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Dechlorination of Chlorinated Benzenes by an Anaerobic Microbial Consortium That Selectively Mediates the Thermodynamic Most Favorable Reactions

Jacobus E. M. Beurskens,* Connie G. C. Dekker, Henny van den Heuvel, Martien Swart, and Johan de Wolf

Institute for Inland Water Management and Waste Water Treatment (RIZA), P.O. Box 17, NL-8200 AA Lelystad, The Netherlands

Jan Dolfing

Research Institute for Agrobiology and Soil Fertility (AB-DLO), P.O. Box 129, NL-9750 AC Haren, The Netherlands

A chlorinated benzene dechlorinating anaerobic microbial consortium was obtained by selective enrichment with hexachlorobenzene (HCB) and lactate from a freshwater sediment sample that originated from an area with proven in situ HCB dechlorination. The consortium was used to determine compound selectivity and relative dechlorination rates by incubation with the individual chlorinated benzenes under methanogenic conditions. The dechlorinating activity was restricted to benzenes with at least three adjacent chlorines, except for a relative slow transformation of 1,2,4,5-tetrachlorobenzene to 1,2,4-trichlorobenzene. Optimal temperature for dechlorination was around 30 °C, significant dechlorinating activity was still observed at a temperature of 3 °C. The selectivity of the enrichment culture showed an interesting correlation with the thermodynamics of the various dechlorination steps: from the 19 dechlorination reactions possible with benzenes that contain at least two chlorines, only the seven reactions with the highest energy release took place.

Introduction

Chlorinated benzenes (CBs) can enter the aquatic environment as solvents, pesticides, dielectric fluids, deodorants, and chemical contaminants or intermediates. They are prevalent in both solid and liquid industrial effluents and in atmospheric discharges. As a result of their widespread use during several decades, CBs have become ubiquitous in the aquatic environment; they have been detected in water, sediments, and aquatic biota (*1*, *2*).

Elimination of CBs from the aquatic environment may be caused by volatilization, photodegradation, and biodegradation. Volatilization seems to be a major loss mechanism for mono- (MCB) and dichlorobenzenes (DCBs) (*3*, *4*). Photochemical transformation of some CBs has been demonstrated (*5*, *6*). Microbial mineralization of the lower chlorinated benzenes in the aerobic water column may occur (*7–14*), although reported degradation rates are slow in surface waters (*15*). Due to the incompleteness of the above-mentioned removal processes and the relative hydrophobic character of CBs, a substantial amount ends up in sediments.

A long-term persistence of CBs in anaerobic sediments was assumed until reductive dechlorination of hexachlorobenzene (HCB) was first reported in 1987 (*16*). Since then, laboratory studies have demonstrated the microbial dechlorination of any CB that contains at least two chlorines, resulting in the accumulation of less chlorinated isomers (*17–19*). Calculated half-lifetimes of HCB dechlorination, based on laboratory studies (*17*, *20*), range between a few days to several weeks.

In situ reductive dechlorination of CBs in sediments was subject to speculation in the past (*21–23*). Only recently, dechlorination of HCB in natural sediments was demonstrated unequivocally (*24*). Comparison of HCB concentrations in recently collected core layers deposited around 1970 with 20-year-old stored sediment top layers that reflected the original input revealed an 80% loss of HCB and increases in 1,3,5-trichlorobenzene (1,3,5-TCB) and 1,3-dichlorobenzene (1,3-DCB) during the 20 years of "environmental incubation". Laboratory incubations with sediment from the same area (Lake Ketelmeer, a sedimentation area of the Rhine River (western Europe)) demonstrated that the native anaerobic microbial population was capable of catalyzing this reaction. On the basis of the sediment core data, a maximum half-life of HCB in the anaerobic sediment was estimated to be 7 years (*24*). Two major differences between laboratory studies and field observations can be derived. First, the compound spectrum subject to dechlorination in the environment is not as broad as observed in laboratory studies. Laboratory studies indicate the possibility of a sequential dechlorination leading to the accumulation of MCB as the only end product (*17–19*), instead of 1,3,5-TCB and 1,3-DCB accumulation as observed in the environment. Second, dechlorination rates in the environment seem to be rather slow.

