

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES

JOURNAL OF
ENVIRONMENTAL
SCIENCESwww.jesc.ac.cn

Assessment of aerobic biodegradation of lower-chlorinated benzenes in contaminated groundwater using field-derived microcosms and compound-specific carbon isotope fractionation

Alba Trueba-Santiso¹, Jordi Palau², Jesica M. Soder-Walz¹, Teresa Vicent¹, Ernest Marco-Urrea^{1,*}

¹Department of Chemical, Biological and Environmental Engineering, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Spain

²MAiMA group, SGR Applied Mineralogy, Geochemistry and Geomicrobiology, Department of Mineralogy, Petrology and Applied Geology, Faculty of Earth Sciences, Universitat de Barcelona (UB), Martí Franquès s/n, 08028 Barcelona, Spain.

ARTICLE INFO

Article history:

Received 7 October 2021

Revised 20 December 2021

Accepted 20 December 2021

Available online 3 January 2022

Keywords:

Groundwater

Isotope fractionation

Chlorobenzenes

Aerobic biodegradation

Anaerobic reductive dehalogenation

Natural attenuation

ABSTRACT

Biodegradation of lower chlorinated benzenes (tri-, di- and monochlorobenzene) was assessed at a coastal aquifer contaminated with multiple chlorinated aromatic hydrocarbons. Field-derived microcosms, established with groundwater from the source zone and amended with a mixture of lower chlorinated benzenes, evidenced biodegradation of monochlorobenzene (MCB) and 1,4-dichlorobenzene (1,4-DCB) in aerobic microcosms, whereas the addition of lactate in anaerobic microcosms did not enhance anaerobic reductive dechlorination. Aerobic microcosms established with groundwater from the plume consumed several doses of MCB and concomitantly degraded the three isomers of dichlorobenzene with no observable inhibitory effect. In the light of these results, we assessed the applicability of compound stable isotope analysis to monitor a potential aerobic remediation treatment of MCB and 1,4-DCB in this site. The carbon isotopic fractionation factors (ϵ) obtained from field-derived microcosms were $-0.7\% \pm 0.1\%$ and $-1.0\% \pm 0.2\%$ for MCB and 1,4-DCB, respectively. For 1,4-DCB, the carbon isotope fractionation during aerobic biodegradation was reported for the first time. The weak carbon isotope fractionation values for the aerobic pathway would only allow tracing of *in situ* degradation in aquifer parts with high extent of biodegradation. However, based on the carbon isotope effects measured in this and previous studies, relatively high carbon isotope shifts (i.e., $\Delta\delta^{13}\text{C} > 4.0\%$) of MCB or 1,4-DCB in contaminated groundwater would suggest that their biodegradation is controlled by anaerobic reductive dechlorination.

© 2022 The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Published by Elsevier B.V.

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

* Corresponding author.

E-mail: ernest.marco@uab.es (E. Marco-Urrea).

<https://doi.org/10.1016/j.jes.2021.12.025>

1001-0742/© 2022 The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

Introduction

Chlorinated benzenes are extensively used worldwide as intermediates for the synthesis of pesticides and other chemicals such as dyes, drugs and plastic materials (Field and Sierra-Alvarez, 2008). These compounds are frequently detected in the subsurface nearby industrial areas, as result of its improper disposal or accidental spills, and they are included in the list of priority pollutants in the 2019 US EPA's Priority List of Hazardous Substances (ATSDR, 2019). Their widespread detection in contaminated sites has led to increased public concern and the need for developing efficient remediation strategies.

When leaching into groundwater, chlorinated benzenes can undergo both aerobic and anaerobic biodegradation processes, depending on the number and position of chlorine substituents on the benzene ring and the redox conditions of groundwater (Hölscher et al., 2010). In general, chlorinated benzenes are susceptible to oxidative transformation processes with decreasing chlorination degree but the potential for anaerobic reductive dechlorination increases with increasing number of chlorine substituents. Anaerobic reductive dechlorination of hexachlorobenzene (HCB) via pentachlorobenzene (PCB) and trichlorobenzenes (TCBs) to dichlorobenzene (DCBs) has been reported in anoxic groundwaters and sediments (Capozzi et al., 2018; Kranzioch-Seipel et al., 2016; Sohn and Häggblom, 2016).

