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# Microbial community shifts as a response to efficient degradation of chlorobenzene under hypoxic conditions

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**Abstract** Limitations in the availability of oxygen restrict aerobic biodegradation of chloroaromatic compounds in groundwater ecosystems. In this context the activity of ring-cleaving chlorocatechol dioxygenases (CC12O) is crucial for effective mineralization. Previously we demonstrated that oxygen-related enzyme characteristics of CC12O can vary widely among the Proteobacteria (Balcke et al. submitted). Here, we investigated how strains with different ability to transform intermediary 3-chlorocatechol integrate into biodegradation of chlorobenzene (CB) under low or high oxygen availability. *Pseudomonas veronii* UFZ B549 and *Acidovorax facilis* UFZ B530, which had differing oxygen affinities for CC12O, were mixed together at different proportions (20:80; 80:20), and compared for degradation of chlorobenzene under oxic (215 μM O2) and hypoxic (11 μM O2) conditions. Changes in community composition in binary mixed cultures were determined and compared with an indigenous groundwater community, cultivated under comparable conditions. Community shifts were determined by FISH (fluorescent in situ hybridization) in our model system and SSCP (single stranded conformation polymorphism) fingerprinting in the groundwater community, as well as by analysis of respiratory quinones of taxonomic value. Hypoxia led to enrichment of *Acidovoracae* in the groundwater and binary cultures. Under hypoxic conditions *cis*,*cis*-2-chloromuconate released to the medium by *A. facilis* allowed for concomitant growth of *P. veronii*, although its low-affinity type CC12O would not imply growth on CB. Vice versa, increasing abundance of *P. veronii* induced intermediary 3-chlorocatechol accumulation, which was reduced by growth of *A. facilis*. Thus, reduced oxygen availability caused syntrophic rather than competitive interactions.

**Keywords** Chlorobenzene · Hypoxic · Kinetics · Microaerophilic · Catechol dioxygenase

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**Abbreviations**

| | |
|---|---|
| CB | Chlorobenzene |
| CC | Chlorocatechol |

Cmuc Chloromuconate
CC12O Chlorocatechol-1,2-dioxygenase

## Introduction

CB can become rapidly degraded by a number of microorganisms via formation of 3-chlorocatechol (3CC) and ring cleavage to *cis*,*cis*-2-chloromuconate (2CMuc) (Reineke 2001). However, efficient degradation of CB in the natural environment is supposed to be limited by the availability of oxygen, whereas the provision of microorganisms with oxygen can strongly vary over time or space. This contributes to distinct ecological niches for bacteria with different metabolic activities and enzymatic characteristics adapted to e.g. high or low concentrations of dissolved oxygen (DO). Because oxygen is inadequately provided in subsurface compartments, formation of metabolites, i.e. toxic 3-chlorocatechol in the case of CB, is often observed as a direct consequence of oxygen limitation (Vogt et al. 2004). On the other hand, microaerophilic strains can repress low-affinity type community members and mineralize CB upon persisting oxygen deficiency within a short time scale (Nestler et al. 2007). Limitations in the oxygen availability (hypoxic conditions) therefore have far-reaching consequences for aerobic microorganisms degrading (chloro-)aromatic compounds.

When bioremediation strategies put large amounts of oxygen into contaminated aquifers the availability of oxygen will change. This significantly alters the composition of the indigenous microbial community, adapted to the respective natural conditions, by promoting or disadvantaging certain members (Balcke et al. 2004). In turn, this influences the biodegradation efficiency, since the metabolic activity of individual strains, which preferentially grow under aerobic conditions, may become insufficient under hypoxic conditions.

In case of CB, efficient biodegradation under oxygen-deficient conditions parallels enzyme characteristics of the oxygen-dependent key enzyme chlorocatechol-1,2-dioxygenase (CC12O) in different pure microorganisms, e.g. widely differing oxygen half-saturation constants (Balcke et al. submitted). For this reason, specifically the activity of ring-cleaving dioxygenase is expected to drive the biodegradability of CB and also the abundance of key organisms in a microbial community exposed to oxygen limitation.

Thererfore, in the present study binary cultures of *Pseudomonas veronii* UFZ B549 and *Acidovorax facilis* UFZ B530 served as model community to analyze how strains with different enzyme characteristics (Balcke et al. submitted) interact in the CB degradation under hypoxic (oxygen-deficient) compared to oxic (oxygen-saturated) conditions. A. facilis possesses a high-affinity type CC12O and was demonstrated recently to be capable of transforming 3-chlorocatechol under oxygen limitation, whereas *P. veronii* has a low-affinity type CC12O and accumulates toxic 3CC as oxygen is limiting (Vogt et al. 2004). Thus, it can be hypothesized that prevalent growth of A. facilis will be associated with successful biodegradation of CB under oxygen limitation. Biodegradation and community composition were surveyed during the course of the experiment by combining metabolite profiling with FISH probing (fluorescent in situ hybridisation) capable of resolving both species by rRNA hybridization. The results on the binary mixtures were compared to those obtained on a complex groundwater community, obtained from a CB-polluted aquifer, and equally cultivated. The community structure at the beginning and at the end of the experiments was described by single stranded conformation polymorphism (SSCP) fingerprinting (16S rDNA and rRNA) and sequencing.

