigations were focused on the quarternary aquifer.

Table 1 *Pollutants of the groundwater from the quarternary aquifer at the Bitterfeld test area (data from Popp, P. and Möder, M., 1997 and 1998, internal UFZ reports).*

Substance	Concentration (µg/l)
Monochlorobenzene	3,130 – 33,000
	(18,000 - 32,000)[1]
1,4- Dichlorobenzene	90 – 1,000
	(200 - 400)[1]
1,2- Dichlorobenzene	20 – 180
	(30 - 80)[1]
Benzene	20 – 180
Trichloroethene	< 10 – 460
1,2- cis- Dichloroethene	10 – 280
1,2- trans- Dichloroethene	10 – 60
Chloromethylphenols	43.5
Trichlorophenols	9.2
2,4- Dichlorophenol	3.3
Dimethylphenols	1.4
Detected mass- spectrometrically after enrichment:	
1,1,2- Trichloroethane	
1,1,2,2- Tetrachloroethane	
Bromobenzene	
1- Chloro- 2- methylbenzene	
1,3- Dichlorobenzene	
Vinyl chloride	
Tetrachloroethene	
Tetrachlorobenzene	
Toluene	
m/p- Xylene	

1 = average values of the investigation period in the mobile test unit.

Characterization of the quarternary aquifer as microbial habitat

Apart from the high pollution with MCB, the quarternary aquifer is characterized by a special combination of environmental factors (Table 2) which should allow the development of a specialized bacterial community. Temperature and pH values are in relatively favourable ranges and almost constant, inorganic nutrients (S, N, P) are available in sufficient amounts. The deficit of available electron acceptors must be regarded as a limiting factor for bacterial growth. Molecular oxygen and nitrate are

practically not detectable. Sulfate is certainly present in a high concentration but it is seemingly not (or only in a very low extent) used by sulfate-reducing bacteria in this environment due most probably to the redox potential suboptimal for this ecophysiological group: in spite of the presence of sulfate-reducing bacteria and practically unlimited amounts of sulfates no formation of metal sulfides in the aquifer was observed; only traces of H_2S were found sporadically. Furthermore, the groundwater contains only very small amounts of potential organic carbon sources others than chloroorganics: The total content of organically bound carbon, based on non-volatile substances, amounts to 6 - 8 mg/l; only a small (unknown) part of it might serve as potential carbon substrates for the groundwater bacteria.

It should be mentioned that the actually measured concentrations of electron acceptors, potential carbon sources, macro- and micronutrients in the groundwater and also the bacterial colonization density of the aquifer must be regarded as steady state concentrations within an open dynamic system.

Table 2 *Ecologically relevant abiotic factors characterizing the groundwater of the quarternary aquifer at the test site.*

TOC (mgl^{-1})	20 – 30
TOC_{nv} (based on non-volatile substances ≅ non- chloroaromatics) (mgl^{-1})	6 – 8
NO_3^- (mgl^{-1})	≈ 0
SO_4^{2-} (mgl^{-1})	700 – 800
Cl^- (mgl^{-1})	390 – 470
PO_4^{3-} (mgl^{-1})	6.5 - 16.5
NH_4^+ (mgl^{-1})	4.2 - 6.6
Dissolved O_2 (% saturation)	≈ 0
E_H (mV)	85 – 215
PH	6.7
T (°C)	14

Microbial colonization density of the quartenary aquifer

The subsurface at the test site is inhabited by bacteria down to the maximum drilling depth (50.5 m below the surface). The content of viable cells of the solid phases (sediments) varied with the depth and reached maximal 10^6 cfu/g sediment dry matter in the quarternary aquifer; the colonization density of the tertiary aquifer was sig-

nificantly lower (Figure 1). The total bacterial density in the groundwater of the quarternary aquifer amounted to approximately 10^5 cells/ml. Yeasts and filamentous fungi were not found in the most samples, a tiny abundance (< 5 x 10^2 cfu/g) was only detected in the lignite- bearing strata and in the topsoil (drilling depth 6 m).