Under aerobic conditions, several bacteria are capable of growing on TCBs (with the exception of 1,3,5-TCB, which is considered recalcitrant), the three isomers of DCB and MCB as the sole source of carbon and energy (Field and Sierra-Alvarez, 2008). The degradative attack of chlorinated benzenes by these aerobic bacteria is initiated with dioxygenases to produce chlorocatechols by dihydrodiol dehydrogenase. The catechols derived via this pathway are subsequently oxidized to chloromuconic acids, which are metabolized further to intermediates of the Krebs cycle (Field and Sierra-Alvarez, 2008). The enhancement of *in situ* aerobic bioremediation in aquifers can be achieved by direct injection of oxygen-release compounds (i.e. peroxides of calcium and magnesium), as used routinely for aerobic biodegradation of hydrocarbons (Gholami et al., 2019). Under anaerobic conditions, organohalide-respiring bacteria belonging to the genera *Dehalococcoides* and *Dehalobacter* can use higher chlorinated benzenes as terminal electron acceptors, transforming them to lesser chlorinated benzenes congeners (Adrian et al., 2000; Nelson et al., 2014). At the heart of this process are membrane-bound reductive dehalogenases, which catalyze dechlorination of chlorinated compounds to generate cellular energy. Anaerobic dechlorination can be enhanced by direct injection of easily fermentable substrates (i.e. lactate, vegetable oils, molasses) into the groundwater that results in the generation of hydrogen (Blázquez-Pallí et al., 2019a). Although complete reductive dechlorination of monochlorobenzene (MCB) via benzene has been demonstrated in methanogenic sediments (Fung et al., 2009; Liang et al., 2013), this dechlorination pathway has been rarely observed in field sites under strong reducing conditions.

Thus, engineered bioremediation systems can exploit the degradation capability of these microorganisms to remediate sites impacted with chlorinated benzenes. A crucial issue in the implementation of such techniques is the selection of a proper tool to monitor the extent of biodegradation. One of the most promising tools for both qualitative and quantitative estimation of biodegradation processes at contaminated field sites is compound-specific stable isotope analysis (CSIA), that has been applied successfully in many studies over the last years (Blázquez-Pallí et al., 2019b; Palau et al., 2017). Typically, molecules containing light isotopes (e.g. ^{12}C) react faster compared to those with heavy isotopes (e.g. ^{13}C), resulting in an enrichment of the heavy isotope in the residual fraction of the contaminant, so called kinetic isotopic fractionation. Nondegradative processes such as dilution, sorption and dispersion in the aquifer are not, or only to a much lower extent, subject to isotope fractionation (Nijenhuis and Richnow, 2016). Thus, for a given contaminant and reaction mechanism, greater extent of biodegradation is expected to provoke greater changes in its isotopic composition. The magnitude of isotope fractionation during biodegradation of a selected contaminant is commonly determined in laboratory experiments with pure or enrichment cultures. The results of these experiments are subsequently interpreted based on the Rayleigh equation, that links degradation-induced shifts in isotope ratios (e.g. $^{13}\text{C}/^{12}\text{C}$) to the degree of degradation (Elsner, 2010). The laboratory-derived isotope fractionation values can be afterwards used to quantify the extent of biodegradation *in situ*, provided that the environmental conditions and biochemical reactions governing the degradation process in the laboratory experiments and at the field site are the same (Elsner, 2010).

Over the last decade, CSIA has been applied to study carbon stable isotope fractionation during the reductive dechlorination of various chlorinated compounds, and particularly chlorinated ethenes (Badin et al., 2014; Blázquez-Pallí et al., 2019a; van Breukelen et al., 2017). However, the CSIA studies reported from pure cultures or field-derived microcosms with chlorinated benzenes have been more limited. No significant changes in the carbon isotope composition have been observed during the dechlorination of highly chlorinated benzenes (HCB, PCB, 1,2,3,5-tetrachlorobenzene) in microcosms derived from sediments (Sohn et al., 2018) whereas significant isotope fractionation during anaerobic reductive dechlorination of 1,3-DCB and 1,4-DCB by methanogenic cultures and 1,2,4-TCB by *Dehalococcoides mccartyi* strain CBDB1 have been reported (Griebler et al., 2004; Liang et al., 2014). The aerobic degradation of MCB and 1,2,4-TCB by groundwater and sediments collected from a MCB-contaminated site and a *Pseudomonas* sp. P51, respectively, was not accompanied by a significant carbon isotope fractionation (Griebler et al., 2004; Marchesi et al., 2018). Compared to chlorinated C_1 - and C_2 -hydrocarbons, a lower bulk carbon isotopic fractionation during compound transformation is typically observed for chlorinated benzenes due to the higher number of C atoms. For the latter, the carbon isotopic fractionation at the reactive position(s) of the molecule is diluted by the relatively high number of C atoms located at the non-reactive ones. Carbon isotope fractionation at non-reactive positions, so called secondary isotope effect, is typically an order of magnitude lower

(Elsner et al., 2005). In addition, the weak isotopic fractionation of chlorinated benzenes has been also attributed to the masking effect associated to their low solubility (Sohn et al., 2018).

In this study, we aimed at assessing the efficacy of a bioremediation treatment in the subsurface of a former chemical plant located at an industrial area of Barcelona where the production of predominantly pesticides caused a release of a large volume of chlorinated compounds, mostly chlorinated benzenes, into the groundwater. Consequently, we assessed the redox conditions of the aquifer and the intrinsic biodegradation potential of this site using field-derived microcosms (established with groundwater from either the source zone or the contaminant plume) and testing for aerobic and anaerobic biostimulation. A second goal of this study was to explore whether carbon isotope signatures of MCB and 1,4-DCB would allow monitoring of their biodegradation at this site.