Microorganisms from different phylogenetic genera can synthesize quinones differing in the length of the isoprenoid side chain ($n$ = 6–14) (Geyer et al. 2004; Hedrick and White 1986; Hiraishi 1999). Since PCR-based data cannot be interpreted without bias in terms of quantitative abundances of certain community members, additionally profiles of isoprenoid quinones served as quantitative biomarkers for both model organisms and microbial taxa of the groundwater community. We used the number of isoprenoid units as a criterion to distinguish between Pseudomonas ($\gamma$-proteobacteria) and Acidovorax ($\beta$-proteobacteria) characterized by the ubiquinones UQ-9 (Yamada et al. 1982) and UQ-8 (Busse et al. 1992) (unpublished results), respectively, when grown under oxic conditions. Quinones are involved in the transport of electrons in the cell membrane, whereas different quinone species are formed depending on the type of the terminal oxidase active

under the respective conditions, i.e. aerobic or anaerobic. As the formation of ubiquinones (UQ) is typically associated with aerobic respiration, formation of menaquinones (MK) and deoxymenaquinones (DMK) is found to relate to anaerobic processes. Thus, quinone profiles were recorded to additionally deliver insights into the microbial respiratory chains that are active at the applied oxygen levels.

## Materials and methods

### Site description and sample collection

The Bitterfeld aquifer and its physicochemical characteristics were described in detail elsewhere (Heidrich et al. 2004; Kaschl et al. 2005). Groundwater was collected from a depth of 19.5 m from a well with average annual CB concentrations of 15–20 mg $l^{-1}$ and <0.1 mg $l^{-1}$ dissolved oxygen. About 12 l groundwater were filtered through sterile 0.2 µm Isopore cellulose acetate filters (Millipore) for enrichment of the indigenous microbial community and further cultivation as described (Balcke et al. submitted). For analysis of the indigenous groundwater community by SSCP and respiratory quinones cells were collected by filtration of 250 ml groundwater through 0.22 µm Durapore filters (Millipore, Bedford, MA, USA). For analysis of the culture compositions centrifugation of 5.0 ml culture medium (2,822*g*, 5 min, 4°C) was taken at the end of each cultivation. For FISH probing 2.0 ml aliquots of the culture media were sampled over the course of the cultivations, fixed with 4% paraformaldehyde, and filtered through 0.22 µm filters as above. Cells were immediately frozen and stored at –20°C until further use.

### Culture conditions

A cell concentrate of the groundwater community and binary mixtures from Acidovorax facilis B530 and *Pseudomonas veronii* B549 were cultivated in duplicates on CB as substrate (600 µM initial concentration). Details of the oxidostat fed batch cultivation have been described elsewhere (Balcke et al. submitted). Briefly, permanently stirred culture media were kept under an atmosphere containing a nearly constant level of oxygen either at concentrations of 0.75% (corresponding to 11 µM dissolved oxygen; hypoxic) or of 14.4% (215 µM dissolved oxygen; oxic). Oxygen levels were adjusted in the gas phase by periodical spikes with sterile oxygen gas while the headspace and the DO levels were monitored non-invasively (optode) (for details see Balcke et al. submitted). Pre-cultivation of *A. facilis* B530 and *P. veronii* B549 was made under oxic conditions prior to inoculation into batches kept under the hypoxic and oxic conditions, respectively. Cells of the pure strains concentrated to approximately $5 \times 10^7$ cells $ml^{-1}$ were mixed in proportions of 80:20 and 20:80 (*A. facilis*:*P. veronii*) before spiking the batches. Colony forming units were not analyzed because plate cultivation with a definite amount of CB as a substrate is impossible. The calculation was done by separate cultivation of pure cultures of both strains, measurement of optical densities (OD600) and calculation of the cell ratio according to a calibration curve. These data were the basis for mixing of both cultures in a definite ratio.

### Metabolic analysis

Analysis of CB and oxygen consumption as well as of 3-chlorocatechol (3CC), 2-chloromuconate (2Cmuc) and $Cl^-$ have been described in detail elsewhere (Balcke et al. submitted).

### Isoprenoid quinones

Duplicate samples were extracted with a single-phase solvent system (Bligh and Dyler 1954) modified to include a phosphate buffer (White et al. 1979). Total lipids were extracted in 19 ml chloroform/methanol/potassium phosphate buffer (1:2:0.8 by volume at pH 7.4) for 6 h including three intervals of ultrasonication for 2 min. Chloroform (5 ml) and nanopure water (5 ml) were added in order to promote phase separation. After clear separation the organic phase was transferred to a new tube and dried by ultra pure nitrogen at ≤37°C. The total lipid extract was separated into neutral-, glycol- and polar-lipids by silica acid column chromatography with chloroform, acetone, and methanol as described elsewhere (Guckert et al. 1985). The neutral lipid fractions

were analysed for major respiratory quinone species by HPLC in conjunction with detection by atmospheric pressure chemical ionization tandem mass spectrometry (HPLC-APCI-MS/MS) using an Agilent capillary LC 1100 system and a 4000QTrap tandem MS (MDS SCIEX, Concord, Canada). The total amount of ubiquinones (UQ) with 6–10 isoprenoid units, menaquinones (MK) with 4–10 isoprenoid units as well as demethylmenaquinone (DMK) with eight isoprenyls in the side chain were quantified using authentic standards and their relative amounts calculated (Geyer et al. 2004).