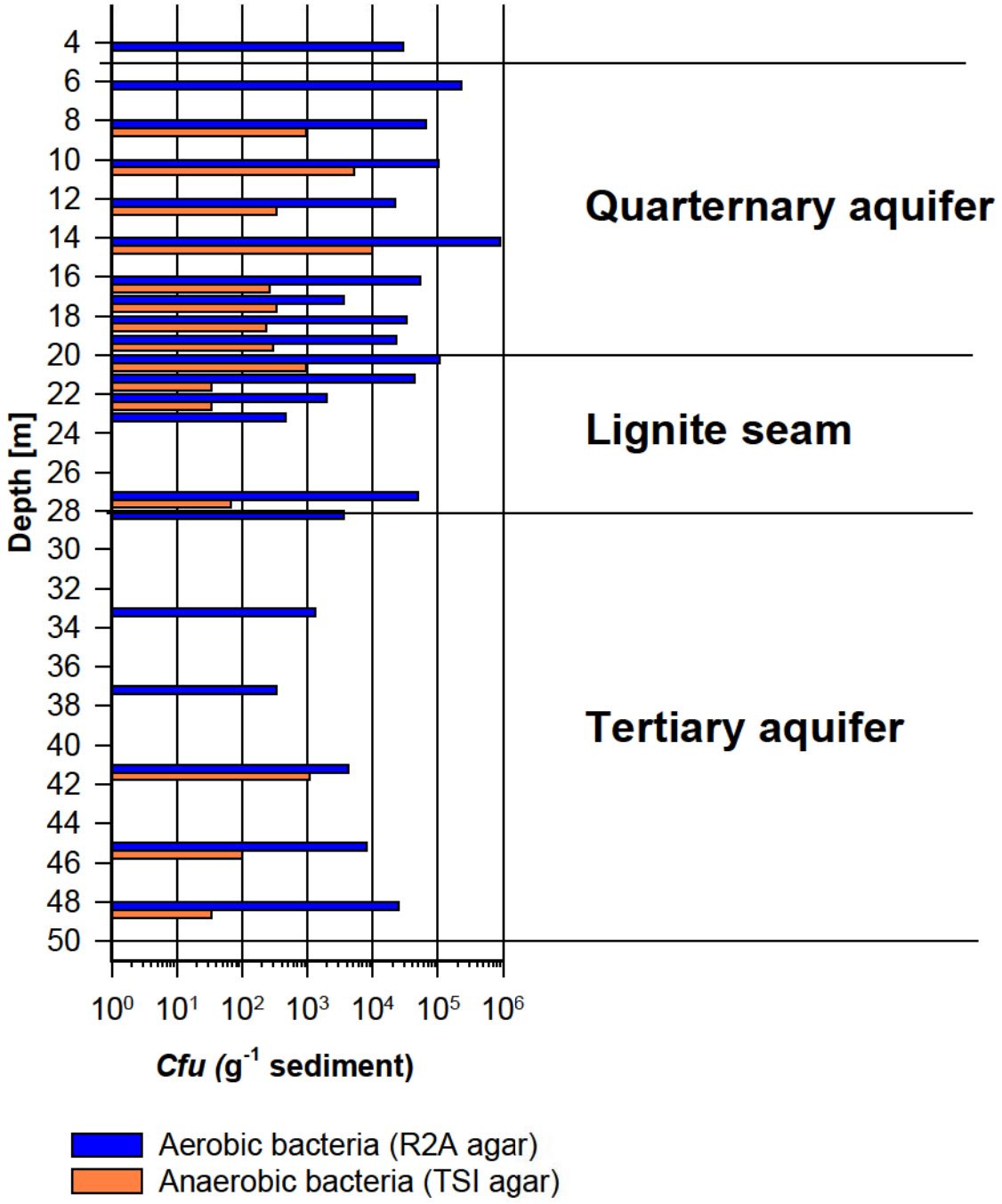

Figure 1 Bacterial colonization of the subsurface at the test site (SAFBIT 1/97 and 2/97).

Although the quarternary aquifer is practically free of molecular oxygen, aerobic bacteria represented, with about 7 x 10^5 cells /ml groundwater and more than 10^6 cfu/g sediment dry matter, the quantitatively predominant ecophysiological group of the autochthonous bacteriocenosis (Table 3). Due to methodological reasons, this group included also facultatively anaerobic bacteria. It could be demonstrated by the replica technique that the aerobic bacteriocenosis consisted at least partially of denitrifying bacteria. Using the MPN method, however, relatively low colonization densities of denitrifying bacteria were found in sediments and groundwater, in the same magnitude than sulfate- reducing bacteria (Table 3). Iron-reducing bacteria were present in significantly higher concentrations than these groups (Table 3), reaching approximately 35 to 65% of the abundance of the aerobic/facultatively anaerobic bacteria. The colonization density of the different ecophysiological groups reflects the influences of the abiotic environment, especially of redox potential and availability of electron acceptors.

Table 3 Colonization of groundwater and sediments of the quarternary aquifer by different ecophysiological groups of bacteria.