1. Materials and methods

1.1. Hydrogeological description of the aquifer

The studied site is located in the province of Barcelona (Spain), at around 400 m NW of the Besòs river and near the coastline. This site was significantly impacted by chlorinated aromatic hydrocarbons contamination due to improper disposal practices during its former use as pesticide manufacturing plant. The lithology at the site consists of Quaternary alluvial deposits (deltaic facies with a maximum thickness of 50 m) overlying Pliocene marls and clays.

For the characterization of the contamination at the studied area, five piezometers (S1, S2, S3, S4, S5) were installed in the source zone where the chemical manufacturing plant was operating and three piezometers (P1, P2, P3) were installed downgradient in the contaminant plume (Appendix A Fig. S1). Groundwater samples were collected from the upper part (up to 9.1 m below ground surface, Appendix A Table S1). The upper part is an unconfined alluvial aquifer mainly composed of sand and gravel layers of elevated hydraulic conductivity. The water table at the site is relatively shallow (from 2.8 to 5.5 m below ground surface, Appendix A Table S1) and the main groundwater flow direction is towards the SW (Appendix A Fig. S1). Previous chemical investigations performed in the period 2009–19 in the source zone and the plume revealed the presence of several chlorinated compounds in groundwater (Appendix A Tables S2 and S3).

1.2. Collection of aquifer samples

Two groundwater sampling campaigns were carried out. The first one was done in November 2019 on monitoring wells from both the source zone (S1, S2, S3) and plume (P1, P2, P3) using a submersible stainless-steel pump (diameter of 1.82 inches and 12 V). After proper purging of the wells, hydrogeochemical parameters (i.e., temperature, pH, electric conductivity, redox potential (Eh) and dissolved oxygen (DO), Appendix A Table S1) were measured using a flowthrough cell connected to the pump (to avoid contact with the atmosphere) and equipped with the corresponding electrodes

(multi-parameter 3430 WTW, Weilheim, Germany). All parameters were recorded once stabilized and the Eh values were reported according to the standard hydrogen electrode system (UH). Afterwards, samples for chemical characterization and microcosms construction were collected. For the establishment of microcosms, groundwater with fine sediments was collected from the bottom of the monitoring wells in amber autoclaved glass bottles, which were previously filled with N₂ gas to reduce bacterial contact with oxygen, and sealed with septum-lined (PTFE) caps to minimise the adsorption of chlorinated compounds. Groundwater for chemical characterization was collected at 1 m above the bottom of the wells to avoid sediments.

The second groundwater sampling campaign was done in February 2020 to collect samples from wells P1 and P2 for CSIA experiments. Samples were collected as described above and the hydrogeochemical parameters of groundwater were also measured *in situ* following the same procedure (Appendix A Table S1).

All groundwater samples were kept in the dark at 4°C until analysis.

1.3. Laboratory microcosms

Each microcosm consisted of 160 mL glass serum sterile bottles sealed with Teflon-coated butyl rubber septa and aluminium crimp caps and contained 115 mL of sampled groundwater and fine sediments.

1.3.1. Assessment of the intrinsic biodegradation potential of the site

Three treatments (aerobic, anaerobic and abiotic control) were prepared in parallel sets of microcosms constructed with groundwater from the source zone (wells S1, S2 and S3). Each treatment consisted of four replicates. To investigate whether the addition of lactate could enhance the anaerobic reductive dichlorination of lower-chlorinated benzenes in the source zone, sodium lactate (3 mmol/L) was added into microcosms prepared in an anaerobic glovebox (anaerobic treatment). The effect of oxygen on the aerobic degradation of lower-chlorinated benzenes was investigated in parallel microcosms gassed with oxygen to overpressure (+1.4 bar) (aerobic treatment). Abiotic controls consisted of microcosms inactivated with nitric acid (HNO₃ 70%) to pH 2. Each microcosm was spiked with a mixture of lower-chlorinated benzenes prepared in ethanol to reach 50 µmol/L (nominal concentration) of MCB, 1,2-DCB, 1,3-DCB, 1,4-DCB, 1,2,3-TCB, 1,2,4-TCB, and 1,3,5-TCB. When either MCB or 1,4-DCB were supplied alone, the stock solutions were prepared with acetone.

To study whether oxygen could enhance the aerobic biodegradation of MCB in the contaminant plume downgradient from the source, two treatments (aerobic and abiotic control) were prepared in parallel sets of microcosms constructed with groundwater from the plume (wells P1, P2 and P3). Each treatment consisted of four replicates. Aerobic microcosms were gassed with oxygen to overpressure (+1.4 bar) and abiotic controls consisted of microcosms inactivated with acid (70% HNO₃) to pH 2. Each microcosm was spiked with 50 µmol/L (nominal concentration) of MCB unless otherwise stated.

All microcosms were incubated at 25 °C in the dark without agitation.