## Whole-cell fluorescence in situ hybridization (FISH)

Following the description by Glöckner et al. (1996) the hybridization was first optimized for both pure cultures with the probes BETA42a (Manz et al. 1992) and GAMA42a (Manz et al. 1992) labelled with indocarbocyanine fluorescent dye CY3 (MWG-Biotech AG, Ebersberg, Germany) and used for FISH analysis of the binary mixed cultures. Additionally, the filters were stained with DAPI (final concentration, 2–5 mg $ml^{-1}$), embedded in Citifluor solution (Citifluor Ltd., London, UK) for whole cell counts. Samples of the binary cultures were analyzed thereafter in the same manner. Stained cells were inspected by epifluorescence microscopy (Axioscop 20; Zeiss, Jena, Germany) and amounts of hybridized cells calculated as in Kirchman et al. (1993).

For pre-cultures of A. facilis B 530 repeat counts ($n = 21$) resulted in a cell number of $4.45 \times 10^7$ cell $ml^{-1}$ (DAPI counts) and $3.1 \times 10^7$ cells $ml^{-1}$ after hybridization with the BET42a probe (standard deviation 0.9%). In the P. veronii B549 pure culture (repeat counts $n = 13$) we found $5.3 \times 10^7$ cells $ml^{-1}$ by DAPI staining and $3.03 \times 10^7$ cells $ml^{-1}$ with the GAM42a probe (standard deviation 1.6%). Aliquotes of the pure cultures were the used for the binary mixed cultures in the experiments.

## Nucleic acid extraction and amplification

DNA of frozen cells were extracted in duplicates by a bead beating method using the Fast DNA® SPIN Kit for Soil and the protocol therein (Q•BIOgene, Germany). For efficient lysis, 10 µl $\beta$-mercaptoethanol was added per tube. DNA concentrations were calculated with the NanoDrop® ND-1000 Spectrophotometer (Peqlab Biotechnologie GmBH, Germany), the DNA was stored at –20°C. PCR was carried out in a PTC-200 Thermal Cycler (MJ Research, MA, USA) with PCR Taq polymerase Master Mix (Promega, Madison, USA) and oligonucleotides from MWG-Biotech AG (Ebersberg, Germany). For all reactions the PCR master mixes were used with 1.5 mM $MgCl_2$, 0.3 µM of each primer, 2.5 µl DMSO (SIGMA, Deisenhofen, Germany) and corresponding DNA aliquots. The PCR conditions for total 16S rDNA amplification with the primer pair UniBac 27f and Univ1492r (Lane 1991) were: 94°C, 3 min (initial denaturation); 94°C, 25 s. (denaturation); 53°C, 30 s (annealing); 72°C, 75 s (elongation) for 35 cycles followed by a 5 min completion at 72°C. For amplification of a highly variable region (V4/V5) of the 16S rDNA the primers UniBac 515f and UniBac 927r (with a 5′-terminal phosphate group) (Lane 1991) were used at an annealing temperature of 53°C for 15 cycles only. The PCR products were checked for size on 1.5% (w/v) agarose gels (Cambrex Bio Science, Rockland, USA) with a 100 bp ladder (TaKaRa Biomedicals, Japan) as standard. Phosphorylated products were purified using the E.Z.N.A. Cycle Pure Kit (Pequlab Biotechnologie GmbH, Erlangen, Germany).

## SSCP analysis and DNA reamplification

SSCP for molecular fingerprinting instead of the widely used Denaturising Gradient Gel Electrophoresis (DGGE) was used, since in contrast to DGGE, the products for SSCP analysis do not need GC-clamps, a step making the choice of primer pairs for SSCP easier. The removal of one of the complementary strands in SSCP additionally avoids the problem of heteroduplex formation, a problem often encountered with DGGE.

The amplified and purified V4–V5 regions of the 16S rDNA were digested with $\lambda$-exonuclease (New England Biolabs, Germany) at 37°C for 2 h, mixed with one volume of SSCP loading buffer (95% formamide, 10 mM NaOH, 0.025% bromophenol blue – xylene cyanol), denatured by heating to 95°C for 2 min and immediately placed on ice for at least

5 min as recommended by Dohrmann and Tebbe (2004). The SSCP electrophoresis was performed on a TGGE Maxi System (Biometra, Germany) at 400 V and 28°C for 12 h. The gel matrix (0.4 × MDE stock solution (Biozym, Germany) in 1 × TBE buffer (Sambrook and Russell 2001) was polymerized with 0.04% TEMED and 0.69% ammonium. Gels were silver stained (Bassam 1991) dried for at least 24 h at room temperature and then scanned.

Interesting bands were excised, rehydrated within 150 µl sterile water, reextracted with 150 µl elution buffer (0.5 M ammonium-acetate, 10 mM magnesia-acetate, 1 mM EDTA, 0.1% SDS) by incubation at 37°C for 3 h, followed by ethanol precipitation (Tebbe et al. 2001). The extracted DNA was reamplified as described above, but without phosphorylation. PCR products were purified with the E.Z.N.A.® Cycle-Pure Kit and sequenced.

### DNA sequencing and phylogenetic analysis

Sequencing was performed using a BigDye RR Terminator AmpliTaq™, FS Kit version 3.1 (Applied Biosystems, Germany) and the same V4–V5 universal primers (without phosphorylation) following supplier recommendations. Electrophoresis and data collection were carried out on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Germany). Data were analyzed by the ABI PRISM® DNA Sequencing Analysis software, and sequences of both complementary strands were assembled by the ABI PRISM® Autoassembler software. The BLASTN program (http://www.ncbi.nlm.nih.gov/blast/; version 2.0; (Altschul et al. 1990) was used to search for similar sequences in the nucleotide sequence databases, and the Sequence Match tool (version 2.7) to search for similar sequences in the Ribosomal Database Project II (http://rdp.cme.msu.edu/html/) (Maidak et al. 1999).