The objective of the present study was to determine compound selectivity, relative dechlorination rates of the different CBs, and the influence of temperature on the dechlorination rate carried out by an anaerobic microbial consortium from Lake Ketelmeer sediment where *in situ* dechlorination was observed. In order to make experiments less time-consuming and simplify analytical procedures, an anaerobic enrichment culture was obtained from Lake Ketelmeer sediment, and all experiments were carried out in liquid media without sediment.

Experimental Section

Chemicals. HCB and pentachlorobenzene (QCB) were obtained from Aldrich Chemie N. V. (Brussels, Belgium). 1,2,3,4-Tetrachlorobenzene (1,2,3,4-TeCB), 1,2,4,5-TeCB, and all three DCBs were purchased from Janssen Chimica (Beerse, Belgium). All trichlorobenzenes (TCBs) were obtained from Merck (Amsterdam, The Netherlands); 1,2,3,5-TeCB was obtained from Promochem (Wesel,

* Address correspondence to this author at his present address: RIVM-LWD, P.O. Box 1, NL-3720 BA Bilthoven, The Netherlands.

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Germany). Gases were from Hoekloos (Schiedam, The Netherlands).

Source and Preparation of the Inoculum. CB-contaminated sediment was collected from Lake Ketelmeer, a shallow freshwater lake in the central part of The Netherlands. The lake acts as a sedimentation area for the suspended solids from the Rhine River. Microbial dechlorination of HCB in sediment slurries, containing 30% sediment (wt/wt, on a dry weight basis), from Lake Ketelmeer has been demonstrated earlier (*24*). With one of these sediment slurries, an enrichment procedure was started by shaking the slurry and taking a 80-mL sample to inoculate a 1-L serum bottle with 720 mL of anaerobic medium (see below), containing lactate (17.5 mM) and HCB (70 nM, added as a methanolic solution). The transfer of 80-mL samples into 720 mL of fresh medium was repeated eight times after completion of the HCB dechlorination in order to get a highly active microbial consortium in the absence of sediment. In order to maintain a highly active culture for the inoculation of several experiments, repeated additions of HCB (approximately 100 nM) immediately after depletion were carried out, up to a maximum of six times in a single bottle. Methane was produced in these bottles, confirming the methanogenic conditions, but was not proportional to HCB dechlorination rates (data not shown).

Anaerobic Medium. The phosphate-buffered mineral medium (pH 7.0) was prepared after Holliger et al. (*19*) with a small modification, 0.50 g/L instead of 0.24 g/L $Na_2S{\cdot}9H_2O$. The headspaces were flushed with O_2-free N_2 and sealed with Viton stoppers (Eriks b.v., Alkmaar, The Netherlands) before autoclaving. Filter-sterilized stock solutions of trace elements (*25*) and vitamins (*19*) were added, both 1 mL/L, to the autoclaved mineral medium.

Incubation Methods. All experiments were carried out in 1-L serum bottles, containing 800 mL of the methanogenic medium. The general incubation protocol consisted of three phases: (A) Addition of sodium lactate (2 mL of 60% syrup; initial concentration in medium, 17.5 mM), HCB dissolved in methanol (200 μL; initial HCB concentration in medium, approximately 70 nM), and 50 mL of inoculum. In this phase, encompassing a period of about 3 weeks, growth of organisms took place and HCB dechlorination was generally slow. (B) When the concentration of HCB fell below 4 nM, a second amount of HCB was added. This amount was readily dechlorinated as a result of increased cell densities. This phase was used to characterize rate differences between bottles. (C) The actual experimental phase started when the concentration of HCB again fell below 4 nM. CBs were added to determine activity toward chlorobenzenes other than HCB, or bottles were given HCB and incubated at different temperatures in order to determine temperature influences. All tests consisted of singular incubations for the individual CBs or for HCB at different temperatures.

Sterile controls were prepared by adding formalin (final concentration 4%) to the bottles at the end of phase B, prior to the third CB addition. All incubations were in the dark at 25 °C, unless otherwise stated.