1.3.2. Biodegradation experiments for CSIA

Groundwater samples collected from the plume (wells P1 and P2) were purged with nitrogen for 20 min to remove chlorinated solvents. Each experimental bottle consisted of 160 mL glass serum sterile bottles sealed with Teflon-coated butyl rubber septa and aluminium crimp caps and contained 100 mL of a mixture of groundwater and fine sediments from wells P1 and P2 (50 mL each). Two sets of experiments were established with fourteen microcosms each containing 200 $\mu\text{mol/L}$ MCB and 150 $\mu\text{mol/L}$ 1,4-DCB (nominal concentration), respectively. The concentration of MCB and 1,4-DCB was monitored periodically through the experiment by headspace analysis as described in section 1.4 and each microcosm was sacrificed at different extent of degradation by adding 300 μL HNO_3 (70%) through the septum.

Two different controls were included: (i) controls killed with 300 μL HNO_3 (70%) containing groundwater and sediments without addition of MCB and 1,4-DCB to check that both compounds were efficiently removed after purging with nitrogen, and (ii) controls containing sterilized distilled water with MCB and 1,4-DCB to account for abiotic transformations and potential impurities from the stock solutions. All microcosms were incubated at 25 °C in the dark without agitation.

1.4. Analytical methods

The concentration of MCB, DCBs and TCBs in liquid samples from the microcosms was determined by static headspace gas chromatography. For this, 3-mL liquid samples from each microcosm were withdrawn and filtered through a 0.22 μm filter (Millex-GV, Millipore) and subsequently transferred to a 10 mL vial containing 3.5 mL saline solution (NaCl, 1 mol/L), sealed immediately with a Teflon-coated stopper. The vial was placed in a headspace sampler Agilent 7964 (Agilent Technologies, Palo Alto, CA) and heated to 85 °C for 25 min. Subsequently, a 1-mL headspace sample was injected automatically into a gas chromatograph (GC, Agilent 6890 N) equipped with a flame ionization detector (FID) operated at 300 °C, and a column DB-624 (30 m \times 0.32 mm). The initial column temperature was 40 °C (held for 2 min); and then temperature slopes were applied as follows: slope 20 °C/min (2 min); slope 10 °C/min (2 min), slope 20 °C/min (3 min), slope 1 °C/min (4 min), slope 5 °C/min (2 min), slope 10 °C/min (3 min), slope 20 °C/min (1 min), slope 40 °C/min (2 min), final temperature: 260 °C (maintained 2 min). The injector was set at 250 °C in split mode (1:2). The carrier gas was helium at 40 mL/min.

The concentrations of hydrogen, carbon dioxide, methane and oxygen were measured on 0.1-mL headspace samples using an Agilent 7820A GC equipped with a thermal conductivity detector as described elsewhere (Trueba-Santiso et al., 2020).

The concentration of anions (SO_4^{2-} , NO_3^- , Cl^-) was measured on a Dionex ICS-2000 ion chromatography system (Dionex Corp., Sunnyvale, CA, USA) equipped with an IonPac AS18 anion-exchange column. The column was operated at 30 °C and a flow rate of 1 mL/min. The injection volume was 25 μL . The potassium hydroxide concentration of the eluent varied from 25 mmol/L to 50 mmol/L along the 10 min analysis.

Samples for ^{13}C -CSIA of MCB and 1,4-DCB were prepared at the laboratories of the research group on Stable Isotopes and Mineralogy (MAiMA) at the University of Barcelona (UB) and analyzed with a GC (Agilent 6890) coupled to an IRMS (ThermoFinnigan Delta Plus) at “Centres Científics i Tecnològics de la UB” (CCiT-UB). For each compound, aqueous samples were diluted to a similar concentration in 20 mL vials containing a 30 mm PTFE-coated stir bar. The vials were immediately sealed with PTFE/Silicone septa and aluminum crimp caps. Then, the sample solution was stirred at room temperature and the corresponding analyte was extracted during 20 min by headspace solid-phase micro extraction (SPME) using a manual sampler holder equipped with a 75 μm Carboxen-PDMS fiber (Supelco, Bellefonte, PA) (Palau et al 2017). The GC was equipped with a Supelco SPB-624 column (60 m \times 0.32 mm, 1.8 μm film thickness; Bellefonte, PA). The injector was set at 250 °C in split mode (1:10) and the oven temperature program was kept at 60 °C for 2 min, heated to 220 °C at a rate of 8 °C/min and finally held at 220 °C for 5 min. Helium was used as a carrier gas with a gas flow rate of 1.8 mL/min. Isotopic working standards of MCB and 1,4-DCB were used to ensure accuracy of the isotopic measurements during the course of samples analysis and were measured interspersed in the same sequence. The working standards had been referenced isotopically beforehand towards the international standard Vienne Pee Dee Belemnite (VPDB) with an elemental analyzer (EA) coupled to an IRMS. Standards and samples were analyzed by duplicate as a quality control and the precision (1σ) of $\delta^{13}\text{C}$ values based on the analysis of the standards was $\leq \pm 0.3\%$ for both compounds.