## Results and discussion

Based on our results on pure isolates of *Acidovorax facilis* B530 and *Pseudomonas veronii* B549 (Balcke et al. submitted) we used both strains here in binary culture as model community to evaluate how two chlorobenzene (CB) degrading species integrate into the biodegradation of CB as a function of the oxygen availability. The observed shifts in the abundance of each individual strain in response to different oxygen availability were compared to a more complex microbial community, obtained from a CB-contaminated aquifer.

### CB degradation under hypoxic and oxic conditions

Binary cultures of *P. veronii* and *A. facilis* (mixed at ratios of approx. 80:20 and 20:80) were cultivated and analyzed for their capability to degrade 600 µM CB under oxic or hypoxic conditions (whereas oxygen concentrations in the headspace were adjusted by periodical spikes with oxygen to give an equivalent to 215 ± 10 µM or 11 ± 2 µM dissolved oxygen). Since inter-phase mass transfer of oxygen may lack behind the oxygen consumption, dissolved oxygen concentrations actually achieved in solution can become lower, depending on the activity of oxygen requiring transformations.

At high and at low oxygen availability CB was transformed by the binary cultures (Figs. 1 and 2). The time required for CB degradation was higher for hypoxic than for oxic growth conditions, whereas oxygen consumption rates (mol oxygen per hour; data not shown) as well as the total amount of oxygen consumed were lower (Table 1). Under oxic conditions all cultures readily released chloride (82–100% within 5 days) verifying the rapid transformation and dechlorination of CB. No excessive accumulation of the metabolites 3CC or 2Cmuc was observed. By contrast, hypoxic cultivations showed strong differences in the metabolism of CB, depending on which strain initially dominated. Rapid intermediary 3CC accumulation (180 µM, 42 h) besides moderate formation of 2Cmuc was measured for the *P. veronii* surplus culture. The culture turned first pink, later black, indicating intracellular accumulation of toxic 3CC. CB was transformed incompletely within 10 days, and only 35% of the theoretically possible chloride was released.

By contrast, no accumulation of 3CC but a steadily increasing 2Cmuc concentration (maximum at the late stage of cultivation 430 µM, 160 h) indicated higher activity of CC12Os in the consortium initially dominated by *A. facilis*. CB was completely

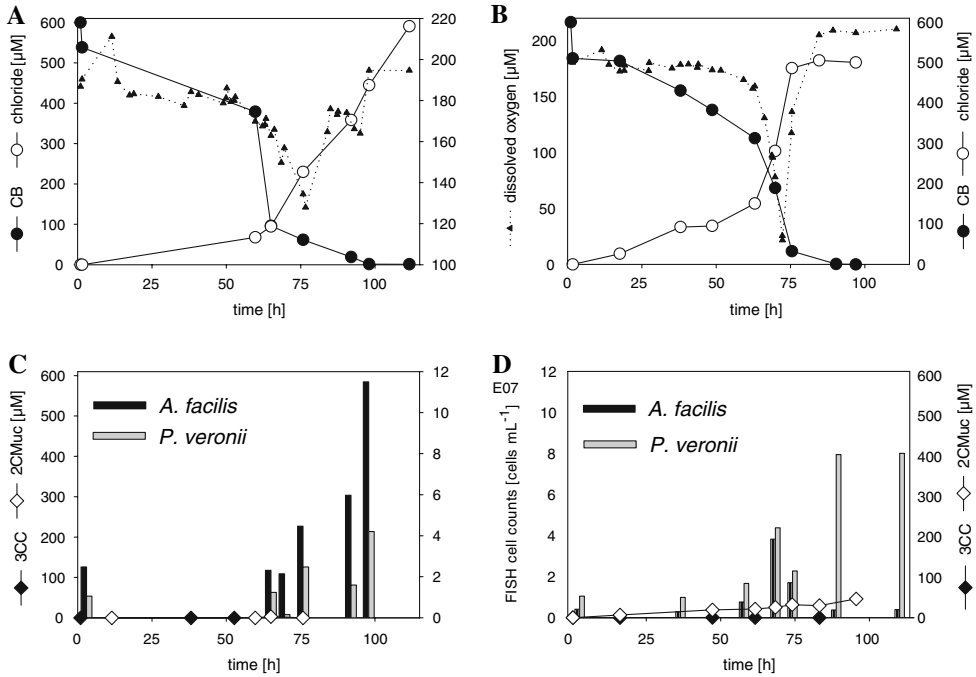


**Fig. 1** Oxic cultivation of binary mixed cultures A. facilis:P. veronii. Time courses of CB degradation, oxygen consumption and chloride release (A, B) and community compositions and metabolite release (C, D). Left hand side: starting with a surplus of A. facilis, right hand side: starting with a surplus of P. veronii