Incubations with the 11 individual CBs (monochlorobenzene was excluded) to determine compound selectivity, dechlorination pathways, and rate constants followed the general protocol as described above. However, the added amounts of CBs in phase C were elevated to obtain an initial concentration of approximately 200 nM. The dechlorination rate of HCB was determined in phase B, and the dechlorination rate of the specific test compound was determined in phase C. Subsequently, the rate of the test compound was standardized with the aid of the HCB dechlorination rate in phase B. Dechlorination rate constants and half-lifetimes were determined by nonlinear regression analyses of CB disappearance curves assuming first-order kinetics.

Chemical Analysis. At intervals, singular 10-mL samples were removed aseptically from each active and control incubation while swirling the medium to ensure a uniform suspension. The samples were combined with 2 mL of isooctane and shaken for 2 h. Separation of water and the isooctane fraction was obtained by mixing on a vortex mixer and subsequent storage for at least 2 h at −20 °C. The extraction efficiency was highly reproducible and ranged between 55 and 66% for the individual isomers. Samples of the isooctane fraction were analyzed with a HP 5890A gas chromatograph, equipped with a ^{63}Ni electron capture detector using automated splitless injection (HP 7673A). Compound separation was accomplished with a fused silica wall coated open tubular capillary column (50 m × 0.25 mm i.d.) CP Sil 19CB (0.20 μm film thickness of dimethyl- (86%), phenyl- (7%), cyanopropyl- (7%)silicone polymer; Chrompack, Bergen op Zoom, The Netherlands). Helium was used as a carrier gas, and nitrogen was used as a detector-quench gas. The operating temperatures of the injector and detector were 225 and 300 °C, respectively. The oven temperature program was as follows: 90 °C initially for 2 min, increase to 140 °C at 2 °C/min with a 5 min isothermal period at 140 °C before temperature increased to 225 °C at 5 °C/min, with an isothermal period of 90 min at the end. Retention times and peak areas were determined by using the HP 3365 ChemStation software. Peaks were identified and quantified by comparing injections with authentic external standards prepared in isooctane.

Results and Discussion

Repeated Additions of HCB. The maintenance of dechlorinating activity during the successive additions of CBs to the same bottles was verified in order to demonstrate the suitability of the selected experimental procedure. For that purpose, the dechlorination kinetics were determined for six consecutive HCB additions to a single bottle, with only one lactate addition at the beginning of the experiment. The first amount of HCB disappeared slowly, the subsequent additions of HCB disappeared rapidly (Figure 1). Disappearance of HCB coincided with an accumulation of lower chlorinated benzenes (see below). A sterile control showed neither any significant loss of HCB nor any formation of lower chlorinated benzenes. This indicates that the disappearance of HCB is mediated by an anaerobic microbial consortium that carried out a dechlorination reaction. After growth of the dechlorinating population in phase A, a stable consortium seemed to be active, and a constant dechlorination capacity was observed during several successive additions. The dechlorination rate constants for periods B–F were rather constant: the mean dechlorination rate constant (k) was 0.071 h^{-1} ± 0.015 (standard deviation). The coefficient of variance was 21%, which is acceptable for a repeated reaction in a batch system.

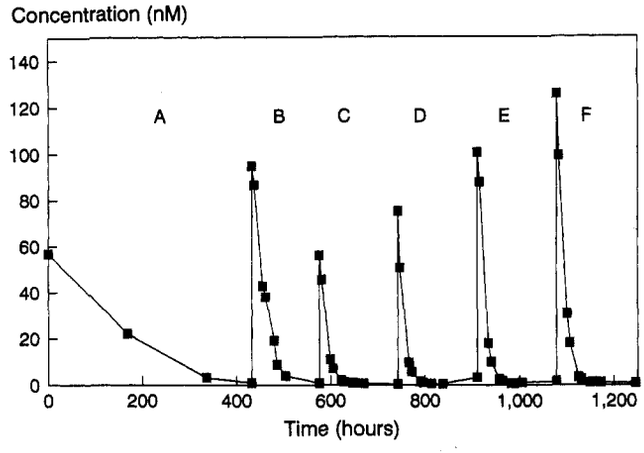

Figure 1. Repeated additions of HCB to a single serum bottle with subsequent removal by the enrichment culture.

Repeated lactate additions combined with HCB additions in each period lowered the successive dechlorination rates, resulting in a k value of 0.030 h^{-1} in period F (results not shown). Therefore, lactate was added only once at the start of all following experiments.