1.5. Isotope data evaluation

Carbon isotope ratios of MCB and 1,4-DCB were measured at natural abundance and were reported using the delta notation Eq. (1):

$$\delta^{13}\text{C}_{\text{sample}} = \frac{R(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{R(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} - 1 \quad (1)$$

where R is the isotope ratio ($^{13}\text{C}/^{12}\text{C}$). The relationship between isotope fractionation and the extent of MCB and 1,4-DCB biodegradation in laboratory experiments was evaluated by a modified form of the Rayleigh distillation Eq. (2):

$$\ln \frac{R_t}{R_0} = \ln \left(\frac{\delta^{13}\text{C}_t + 1}{\delta^{13}\text{C}_0 + 1} \right) = \epsilon_{\text{bulk}} \cdot \ln f \quad (2)$$

where R_0 is the initial isotope ratio at the beginning of the transformation process and R_t and f the isotope ratio and the compound remaining fraction ($f = C/C_0$), respectively, at a point in time t . The bulk (i.e., compound-average) isotope fractionation values (ϵ_{bulk}) were quantified by least squares linear regression of Eq. (2) without forcing the regression through the origin (Scott et al., 2004) and the uncertainty corresponds to the 95% confidence interval (C.I.) derived from the standard deviation of the regression slope.

For a given substrate, intrinsic kinetic isotopic effects (KIEs) during compound transformation are position specific whereas ϵ_{bulk} values are calculated from compound-average isotope data Eq. (2)). Hence, observable ϵ_{bulk} values

must be converted into apparent KIEs (AKIEs) in order to obtain information about the underlying reaction mechanisms (Elsner et al., 2005). For the calculation and interpretation of AKIEs a hypothesis about the reaction mechanism, or assumed reaction mechanism, is thus necessary. The effects of non-reacting positions within the molecule, as well as of intramolecular competition, are then taken into account with the Eqs. (3) and (4), respectively (Elsner et al., 2005),

$$\varepsilon_{\text{rp}} \approx \frac{n}{x} \cdot \varepsilon_{\text{bulk}} \quad (3)$$

$$\text{AKIE}_{\text{C}} = \frac{1}{z \cdot \left(\frac{\varepsilon_{\text{rp}}}{1000} \right) + 1} \quad (4)$$

where ε_{rp} is the isotopic fractionation at the reactive position, “n” is the number of C atoms, “x” is the number of C atoms at reactive sites (i.e., atoms that would experience isotope effects in the given reaction) and “z” is the number of identical reactive sites undergoing intramolecular competition. These equations assume the absence of secondary isotope effects. As mentioned above, this is a valid assumption for carbon because secondary carbon isotope effects are usually not significant compared to the primary ones (Elsner et al., 2005).

2. Results and discussion

2.1. Site characterization

The hydrogeochemical data collected from the wells during the two sampling campaigns is summarized in Appendix A Table S1. In the monitored wells, the water table was located at depths ranging between 2.8 and 5.5 m below ground surface. The pH and temperature values were on average 7.1 ± 0.1 and 20.3 ± 0.9 °C, respectively, suitable for microbial activity. Dissolved oxygen concentration ranged from 0.04 to 0.33 mg/L, with exception of the value of 1.2 mg/L in well P1 (plume zone) in February 2020. This result suggests that groundwater at the site is mainly anoxic. The concentration of nitrate and sulphate was below the method detection limit (≤ 0.003 mg/L), which correlates well with the very low dissolved oxygen concentrations and the negative standard redox potentials measured in wells S1, S3 and P1. The low E_{hSTD} value (-39.7 mV) obtained in P1 in February 2020 suggest that the relatively high DO concentration detected in this well (1.2 mg/L) might be due to atmospheric oxygen contamination during the measurement. A lower DO value of 0.33 mg/L was measured in P1 in November 2020. Positive standard redox potentials were found in well S2, P2 and P3, which were in the typical range of iron (III)-reducing conditions (0–100 mV).

Determination of the redox conditions can help to elucidate the contaminant degradation pathways, which is essential information for (i) evaluating the natural attenuation of chlorinated benzenes at contaminated sites and (ii) predicting potential accumulation of toxic products. However, aerobic biodegradation of chlorinated benzenes might be sustained at very low DO concentrations. For instance, Gossett (2010) reported VC oxidation in transfer cultures with DO concentrations below < 0.02 mg/L, low enough

to be considered anaerobic in field assessments. Furthermore, anaerobic oxidation of compounds such as benzene or 1,2-dichloroethane was demonstrated under iron (III)- and nitrate-reducing conditions, respectively (Anderson et al., 1998, Dinglasan-Panlilio et al., 2006). Hence, additional information is necessary for better characterization of chlorinated benzenes biodegradation in the field.