Sample	Total cell counts (DAPI staining)	Aerobic bacteria (colony forming units on R2A/100, agar)	Nitrate-reducing bacteria (MPN)	Sulfate-reducing bacteria (MPN)	Iron-reducing bacteria (MPN)
Groundwater SAFBIT 7/97	6.9×10^5 ml^{-1}	1.2×10^5 ml^{-1}	2.0×10^4 ml^{-1}	8.0×10^3 ml^{-1}	not determined
Sediment 18-19 m SAFBIT 30/98	8.8×10^5 g^{-1}	7.1×10^4 g^{-1}	1.2×10^3 g^{-1}	1.6×10^3 g^{-1}	2.4×10^4 g^{-1}
Sediment 20-21 m SAFBIT 30/98	1.1×10^6 g^{-1}	1.0×10^5 g^{-1}	6.3×10^2 g^{-1}	1.1×10^3 g^{-1}	6.7×10^4 g^{-1}

Potential of the autochthonous bacteriocenosis for the degradation of chlorobenzenes

The biodegradation of chlorobenzenes has been intensively studied in the last decades. Under anaerobic conditions high chlorinated benzenes were reductively dechlorinated to MCB and the three isomers of dichlorobenzene (DCB) which are regarded as indegradable and, therefore, persisting substances under anaerobic conditions.

There are only some indications in the literature that benzenes with one or two Cl-atoms might be degraded anaerobically, but the mechanisms are completely unknown. The aerobic pathways of the degradation/mineralization of low chlorinated benzenes, however, are elucidated in detail and well understood (Figure 2).

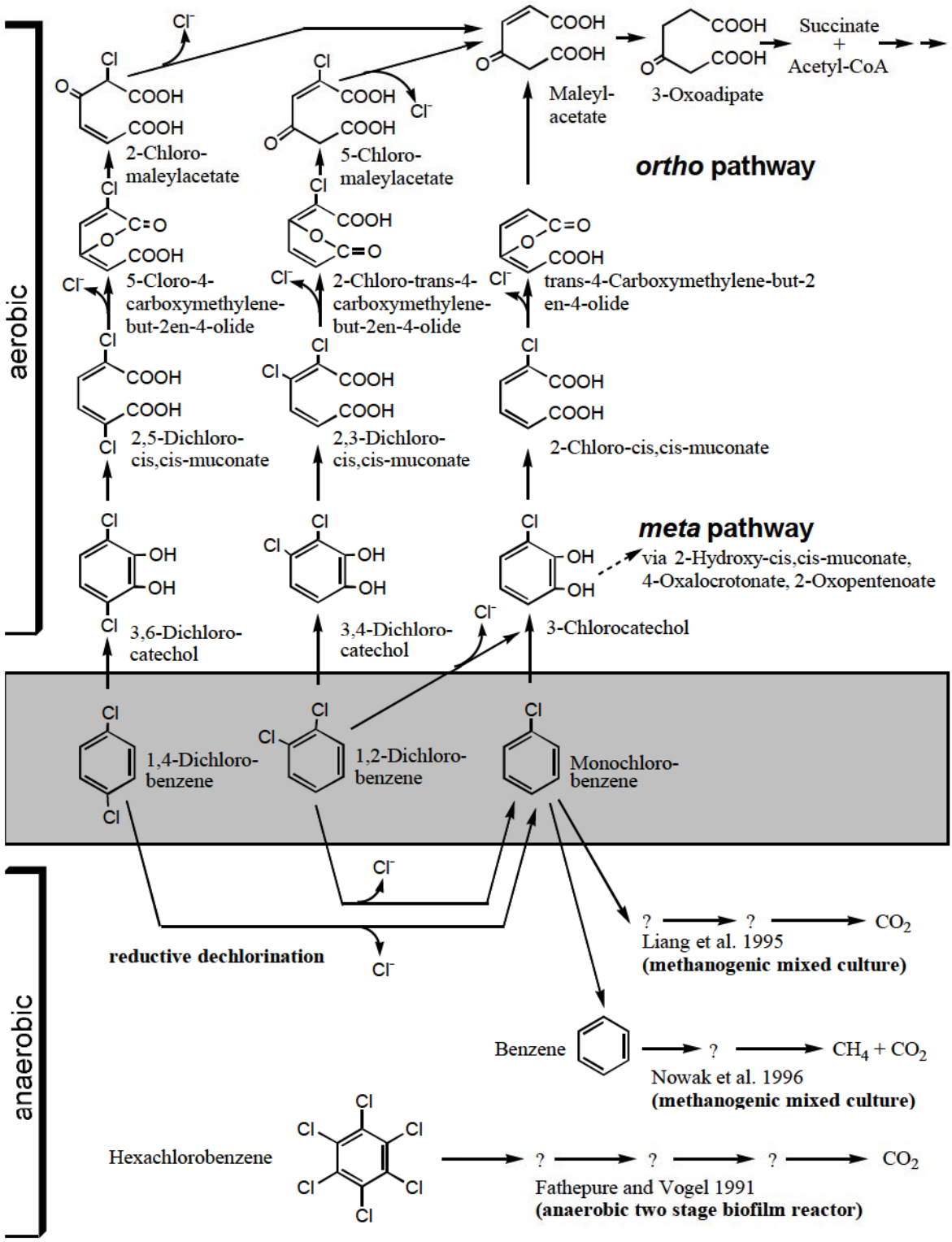

Figure 2 Pathways for the degradation of the main chloroaromatic contaminants (MCB,1,4- and 1,2-DCB) of the quaternary aquifer.