A substantial amount of methanol, yielding an initial concentration of 6 mM, was introduced with each addition of HCB, since HCB was dissolved in methanol. Methanol may serve as a source of reducing equivalents, carbon, and energy for the dechlorinating consortium in these incubations. Stimulation of dechlorinating activity by methanol has been reported for PCBs (*26*).

Dechlorination of the Individual Compounds. Sterile controls with individual compounds did not show any significant loss of the test compounds or any product formation. Therefore, transformations in the biologically active incubations can be attributed to microbial activity. The transformation of HCB and resulting product formation is shown in Figure 2A. The initial concentrations of 1,2,4- and 1,3,5-TCB were approximately 40 nM in period C as a result of HCB dechlorination in the preceding periods A and B. The transformation of HCB resulted in a transient accumulation of QCB, 1,2,3,5-, and 1,2,4,5-TeCB. After 380 h of incubation, a substantial amount of 1,2,4,5-TeCB was still present. The subsequent transformation of 1,2,4,5-TeCB was less rapid than the transformation of 1,2,3,5-TeCB. 1,3,5- and 1,2,4-TCB accumulated further within the experimental period. At the end of the experiment, 87% of the loss in HCB was recovered as lower chlorinated benzenes.

The incubation with QCB showed a rapid dechlorination to 1,2,3,5- and 1,2,4,5-TeCB (Figure 2B). Again 1,2,4,5-TeCB disappeared more slowly than 1,2,3,5-TeCB. The 1,3,5- and 1,2,4-TCB accumulated in a ratio of 60:40 after 380 h of incubation. The contribution of 1,2,4-TCB as an end product is clearly higher when produced from QCB than from HCB. About 75% of the parent QCB was recovered as lower chlorinated benzenes at the end of the experiment.

The incubation of 1,2,3,4-TeCB showed an exclusive transformation into 1,2,4-TCB (78% recovery) that was resistent to further dechlorination (Figure 2C).

The incubation of 1,2,3,5-TeCB resulted in a rapid accumulation of 1,3,5-TCB (Figure 2D). The amount of 1,2,4-TCB seemed to increase slightly during the experiment, but this increase was insufficient to support a clear conclusion regarding the dechlorination of 1,2,3,5-TeCB

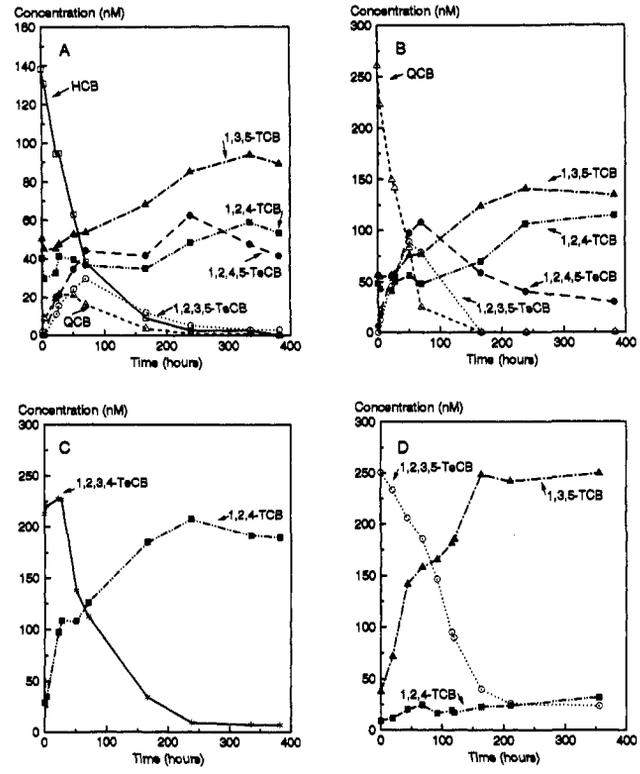

Figure 2. Dechlorination of hexachlorobenzene (A), pentachlorobenzene (B), 1,2,3,4-tetrachlorobenzene (C), and 1,2,3,5-tetrachlorobenzene (D) by the enrichment culture.

to 1,2,4-TCB. At the end of the experiment, 105% of the loss in 1,2,3,5-TeCB was recoverd as lower chlorinated benzenes.