A clear downward trend of TCBs, DCBs, MCB towards lower concentration levels is observed between 2009–2019 in well S1 and between 2015–2019 in well S2 (Appendix A Table S2). Despite this, the concentration of MCB, 1,4-DCB, 1,2,3-TCB and 1,2,4-TCB (only in well S2) in 2019 exceeded the groundwater remediation goals established by the Catalan authorities (Appendix A Table S2). In contrast, the concentration of lower-chlorinated benzenes in well S4 and S5 in 2019 complied with the legal requirements (Appendix A Table S2). In 2019, all monitoring wells with the exception of S5, P1 and P2 exceeded the groundwater remediation goals for lindane, 2,4'-dichlorodiphenyldichloroethane (DDD), and 4,4'-DDD (Appendix A Tables S2 and S3). The concentration of lindane decreased in the period 2015 to 2019 in wells S1, S2 and S4 (Appendix A Table S2). Although the concentration of lindane was relatively low, anaerobic reductive dechlorination of lindane might slightly contribute to the generation of lower chlorinated benzenes (mainly 1,2-DCB, 1,3-DCB, MCB and benzene) in this anoxic aquifer (Zhang et al., 2020).

The analyses of chlorinated compounds across the site showed that in the wells located in the source (S1, S2 and S3), MCB, the three dichlorobenzene isomers (1,2-, 1,3-, and 1,4-DCB), and 1,2,3- and 1,2,4-TCB were detected at the highest concentration whereas the plume (P1, P2, P3) mainly consisted of MCB and 1,4-DCB (Appendix A Tables S2 and S3). The observed spatial distribution of contaminants can be explained by the different physicochemical characteristics of the higher chlorinated benzenes and their dechlorinating products. Under strong reducing conditions, organohalide respiration of higher chlorinated benzenes leads to lower chlorinated benzenes associated with an accumulation of MCB. The low water solubility and strong retardation of higher chlorinated compounds reduces their mobility in the by-passing water but favors the down gradient transport of DCBs and MCB (Stelzer et al., 2009).

The isomer 1,2,4-TCB, which is a transient intermediate during the anaerobic reductive dechlorination of higher chlorinated benzenes, was the most abundant within TCBs in all wells ($< 60\%$ of total TCBs) (Appendix A Tables S2 and S3), suggesting that biodegradation was one of the natural attenuation processes for the higher chlorinated benzenes occurring at the site (Tas et al., 2011). The dechlorination of singly flanked chlorine residue of 1,2,4-TCB producing 1,4-DCB is the thermodynamically more favorable reaction (Hölscher et al., 2010) and, accordingly, this DCB isomer accounts for more than 60% of total DCB in wells P1, P2, P3 and S1. The prevalence of 1,4-DCB can be also explained by the fact that the most adjacent chlorine substituents are preferably used during organohalide respiration of DCBs (Adrian and Gorisch, 2002).

Contaminated sites are usually impacted by mixtures of chlorinated benzenes, which complicates the evaluation of specific DCBs biodegradation from analysis of degradation products because the same products can be formed from dif-

ferent precursors. For instance, under reducing conditions MCB can be formed from 1,4-DCB, 1,2-DCB or 1,2,4-TCB by anaerobic reductive dechlorination (Lawrence, 2006). In addition, end products of the aerobic degradation pathways of chlorinated benzenes, i.e., inorganic carbon and Cl^- , are ubiquitous and often occur at high background concentrations in groundwater. At the investigated site, elevated levels of chloride were detected in all the wells ($\geq 205 \text{ mg/L}$) but they cannot be directly related to dechlorination processes because the natural background concentration of chloride was not determined in non-contaminated groundwater wells. Rather, this concentration of chloride is probably due to the intrinsic characteristics of this coastal aquifer. Therefore, investigation on additional tools, such as CSIA, for improved assessment of chlorinated benzenes biodegradation in contaminated field sites is warranted.

2.2. Aerobic and anaerobic microcosms established with groundwater from the source zone

In the microcosms amended with oxygen, MCB and 1,4-DCB were degraded in all replicates regardless of the groundwater source. Abiotic controls and anaerobic treatments did not degrade any of the investigated chlorobenzenes. Anaerobic microcosms were monitored for 55 days, and methane was not produced during this period, indicating that strongly reducing conditions were not achieved (data not shown). Absence of anaerobic biodegradation of lower chlorinated benzenes might be due to the inhibitory effect of co-contaminants in the medium.

The aerobic microcosms established with groundwater from S1 showed a faster dechlorinating activity, with 71.4% of MCB degraded within 15 days and 81.1% of 1,4-DCB degraded within 45 days (Fig. 1a-b). At this degradation point, MCB was reamended with $50 \mu\text{mol/L}$, and 62% of MCB was degraded in 15 days, but the third dose of MCB, amended at a higher concentration ($200 \mu\text{mol/L}$), only started degradation after the addition of a mixture of sodium phosphate (20 mg/L), ammonium sulphate (50 mg/L) and oxygen (Fig. 1a). The second dose of $50 \mu\text{mol/L}$ 1,4-DCB, which was added at day 60, was degraded at 80.6% in 30 days (Fig. 1b). The standard deviation became more significant in the later stages of the degradation, likely due to the heterogeneity in the microbial ecology of the real groundwater used in these microcosms. A longer lag time and slower degradation rate of MCB and 1,4-DCB were observed in microcosms constructed with groundwaters from wells S2 and S3 (Appendix A Fig. S2). MCB and 1,4-DCB can be both utilized as sole source of carbon and energy by a variety of bacteria, including the genera *Pseudomonas*, *Burkholderia*, *Xanthobacter*, and *Rhodococcus*, among others, initiating their degradation with dioxygenases (Field and Sierra-Alvarez, 2008).