An extended screening programme at the beginning of our investigations showed that the autochthonous bacteriocenosis of the quarternary aquifer of the Bitterfeld test site is capable of degrading the main pollutants MCB and 1,4- DCB both under aerobic and anaerobic conditions. Basically, the anaerobic degradation processes proceeded significantly slower than the aerobic mineralization, the degradation of MCB under aerobic and denitrifying conditions (Figure 3) is quoted as an illustration of this general statement.

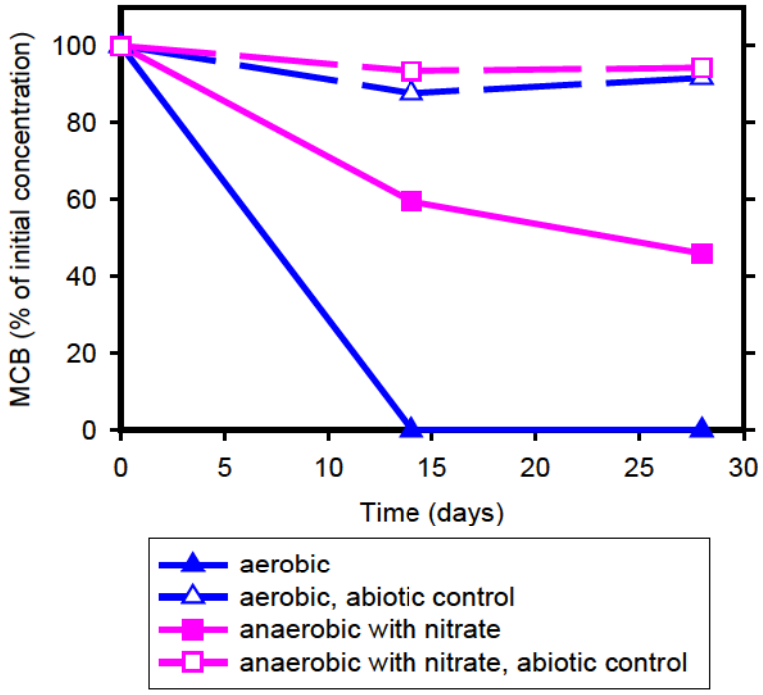

Figure 3 *Degradation of MCB by the autochthonous bactericenosis of the Bitterfeld aquifer under aerobic and anaerobic (denitrifying) conditions. Initial MCB-concentration: 18 mg/l (n=3).*

Aerobic degradation of MCB

More than 10 strains could be isolated from the quarternary aquifer which were capable of growing aerobically on MCB as sole source of carbon and energy. Strains with the best degradation properties (Figure 4) utilized 80 – 100 mg/l MCB within 48 h at 14°C (corresponding to the in situ-temperature of the aquifer). Members of the genus *Rhodococcus* predominated among the active MCB-degrading strains, but also Gram- negative bacteria with high degradation potential were isolated.

By feeding ^{14}C- labeled MCB it was shown that the autochthonous bacteriocenosis of the quarternary aquifer mineralized MCB: After 5 days of incubation about 7% of the fed radioactivity were found in the biomass, approximately 47% in the formed CO_2 (Figure 5).