The incubations with 1,2,4,5-TeCB, all TCBs, and DCBs did not show any substantial substrate disappearance or any product formation within the experimental period of 380 h. An extended incubation period only showed a slow dechlorination of 1,2,3-TCB. After a lag phase of about 1 month, 1,3-DCB was produced. The lack of mass balance in three incubations that showed reactivity does not dramatically exceed the variation in HCB quantitation in sterile controls (≤15%). Formation of DCBs can not explain the lack of mass balance, since detection limits for these compounds were 2.3, 2.3, and 4 nM for 1,3-, 1,4-, and 1,2-DCB, respectively.

For parent compounds that showed an instantaneous reactivity (HCB, QCB, 1,2,3,4-, and 1,2,3,5-TeCB) half-lifetimes did not differ significantly and were in the range of 35–63 h (data not shown), indicating a high and similar affinity of the consortium for these compounds.

A striking phenomenon was observed with 1,2,4,5-TeCB. If this compound was produced from QCB, it was clearly dechlorinated (Figure 2B), while 1,2,4,5-TeCB added as the parent compound resisted dechlorination. Thus, there is a difference in dechlorination reactivity between compounds that are formed as an intermediate during the dechlorination and compounds that are present as a parent substrate.

The observed dechlorination pathways are summarized in Figure 3. HCB and QCB were dechlorinated via 1,2,3,5- and 1,2,4,5-TeCB and as final dechlorination products 1,3,5- and 1,2,4-TCB accumulated. The latter contributed at least 30% to the total amount of TCB products. The ratio between these two products deviates from earlier reports, where 1,2,4-TCB did not exceed the 10% level

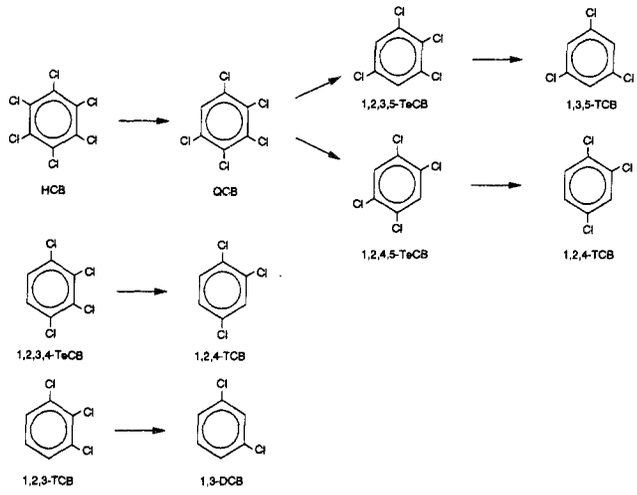

Figure 3. Reductive dechlorination pathways of hexachlorobenzene, 1,2,3,4-tetrachlorobenzene, and 1,2,3-trichlorobenzene catalyzed by the anaerobic enrichment culture.

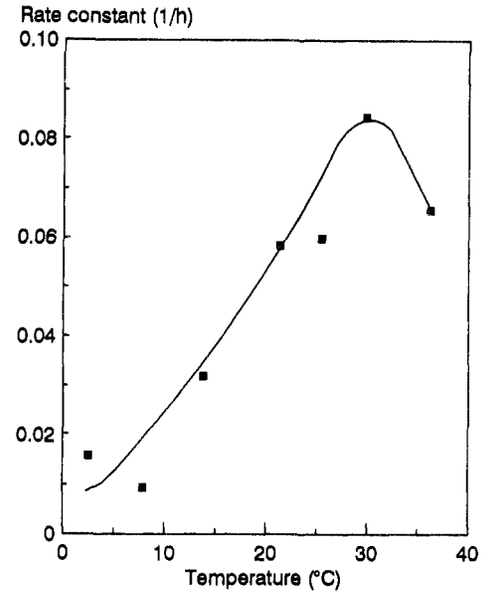

Figure 4. Temperature dependence of relative dechlorination rate of hexachlorobenzene by the enrichment culture. Rate constants were calculated for HCB disappearance curves at the selected temperatures. The curves consisted of at least six measurements (correlation coefficients (r^2) ≥0.99).