Degradation of 1,4-DCB and MCB was concomitant with a net increase of CO_2 in the headspace (Fig. 1c and Appendix A Fig. S2). According to the stoichiometry of the oxidation reaction, the amount of CO_2 generated per each μmol of chlorinated benzene consumed would be $6 \mu\text{mol}$ (i.e., $\text{C}_6\text{H}_6\text{Cl}_x + 7.5\text{O}_2 \rightarrow 6\text{CO}_2 + 3\text{H}_2\text{O} + \text{XCl}^-$). The observed ratio of CO_2 produced against the CO_2 expected from complete oxidation of chlorobenzenes degraded indicated that other carbon

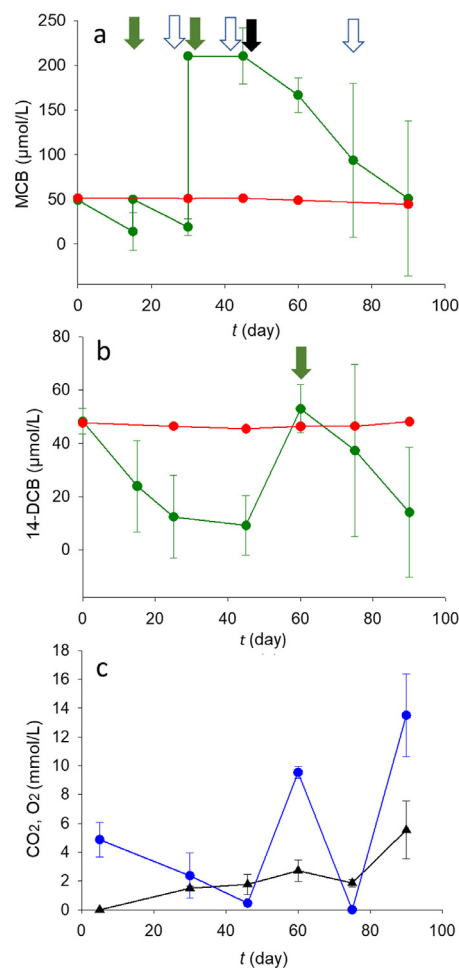


Fig. 1 – Degradation of MCB (Panel a) and 1,4-DCB (Panel b) in aerobic microcosms containing groundwater from well S1. Red and green lines represent concentration of the respective chlorinated benzene in abiotic and aerobic treatments, respectively. Blue and black lines represent oxygen and carbon dioxide concentration in aerobic treatments, respectively (Panel c). Arrows indicate reamendment of the corresponding chlorinated benzene (green), oxygen (white) and nitrogen/phosphorous source (black). Each curve shows the mean value of four replicates. Error bars indicate standard deviations of four replicates.

sources were mineralized in the microcosms, and therefore it cannot be assigned -at least, not exclusively- to MCB degradation (Appendix A Table S4). For instance, acetone and ethanol used as solvent in stock solutions were completely consumed in all microcosms (data not shown), and other chlorinated compounds and labile organic matter present in groundwater but not monitored in this study would be susceptible to aerobic degradation as well.

In parallel, four culture bottles per monitoring well were amended with HCB crystals in anaerobic microcosms with lactate to study whether lower-chlorinated benzenes were generated in this site from the anaerobic reductive dechloro-

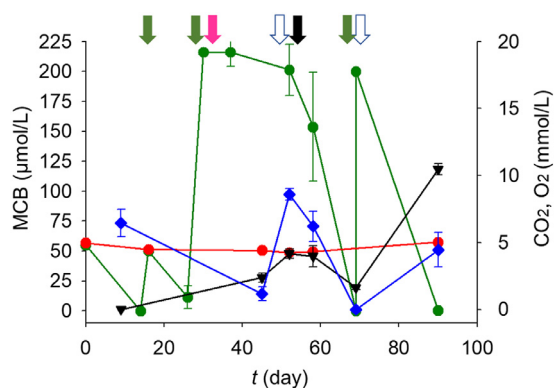


Fig. 2 – Degradation of MCB in aerobic microcosms containing groundwater from well P2. Red and green lines represent concentration of MCB in abiotic and aerobic treatments, respectively. Blue and black lines represent oxygen and carbon dioxide in aerobic treatments, respectively. Arrows indicate reamendment of the corresponding chlorinated benzene (green), oxygen (white) and nitrogen/phosphorous source (black). The pink arrow indicates that at this point the first replicate received 50 $\mu\text{mol/L}$ 1,2-DCB, the second replicate 50 $\mu\text{mol/L}$ 1,3-DCB, and the third replicate 50 $\mu\text{mol/L}$ 1,4-DCB. The fourth replicate did not receive DCB and acted as a control. Each curve shows the mean value of four replicates. Error bars indicate standard deviations of four replicates.