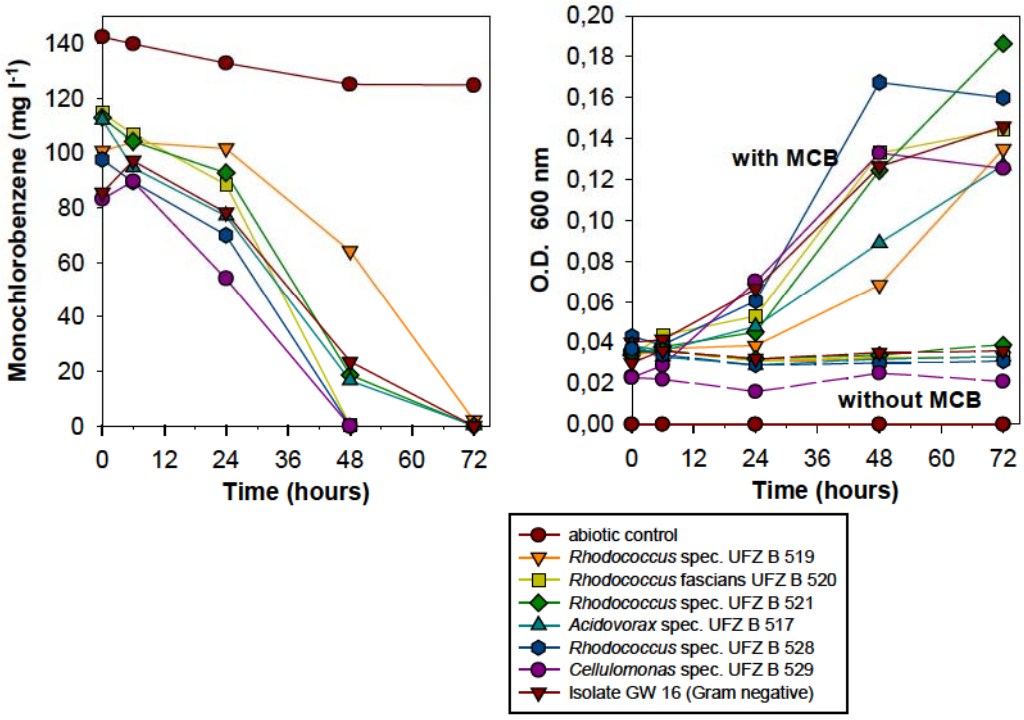

Figure 4 *Degradation of MCB under aerobic conditions at 14 °C - isolates from the autochthonous bacteriocenoses.*

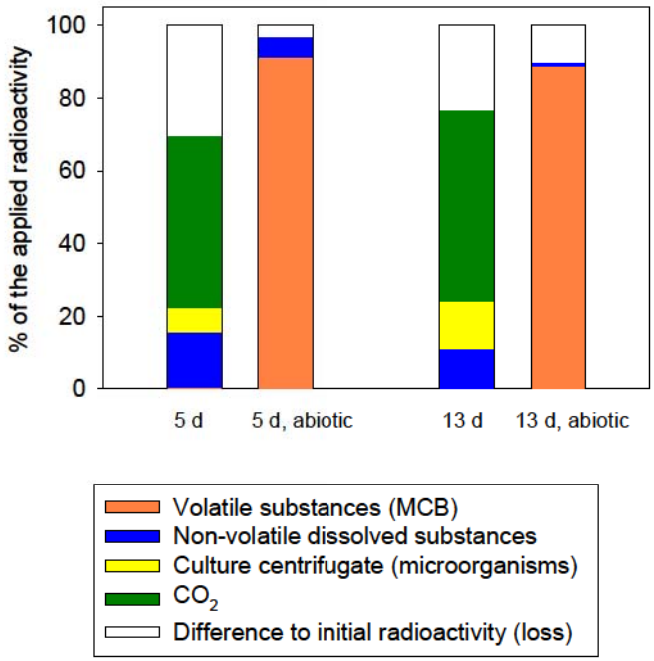

Figure 5 *Complete mineralization of monochlorobenzene by the autochthonous bacteria of the Bitterfeld bacteriocenosis under aerobic conditions - Application of ^{14}C-labelled monochlorobenzene.*

Anaerobic degradation of MCB

Using nitrate and sulfate as alternative electron acceptors a significant reduction of the MCB concentrations in the culture suspensions was measured under different laboratory conditions. In all experiments the chlorobenzenes (MCB, in some cases also 1,4- DCB) were the sole growth substrate. In contrast to processes with molecular oxygen as electron acceptor, the degradation processes were much slower even under optimized conditions. For example, in fed batch cultivation under denitrifying conditions the fed MCB (between 14 and 18 mg/l) was no more detectable in the culture suspension after 10-32 days of incubation (Figure 6).