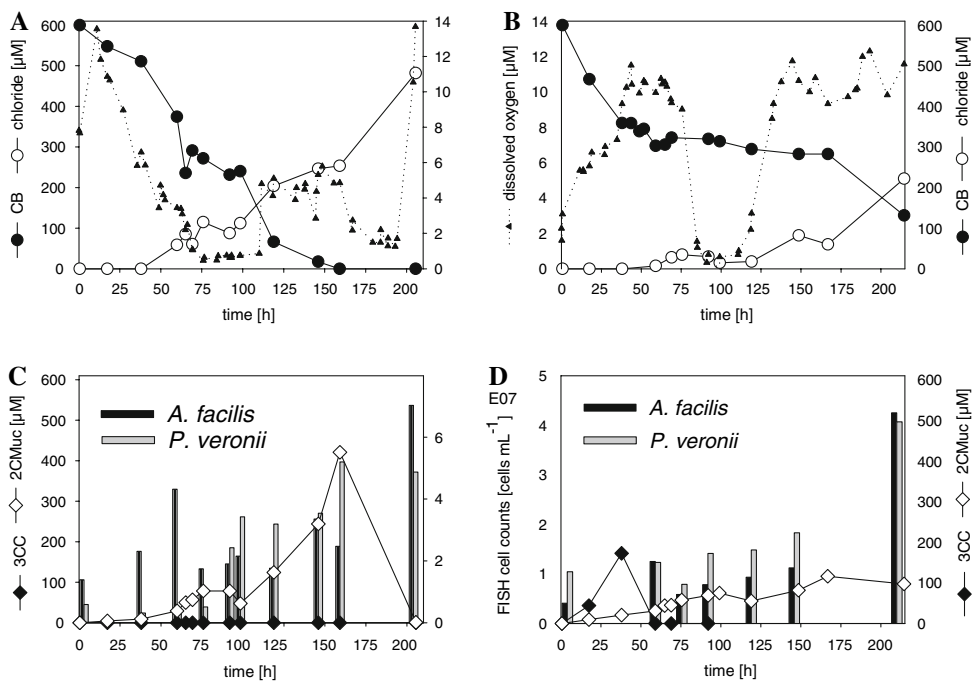


**Fig. 2** Hypoxic cultivation of binary mixed cultures A. facilis:P. veronii. Time courses of CB degradation, oxygen consumption and chloride release (A, B) and community compositions and metabolite release (C, D). Left hand side: starting with a surplus of A. facilis, right hand side: starting with a surplus of P. veronii

**Table 1** End point analysis of CB degradation, formation of extracellular metabolites and $Cl^-$ in a groundwater community and two binary mixtures cultivated under high and low oxygen concentrations

| Sample | $O_2$–Regime | CB[a] (%) | Degradation hours | $O_2$ consumption (mmol $l^{-1}$)[b] | $Cl^-$ release (%) | 3-Chloro-catechol | 2-Chloro-muconate |
|---|---|---|---|---|---|---|---|
| *P. veronii: A. facilis* 80:20 | Hypoxic | 73 | 216 | 1.2 | 35 | Yes | Yes |
| *A. facilis: P. veronii* 80:20 | Hypoxic | 100 | 163 | 1.9 | 72 | No | Yes |
| Groundwater community | Hypoxic | 100 | 204 | 1.9 | 75 | No | No |
| *P. veronii: A. facilis* 80:20 | Oxic | 100 | 84 | 3.9 | 82 | No | Yes[c] |
| *A. facilis: P. veronii* 80:20 | Oxic | 100 | 98 | 3.2 | 90 | No | No |
| Groundwater community | Oxic | 100 | 100 | 4.0 | 100 | No | No |

[a] 0.6 mM CB applied; [b]calculated from integral oxygen data pairs (headspace/aqueous phase) measured before and after oxygen gas spikes; [c]maximum ≤10 μM

transformed within 9 days, but the chloride release amounted to only 72%, showing a lower mineralization compared to oxic conditions. Incomplete dechlorination was also shown by chloromuconate excretion, which suggests a bottleneck in *A. facilis* chloromuconate cycloisomerase. Obviously, hypoxic conditions caused different accumulation of intermediary metabolites in both hypoxic cultures. Since chloride and metabolites were measured only in cell-free media, the fate of chloride or chlorinated metabolites, which were not released from the cells, remains unsolved. They are, however, anticipated to close the gap in the chloride mass balance. As for both strains the clc operon is involved (Balcke et al. submitted), lactonization of 2-chloromuconate might not lead to spontaneous elimination of chloride. Instead, 5-chloromuconolactone may accumulate (see Reineke 2001 for details). This intermediate also might account for the lack of chloride balance but was not considered. Also, further increase of chloride in the media would be expected as the experimental course had been prolonged.

## Composition of binary cultures under oxic conditions

After oxic cultivation (100 h, A. facilis surplus, 85 h, *P. veronii* surplus) the strain present at the beginning with the higher inoculum density was still dominant (Fig. 1C, D). The dissolved oxygen (DO) concentrations remained above 150 μM most of the time assuring full saturation of the CC12Os of both strains. Notably, as the dissolved oxygen level dropped to values <100 μM an intermediary increase in the abundance of *A. facilis* in batches initially dominated by *P. veronii* was detected via specific FISH counts (Fig. 1D). Thereafter (90 h), when CB as the substrate was no longer available cell numbers of *A. facilis* declined and only the *P. veronii* cell counts increased further to $8 \times 10^7$ cells $ml^{-1}$, thus, resulting in a dramatic change in the relative abundance (5%/95%). By contrast, in the *A. facilis* surplus culture cell numbers of *P. veronii* significantly increased. The high abundance (27% of all cells) is attributed to growth on 2CMuc, as this expected metabolite, observed for pure *A. facilis*, never accumulated (Balcke et al. submitted; Nestler et al. 2007).

## Composition of binary cultures under hypoxic conditions

Hypoxic cultivation conditions promoted competition between the strains for the available oxygen necessary to perform the dioxygenase reactions and respiration. More pronounced effects of accumulating metabolites promoting shifts in the community composition were expected. Indeed, depending on the starting ratio, the binary cultures exhibited different efficacy in the degradation of CB and accumulated 3-CC or 2CMuc to a different extent (Fig. 2).