(*17, 19*). In the HCB and QCB incubations, 1,2,3,4-TeCB was not detected as an intermediate, in agreement with the results of Fathepure et al. (*17*) and Holliger et al. (*19*). In incubations with Lake Ketelmeer sediment, HCB dechlorination resulted in an accumulation of 1,3-DCB (*24*). That same sediment served as the source of the enrichment culture used in the present study. During the enrichment procedure somehow the ability to continue dechlorination of HCB to the DCB level was lost. From the dechlorination pathways of HCB, 1,2,3,4-TeCB, and 1,2,3-TCB, it becomes clear that the enrichment culture from Lake Ketelmeer sediment preferentially removes the chlorine that is surrounded by chlorines on both sides. Consequently, the dechlorinating activity of this enrichment culture seems to be restricted to benzenes with at least three adjacent chlorines. The slow transformation of 1,2,4,5-TeCB into 1,2,4-TCB is the only exception.

Influence of Temperature. The relationship between temperature and the rate of HCB dechlorination is shown in Figure 4. Possible differences between bottles due to differences in cell densities or activities were taken into account by determing HCB dechlorination rates in phase B at an identical temperature for all bottles (25 °C). This enabled us to calculate normalized rate constants for the HCB dechlorination in phase C at different temperatures. An optimum was observed around 30 °C, with a half-life of 8 h. At temperatures relevant for field conditions during the summer, 8–15 °C, dechlorination rates were 2–6 times below the rates at temperatures generally applied in laboratory research (20–25 °C). Holliger et al. (*19*) found a similar optimum between 25 and 30 °C for the dechlorination of 1,2,3-TCB. These authors, however, did not detect dechlorinating activity below 10 °C. We still observed significant dechlorination at 3 °C. This is relevant because such low temperatures occur during the winter months in Rhine sediments.

Relationship with Thermodynamics of Dechlorination Reactions. It is well-established that chlorinated aromatics can serve as electron acceptors in anaerobic environments and that dechlorinating organisms may conserve energy from this reaction (*18, 19, 27, 28*), as demonstrated for *Desulfomonile tiedjei* (*29*). Holliger et al. (*19*) indicated that for their mixed culture, capable to dechlorinate 1,2,3-TCB, H_2 served as the source of reducing equivalents. Although our incubations started with N_2 as headspace gas, fermentation of lactate in the medium apparently resulted in a sufficient H_2 production to serve as the source of reducing equivalents. Holliger et al. (*19*) demonstrated that 1,2,3-TCB dechlorination under N_2 headspace was obtained in the presence of lactate. Estimates for the Gibbs free energies of CB redox couples with H_2 as the electron donor have recently been reported (*27*) and are listed in decreasing order in Table 1. The dechlorinations observed with the HCB-grown enrichment culture from Lake Ketelmeer sediment are also indicated in Table 1. The seemingly arbitrariness of the selectivity in the catalyzed dechlorination reactions turns out to have certain systematics; from the 19 possible dechlorination reactions with benzenes that contain at least two chlorines,

Table 1. Gibbs Free-Energy Values ($\Delta G^{\circ\prime}$) for Chlorobenzene Dechlorination Reactions with Hydrogen as Electron Donor (Data from Ref 27) and the Presence (+) or Absence (–) of This Reaction in Anaerobic Incubations with Enrichment Culture

$\Delta G^{\circ\prime}$ [a] (kJ/reaction)	parent compd	product	dechlorination
−171.4	HCB	QCB	+
−167.7	QCB	1,2,3,5-TeCB	+
−166.5	1,2,3,4-TeCB	1,2,4-TCB	+
−164.3	1,2,4,5-TeCB	1,2,4-TCB	+
−163.5	1,2,3,5-TeCB	1,3,5-TCB	+
−163.4	QCB	1,2,4,5-TeCB	+
−161.2	1,2,3-TCB	1,3-DCB	+
−161.1	QCB	1,2,3,4-TeCB	–
−159.9	1,2,3,5-TeCB	1,2,4-TCB	–
−158.6	1,2,3-TCB	1,2-DCB	–
−155.2	1,2,3,4-TeCB	1,2,3-TCB	–
−153.4	1,2,4-TCB	1,4-DCB	–
−153.2	1,2-DCB	MCB	–
−150.6	1,3-DCB	MCB	–
−149.9	1,2,4-TCB	1,3-DCB	–
−148.6	1,2,3,5-TeCB	1,2,3-TCB	–
−147.3	1,2,4-TCB	1,2-DCB	–
−147.1	1,4-DCB	MCB	–
−146.2	1,3,5-TCB	1,3-DCB	–