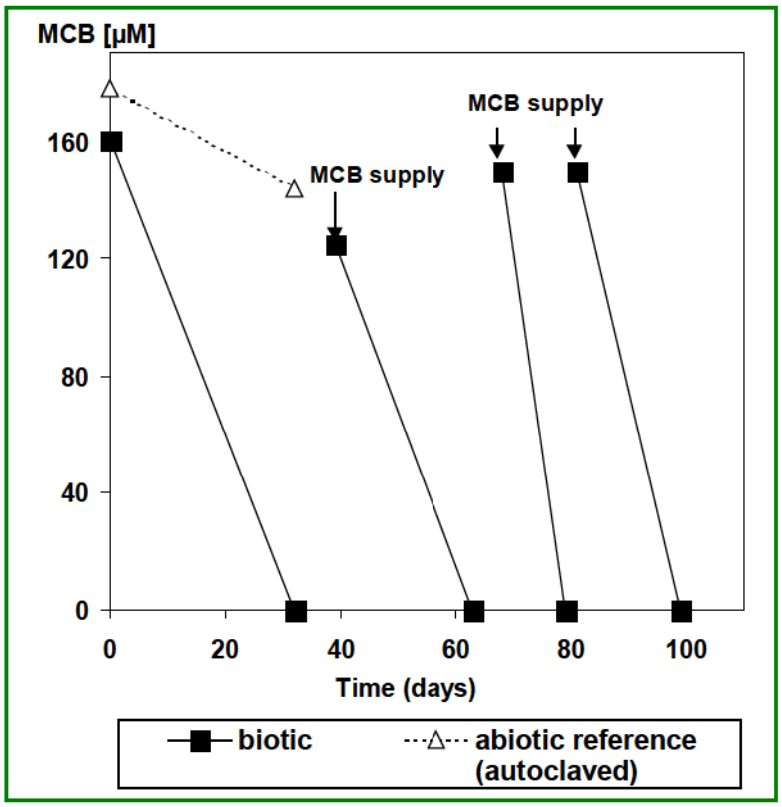

Figure 6 *Degradation of monochlorobenzene by an enrichment culture in fed-batch cultivation under denitrifying conditions.*

Anaerobic degradation processes were only observed when mixed cultures (enrichment cultures from the quarternary aquifer) were used as inoculum. Although denitrifying and sulfate-reducing taxa could be detected within these mixed cultures by analyzing their 16S rDNA (Table 4), pure cultures capable of degradation of MCB could be isolated not yet.

The mechanisms of the anaerobic conversion of MCB are still unknown and under intensive investigation in our laboratories. Both denitrifying and sulfate- reducing enrichment cultures grew with nitrate and sulfate, respectively, as electron acceptors on MCB as sole growth substrate. The expected metabolites CO_2, nitrite (in denitrifying cultures) and sulfide (in sulfate-reducing bacteria) could be detected during the

cultivation processes, but up to now it was impossible to establish complete, reliable mass balances. Although these experiments were performed with the common laboratory equipment for anaerobic work, it can not be excluded absolutely that traces of oxygen got admitted to the culture suspensions (e.g. by diffusion through tubes and plugs, as contaminant of the gas phases and solutions, etc.). These traces of O_2 might be necessary to initiate the degradation process. After that the further degradation might take place via the typical pathways of denitrification and sulfate reduction.

Table 4 Enrichment cultures from the quarternary aquifer capable of metabolizing chlorobenzenes anaerobically.

Denitrifying mixed culture
- growth on monochlorobenzene as sole source of carbon and energy under denitrifying (anaerobic) conditions
- consists of least 5 components which are most probably (according to analyses of their 16S rRNA) strains of
  - Hydrogenophaga palleroni
  - Pseudomonas stutzeri
  - Lactosphaera pasteurii
  - Agrobacterium tumefaciens
  - an iron- oxidizing denitrifying bacterium

Sulfate- reducing mixed culture
- grows on mono-, 1,2- and 1,4- dichlorobenzene as sole source of carbon and energy under sulfate- reducing (anaerobic) conditions
- consists of some Gram- negative and Gram positive sulfate- reducing strains which belong most probably to the genera
  - Desulfotomaculum and
  - Desulfovibrio

Degradation of chlorobenzenes in semi- technical scale

The results of the investigations in bench scale were confirmed successfully in semi-technical scale in the on site- plant (mobile test unit) in Bitterfeld. The microbiological experiments were performed in a column reactor of steel, which was filled by original sediment material from the quarternary aquifer (Figure 7). The column was continuously fed with original groundwater at the in situ- temperature of 14°C. After establishing steady state conditions, concentrated nitrate solution (16 mM KNO_3) was dosed resulting in a final concentration of approximately 1 mM nitrate in the groundwater inflow. The relation KNO_3 solution to groundwater as well as the retention time of this mixture in the column could be varied within a wide range. To avoid an access of air (oxygen) the sytem was completely closed, the gas phases in the sampling bottles and over the nitrate solution in the storage tank consisted of nitrogen. As a consequence of the nitrate feeding the concentration of MCB in the outlet of the column decreased considerably. The nitrogen content in the outlet was also significantly lower than the initial concentration due to formation of nitrite (and, possibly but not analyzed, molecular nitrogen). The degradation rate depended on the

retention time (Figure 8 and 9). At the highest retention time which was investigated (12 days) about 95% of the MCB and 91% of 1,4- DCB were degraded. These values correspond to absolute concentrations of about 1 mg/l MCB and 0.01 mg/l 1,4-DCB in the bioremediated groundwater.