As anticipated, preferential growth of *A. facilis* was observed from 0 to 68 h after inoculation with a surplus of *A. facilis* over *P. veronii*. Concomitantly the CB concentration decreased by 50%, about 15% chloride was released, and the DO concentration in the culture medium dropped from 11 to <1 μM (irrespective of the constant headspace oxygen concentration of 0.75%). As for hypoxic growth of pure *A. facilis*, only

accumulation of 2-chloromuconate (up to 100 μM) was noted (Balcke et al. submitted). During the following 2 days strong oxygen consumption prevailed. However, although the DO remained at levels below 1 μM further CB degradation, chloride release and 2CMuc accumulation stagnated. Interestingly, during this phase the community composition strongly shifted towards *P. veronii* being more abundant than *A. facilis* after 89 h, for unknown reasons. Between 100 and 120 h, paralleled by an increase of the DO concentration to 4–6 μM, metabolic activities and community composition changed again. CB degradation, chloride release and 2Cmuc accumulation reconstituted. After the complete disappearance of CB at about 160 h, intermediary accumulated 2Cmuc was transformed and more chloride released. During this phase the community composition shifted back towards dominance of *A. facilis*.

Starting with a predominance of *P. veronii*, the hypoxic conditions resulted in 3CC accumulation (intermediary release of up to 200 μM) (Fig. 2D) which presumably for more than 150 h confined further bacterial growth and oxygen demand. Transformation of 3CC dissolved in the culture medium after 60 h was associated with preferential growth of *A. facilis*. Higher steady state dissolved oxygen concentrations (10–12 μM), close to the half-saturation constant of *P. veronii* (Balcke et al. submitted), were observed after 150 h. As for pure *P. veronii* (Balcke et al. submitted) less than 40% of chloride was released upon an 80% conversion of CB. However, by contrast to the pure culture of *P. veronii*, accumulating 3CC was rapidly degraded with *A. facilis* present. This suggests *A. facilis* to suppress the level of toxic 3CC. Thus, by contrast to the hypothesis that only microorganisms with catechol dioxygenases favorable to function under oxygen limitation will grow under hypoxic conditions, syntrophic rather than competitive growth of both strains determines the composition of the binary mixture, as is reflected by an almost uniform composition of both strains after 9 days. Oxygen limited growth conditions require dominance of a microorganism with an active (chloro)catechol dioxygenase having a high affinity towards oxygen. This enables a complete conversion of CB in the groundwater system, as it avoids accumulation of chlorocatechol. Acidovorax species possess such high-affinity 3-CC dioxygenases (Nestler et al. 2007) and can therefore grow on CB over a wide range of oxygen tensions. However, incomplete dechlorination and chloromuconate excretion associated with the growth of *A. facilis* under oxygen limitation suggest that *P. veronii* could grow in the mixture on excreted metabolites such as 2CMuc irrespective of its inability to maintain a high transformation rate for 3-CC under oxygen limitation.

## Development of the groundwater community

Although not assigned in detail here, the high number of bands (>30) in our SSCP profiles (Fig. 3, lane 1) pointed to a high phylogenetic diversity in the original groundwater community as analyzed earlier by Alfreider et al. (2002) and Balcke et al. (2004).

In both cases, oxic as well as hypoxic cultivation resulted in a significant reduction in diversity relative to the composition of the original groundwater community. Upon growth on CB under limited oxygen availability the phylogenetic analyses of the community revealed high abundance of *Acidovoracae* (Fig. 3 and Table 2) with two bands in the DNA-based SSCP

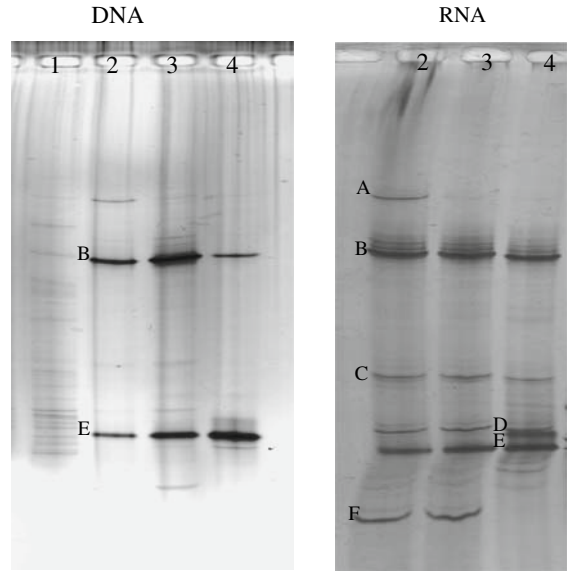


**Fig. 3** DNA and RNA (cDNA) based SSCP pattern of a groundwater community upon cultivation under different O2 pressures. Lane 1, concentrated groundwater community (inoculum); lane 2, pattern after hypoxic cultivation; lane 4, pattern after oxic cultivation. (Lane 3 is from a culture with an oxygen concentration between the oxic and hypoxic level not discussed here)