[a] Values under standard conditions, pH = 7, 25 °C; H_2 in the gaseous state at a partial pressure of 100 kPa; all other compounds in aqueous solution at 1 mol/kg activity.

only the seven reactions with the highest energy release under standard conditions took place. The energy release for those dechlorination steps under standard conditions is more than 161.1 kJ per reaction. There is no immediate explanation for this observation. The energy release of those steps that are not catalyzed by the enrichment are highly exergonic too, and some of them are indeed catalyzed by other CB-dechlorinating consortia (*17, 18, 30*).

The Gibbs free-energy values for chlorobenzene dechlorination in Table 1 are for standard conditions. Under the actual conditions of the consortium, however, concentrations of substrates and products were much lower than the assumed 1 mol/kg under standard conditions and even changed during the experiment. Nevertheless, our observations are consistent with the theory behind the Gibbs free-energy of formation values. These values represent potential energy present in a compound. Location of chlorine substituents in close proximity to each other is energetically unfavorable, and it will be energetically most favorable to remove those substituents first that are close to each other. This is indeed what was observed. By enriching a consortium on HCB, we have enriched for organisms/enzymes that are tailored to removing chlorine substituents in close proximity to other chlorine substituents from benzene rings with multiple adjacent chlorine substituents.

Absence of dechlorinating capabilities toward TCBs and DCBs in our enrichment may be caused by factors like, for example, the source of inoculum or the enrichment technique that consisted of repeated HCB additions. A closer look to all published CBs dechlorination studies may reveal clues as to why HCB dechlorination generally results in TCB accumulation (*16, 17, 19, 24*) instead of MCB accumulation as recently reported by Ramanand et al. (*30*). HCB dechlorination studies that result in an accumulation of 1,3,5-TCB as the main end product were conducted with HCB (*16, 17, 24,* this study) or 1,2,3-TCB (*19*) in the enrichment procedure. Selecting the energetic most profitable reactions (Table 1) HCB dechlorination proceeds via QCB, 1,2,3,5-TeCB, to 1,3,5-TCB. Dechlorination of 1,3,5-TCB to 1,3-DCB is the least attractive step of all reactions listed in Table 1. Dechlorination of 1,3,5-TCB has been demonstrated (*18*), but started only after a lag of 6 months and was preceded by dechlorination of 1,2,3-TCB and 1,2,4-TCB present in the same column. This sequence is in agreement with the order of TCB dechlorination steps in Table 1 and illustrates the recalcitrance of 1,3,5-TCB. Ramanand et al. (*30*) used a culture acclimatized to 1,2,4-TCB to study the dechlorination of HCB. The 1,2,4-TCB was dechlorinated to MCB mainly via 1,4-DCB and in a lesser extent via 1,3-DCB. This is in accordance with the order of 1,2,4-TCB dechlorination steps in Table 1. Incubations with other CBs indicated that their culture preferentially removed chlorines that possess only one chlorine adjacent to the removed chlorine. This culture was simultaneously incubated with a high concentration of 1,2,4-TCB and a low concentration of HCB (concentration ratio 40:1), and dechlorination of HCB only started after depletion of 1,2,4-TCB. The HCB dechlorination proceeded via QCB, 1,2,3,4-TeCB, 1,2,3- and 1,2,4-TCB, and 1,2- and 1,4-DCB to MCB. Reactions that involved the removal of chlorines with only one adjacent chlorine dominated over reactions that are thermodynamic more profitable. Dechlorination of HCB continued to the monochlorinated level presumable as a result of the avoidance of 1,3,5-TCB formation. Under the described specific conditions, microorganisms mediate dechlorination steps that deviate from the thermodynamic most profitable reactions and exhibit a sequential HCB dechlorination that results in MCB accumulation. Thus, with the aid of thermodynamics, the most likely dechlorination pathway and occurrence of possible "dead-end" metabolites can be rationalized, but this does not necessarily mean that this is the only pathway that can be observed.

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