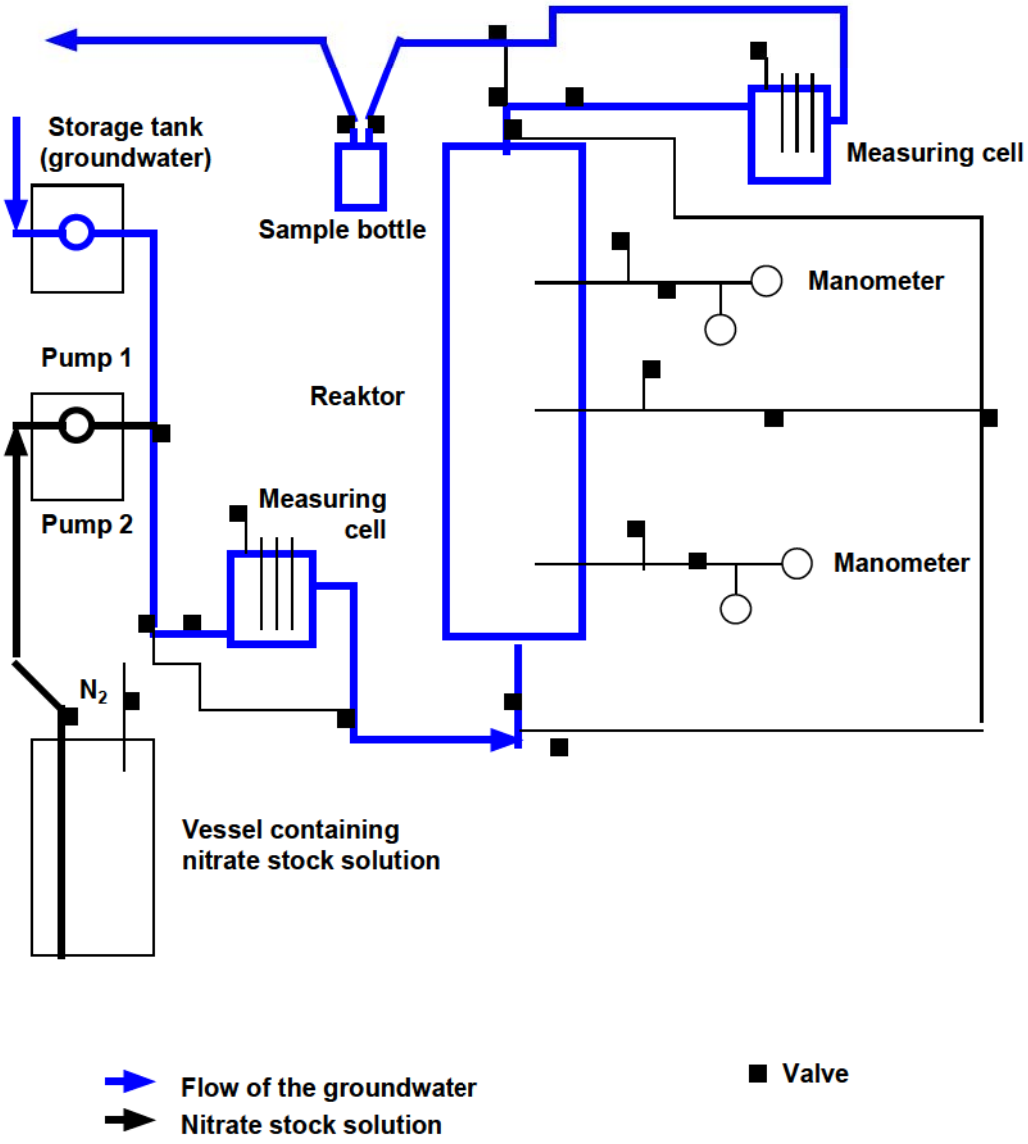

Figure 7 Flow diagram of the on site- plant (mobile test unit), column 3.

The results of our investigations in the mobile test unit in semi- technical scale demonstrate that the in situ- bioremediation of the chlorobenzene contaminated groundwater is possible by supporting the denitrifying autochthonous bacteriocenosis by feeding nitrate solution. The efficiency and long- term stability of the “nitrate procedure” will be investigated in the SAFIRA pilot plant under large- scale conditions.