**Table 2** Results of the identification of SSCP bands by sequence analysis and correlation with public data bases

| Band | Nearest relative | Taxonomy | Accession number | BLAST | RDP |
|---|---|---|---|---|---|
| A | *Acidovorax* sp. *7078* | β-Proteobacteria | AF078767 | 99 | 0.980 |
| B | *Acidovorax* sp. *7078* | β-Proteobacteria | AF078767 | 100 | 1,000 |
| C | *Acidovorax* sp. *7078* | β-Proteobacteria | AF078767 | 100 | 1,000 |
| D | *Flavobacterium* sp. | Flavobacteria | AF433173 | 99 | 0.949 |
| E* | *A. facilis UFZ B530* | β- Proteobacteria | AF235013 | 99 | 0.980 |
| | *P. veronii UFZ B549* | γ- Proteobacteria | AF539745 | 99 | 0.969 |
| | *Pseudomonas* sp. | γ- Proteobacteria | AF134704 | 99 | 0.953 |
| | *Acidovorax* sp. *7078* | β- Proteobacteria | AF078767 | 99 | 0.955 |
| | *Stenotrophomonas* sp. | γ-Proteobacteria | AJ002814 | 99 | 0.977 |
| | *Uncultured Bacterium* | β-Proteobacteria | AY444982 | 99 | 0.932 |
| | *P. veronii UFZ B549* | γ-Proteobacteria | AF539745 | 99 | 0.965 |
| | *Pseudomonas* sp. | γ-Proteobacteria | AY748440 | 92 | 0.523 |
| | *Uncult(γ-Proteobacterium* | γ-Proteobacteria | AY395373 | 99 | 0.967 |
| F | *Acidovorax* sp. *7078* | β-Proteobacteria | AF078767 | 99 | 0.987 |

*Mixed band: result only after cloning

gels (Fig. 3, lanes 4) after oxic cultivation and three (DNA-based) to four bands (RNA-based) (Fig. 3, lanes 2) after hypoxic cultivation. Besides the occurrence of several strains of this genus, additional bands may also be attributed to different rDNA operons in a single host. We also detected sequences closely related to a gram-negative, non-spore forming *Flavobacterium* (Fig. 3, band D and Table 2), clearly seen in the RNA-based pattern. The band E was found to be a mixture comprised of DNA or rRNA of several strains, among these *A. facilis* B530 and γ-Proteobacteria closely related to *P. veronii* B549 (Table 2). As separation by SSCP is based on the folding of a linearized single strand of DNA, a disadvantage of this method is that occasionally a single strand may fold into more than only one conformation. These conformational isomers may result in more than one band per DNA sequence in a community profile and/or in overlapping bands with different sequences but similar conformation.

## Community structure based on quantitative quinone profiles

The aerobically grown pre-cultures of *P. veronii* and *A. facilis* contained either ubiquinone-9 (UQ-9) or ubiquinone-8 (UQ-8) as the dominant electron carrier molecules, characteristic for the γ-proteobacteria *Pseudomonas* (Collins 1985) and the β-proteobacteria *Acidovorax* (Busse et al. 1992), respectively. The relative abundances of UQ-9 and UQ-8 in the mixed cultures were in accordance with the ratio of *Pseudomonas* and *Acidovorax* determined by specific cell counts based on group specific FISH probes at the end of the experiments. The ratio of UQ-9 to UQ-8 showed a clear dominance of *P. veronii* under oxic conditions, whereas growth of *A. facilis* was favored under hypoxic conditions (Table 3).

Unexpected were the significant amounts of menaquinones (MK-7, MK-10 and DMK-8) produced under hypoxic conditions in the culture dominated by *P. veronii*. The occurrence of MK-7 has been reported for one other *Pseudomonas* strain (Rutherford et al. 1991), but not for *P. veronii*, while the occurrence of MK-10 or DMK-8 was not described before, to the best of our knowledge. Presence of MK and DMK is generally attributed to respiratory processes occurring under reduced redox conditions. As oxygen is limiting, slight reduction in the redox potential would seem reasonable. However, the type of respiration activated remains elusive, since only sulphate as potential alternative electron acceptor was present in the media, but hydrogen sulphide formation was not noted (by organoleptic tests after acidification).

Remarkably, the total microbial biomass (quinone content) for *A. facilis* under hypoxic conditions was comparable to that of *P. veronii* under oxic conditions

**Table 3** Relative distribution of respiratory quinones in artificial mixtures of *Pseudomonas veronii* UFZ B549/*Acidovorax facilis* UFZ B530 starting at a estimated ratio of 20:80 and 80:20, followed by cultivation under oxic and hypoxic culture conditions. Quinone profiles were acquired at the beginning and at the end of CB degradation. The enriched groundwater community from the Bitterfeld aquifer was cultivated under the same oxic and hypoxic conditions with CB as the sole source of carbon and energy

| | Ubiquinones(%) | | | | | Menaquinones (%) | | | | | | | UQ & MK pmol flask$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | UQ6 | UQ7 | **UQ8** | **UQ9** | UQ10 | MK4 | MK5 | MK6 | MK7 | MK8 | MK9 | MK10 | DMK8 | |
| 80% *P. veronii* $t_0$ | 1.3 | 3.5 | **15.0** | **74.5** | 1.9 | 0.1 | 0.6 | 0.6 | 0.6 | 0.6 | 0.4 | 0.6 | 0.3 | **869** |
| 80 % *A. facilis* $t_0$ | 0.6 | 3.7 | **86.4** | **4.2** | 0.3 | 0.1 | 0.5 | 0.8 | 0.8 | 0.9 | 0.5 | 0.8 | 0.4 | **1,123** |
| GW community $t_0$ | 0.6 | 2.0 | **18.8** | **9.4** | 5.3 | 1.6 | 4.0 | 7.1 | 11.7 | **25.2** | 6.1 | 4.4 | 3.8 | **75** |
| 80% *P. veronii* oxic | 1.1 | 2.7 | **12.5** | **80.0** | 2.3 | 0.0 | 0.1 | 0.2 | 0.3 | 0.3 | 0.1 | 0.2 | 0.1 | **13,939** |
| 80 % *A. facilis* oxic | 0.6 | 2.1 | **65.1** | **23.9** | 0.9 | 0.1 | 0.3 | 0.5 | 2.3 | 0.8 | 1.8 | 1.1 | 0.5 | **5,107** |
| GW community oxic | 0.7 | 2.4 | **24.7** | **60.8** | 2.0 | 0.1 | 0.2 | 0.6 | 2.4 | 1.7 | 2.4 | 1.3 | 0.5 | **7,931** |
| 80% *P. veronii* hypoxic | 0.5 | 1.2 | **8.8** | **28.0** | 2.8 | 0.7 | 8.4 | 7.9 | **16.5** | 3.0 | 2.2 | **14.0** | **5.8** | **5,220** |
| 80 % *A. facilis* hypoxic | 0.8 | 2.2 | **67.4** | **25.3** | 0.4 | 0.1 | 0.3 | 1.4 | 0.7 | 0.4 | 0.3 | 0.5 | 0.3 | **17,543** |
| GW community hypoxic | 0.5 | 2.6 | **52.5** | **15.3** | 1.5 | 0.5 | 2.4 | 7.8 | 6.5 | 2.9 | 0.8 | 4.4 | 2.4 | **9,299** |

(Table 3). This indicates an efficient metabolic transformation of CB by *A. facilis*/*P.veronii* even at very low dissolved oxygen concentrations (<0.4 μM). The ratios UQ-8 to UQ-9 and UQs/MKs clearly indicated aerobic respiration for Acidovorax even under hypoxic conditions.

The quinone profiles of the groundwater community were similar to profiles of the respective binary culture favored under either oxic or hypoxic conditions (Table 3). Based on the quantitative quinone data and the SSCP profiles, the groundwater community shifted to *Pseudomonas*-related species (high content of UQ-9) under oxic conditions. An increased abundance of UQ8 under hypoxic conditions indicated that an *Acidovorax* species dominated the community. The relative abundance of the *Flavobacterium* in the GW community could be estimated from the quinone profiles as well. *Flavobacterium* usually contains menaquinone-6 (MK-6) as the major respiratory quinone (Wang et al. 2006), although other quinone species have also been reported (WFCC-MIRCEN 2004). *Flavobacterium* spp. degraded a variety of chlorinated compounds according to various reports summarized by Saber and Crawford (1985). Whether these species contributed to the effective conversion of CB directly or use intermediates for growth needs to be investigated further. As in the binary cultures, high UQ/MK ratios clearly indicated aerobic respiration dominating in the GW community also under hypoxic conditions. This indicates, together with the SSCP profiles, that *Acidovorax* and *Pseudomonas* species are indeed key organisms in the GW community enriched from the Bitterfeld aquifer. The GW community responds to the applied oxygen levels in a similar manner as *A. facilis* and *P. veronii* formerly isolated from the same aquifer, proving the feasibility of the binary cultures as model systems. The function of the *Flavobacterium* sp. also abundant in the GW community remains unknown. Quinone profiles and SSCP patterns both indicate that under conditions of oxygen limitation, *Acidovorax* spp. are more effective CB degraders in the groundwater community.

## Conclusions

Under hypoxic conditions the speed of CB degradation may be reduced in a microbial community

(binary culture or more complex) but can otherwise be almost as effective as under strictly oxic conditions. We conclude that in binary cultures under low oxygen concentrations, *A. facilis* plays a key role in 3CC conversion, as the potential metabolite was not detectable throughout the course of the experiments with *A. facilis* being highly abundant. This finding parallels the identification of a high-affinity type catechol 1,2-dioxygenase towards oxygen (Balcke et al. submitted). Differently from our hypothesis, also *P. veronii* (with low-affinity type CC12O) sustained enduring hypoxia and integrated into CB biodegradation by growth on chloromuconate, which was accumulated and partly released by *A. facilis*. These different metabolic preferences may be the major reason for the community shifts observed under hypoxic conditions and may explain why growth of *P. veronii* on CB is observed under severe oxygen limitation, although its enzyme characteristics and pure culture experiments would not imply such behavior.

Under oxic conditions the community composition is mainly determined by strain-specific maximum growth rates, in this case clearly promoting the *Pseudomonas* (Balcke et al. submitted). Comparing a complex groundwater community with the binary mixtures very similar adaptation towards a dominance of *Acidovoracae* besides a lower share of $\gamma$-Proteobacteria under hypoxic conditions and preferential growth of a limited number $\gamma$-Proteobacteria and *Bacteriodetes* under oxic conditions is noted.

The availability of dissolved oxygen is limited in almost all natural subsurface environments. Hence, focussing on the support of hypoxic degradation may be the most suitable strategy for enhanced natural attenuation approaches in CB-contaminated aquifers like Bitterfeld. With a dominant fraction of hypoxic Acidovorax spp., CB can be degraded efficiently at oxygen concentrations as low as 1 μM omitting the accumulation of toxic 3CC. As under oxygen limitation 3CC is accumulated by microorganisms that prevail at high oxygen availability (Vogt et al. 2004), remediation approaches should be adapted or developed that provide consistent hypoxic conditions.

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