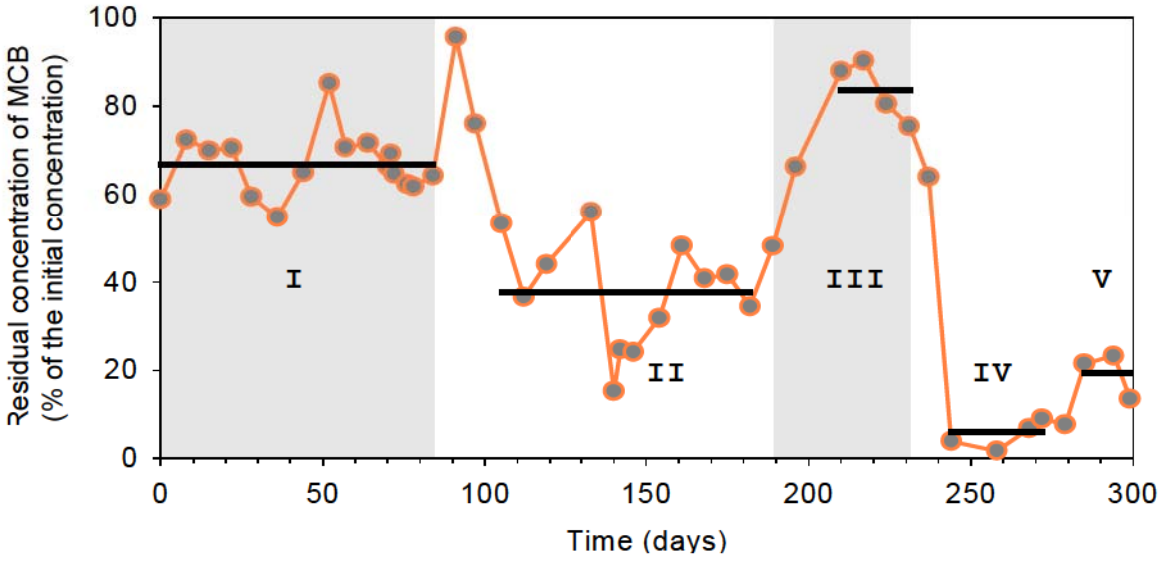

Figure 8 *Degradation of monochlorobenzene in the on-site-reactor (horizontal lines characterize the average value in the test phase during a stable steady state). Test phases:*

I	*Reference phase (without addition of nitrate), retention time 4 days*
II	*Dosage of nitrate solution, retention time 4 days*
III	*Control phase (by-pass)*
IV	*Dosage of nitrate solution, retention time 12 days*
V	*Dosage of nitrate solution, retention time 6 days.*

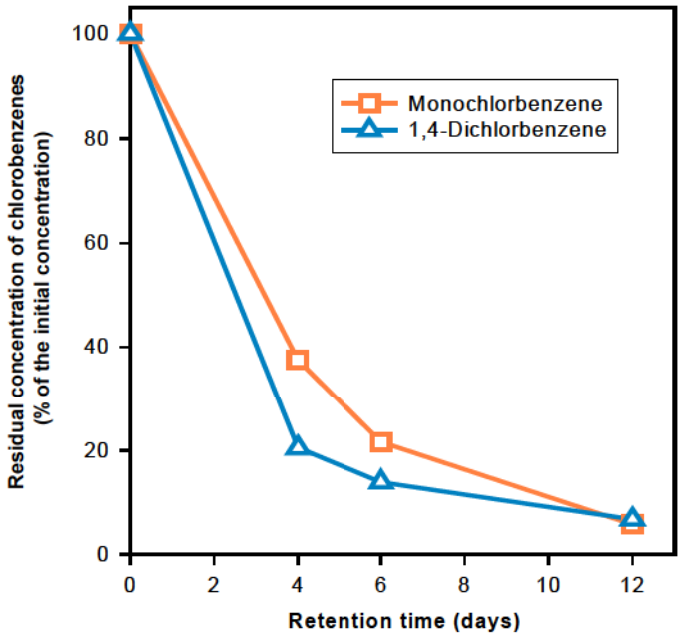

Figure 9 *Degradation of chlorobenzenes in the on-site reactor under denitrifying conditions as function of the retention time.*

Summary

1. The colonization density of the groundwater of the quarternary aquifer amounted to about 10^6 cells/ml. The quantitatively predominating ecophysiological groups are aerobic/facultatively anaerobic and iron- reducing bacteria, the abundance of denitrifying and sulfate-reducing bacteria is significantly lower.

2. The autochthonous bacteriocenosis is capable of degrading mono- and dichlorobenzenes when site-related limitations are removed, e.g. by addition of electron acceptors:
   - Under *aerobic conditions* monochlorobenzene is completely mineralized both by the bacteriocenosis and pure cultures isolated from aquifer material
   - Under *anaerobic conditions* and by application of nitrate and sulfate, respectively, as alternative electron acceptors the degradation processes occurred slower compared to aerobic degradation. The mechanisms of the anaerobic degradation are still unknown. It is possible that a primary ring modification requires traces of oxygen and the following degradation occurs under denitrifying /sulfatereducing conditions.

3. In the mobile test unit at Bitterfeld the almost complete elimination of the chlorobenzenes was demonstrated under in situ- related conditions at semi- technical scale. Continuous supply of nitrate solution to the original groundwater resulted in the degradation of 94% (corresponding to residual concentrations of $\approx$ 1 mg/l) of the original concentration of monochlorobenzene at sufficient retention times (12 days)

SAFIRA

Abstracts of the Workshop

of November 17-18, 1999

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