riation of HCB. In 6 out of 12 bottles we detected 1,3,5-TCB and 1,4-DCB as only byproducts after three months of incubation (see results of well S3 in Appendix A Fig. S3), indicating that anaerobic reductive dechlorination of HCB is feasible under anoxic conditions in this contaminated site. These results are in agreement with several studies showing production of DCB isomers and 1,3,5-TCB as final products of HCB by pure and mixed cultures under anaerobic conditions, with 1,3,5-TCB often as dominant end product (Adrian and Görisch, 2002; Fennell et al., 2004; Wu et al., 2002). If this pathway was active in the field, we would expect production of 1,4-DCB from 1,2,4-TCB in the previous anaerobic microcosms amended with lactate, because 1,2,4-TCB is an obligate intermediate in the production of 1,4-DCB via the HCB dechlorination pathway (Fennell et al., 2004; Hölscher et al., 2010). A feasible explanation is that some of the chlorinated benzenes contained in the mixture, which were absent in the microcosms amended with HCB, might exert an inhibitory impact on the organohalide respiration of these compounds (i.e., 1,2,4-TCB). Self-inhibition by degradation products has been widely reported in the environment and, in the case of chlorinated benzenes, 1,3-DCB, 1,4-DCB and 1,3,5-TCB are known to inhibit HCB dechlorination (Chau et al., 2018).

2.3. Aerobic microcosms established with groundwater from the plume

The microcosms established with groundwater from wells P1, P2 and P3 showed a similar degradation pattern, consuming two consecutive doses of 50 $\mu\text{mol/L}$ MCB in 26 days (Fig. 2 and

Appendix A Fig. S4). No MCB degradation was observed in abiotic controls. Once demonstrated that MCB was degradable under aerobic conditions we aimed to study if DCBs, which were co-contaminants of MCB in these wells, might have an inhibitory effect on aerobic MCB degradation. For this, every bottle was reamended with MCB (200 $\mu\text{mol/L}$) and each of the four replicates constructed for each well were amended with either 1,2-DCB (50 $\mu\text{mol/L}$), 1,3-DCB (50 $\mu\text{mol/L}$) or 1,4-DCB (50 $\mu\text{mol/L}$). One of the replicates was not amended with DCB and remained as a control. The concentration of MCB remained unchanged until a nitrogen and phosphorus source was added (Fig. 2 and Appendix A Fig. S4). From that moment on, MCB and DCB started degradation and were rapidly consumed (Fig. 2, Appendix A Figs. S4, S5). These results show that the three isomers of DCB and MCB were easily degradable under aerobic conditions by the autochthonous bacteria with no observable inhibitory effect. It also raised the question whether the lack of biodegradation of 1,2- and 1,3-DCB in microcosms from the source (section 2.2.) was due to the inhibition provoked by some of the chlorobenzenes contained in the amended mixture.

2.4. Stable isotope fraction during aerobic degradation of MCB and 1,4-DCB

The aerobic degradation of MCB and 1,4-DCB was accompanied by a slight enrichment of ^{13}C in their residual fraction. On the one hand, MCB was enriched in ^{13}C from -28.3‰ to -27.2‰ when 86% was degraded (Fig. 3a). On the other hand, the initial isotope composition of 1,4-DCB was -29.1‰ and when 92% was degraded, the residual 1,4-DCB was enriched in ^{13}C to -26.7‰ (Fig. 3b). A resulting ϵ of $-0.7\text{‰} \pm 0.1\text{‰}$ and $-1.0\text{‰} \pm 0.2\text{‰}$ was calculated for MCB and 1,4-DCB, respectively, by plotting the experimental bottles results on a double-logarithmic plot according to Eq. (2) (Fig. 3c-d). The good linear correlation ($R^2_{\text{MCB}} = 0.96$, $R^2_{1,4\text{-DCB}} = 0.95$) indicated that aerobic degradation of MCB and 1,4-DCB are well described by the Rayleigh model.

The carbon isotopic fractionation (ϵ_{bulk}) during aerobic biodegradation of 1,4-DCB was not available in the literature yet. This is a very valuable information for future studies since knowledge of the compound- and reaction-specific enrichment factor (ϵ_{bulk}) is required to assess the degree of biodegradation using CSIA.

The EPA establishes as a criterion, based on the typical uncertainty of $\pm 0.5\text{‰}$ for $\delta^{13}\text{C}$ measurements, that the values of $\delta^{13}\text{C}$ in the compounds of interest must be enriched (less negative) by 2‰ compared to values of $\delta^{13}\text{C}$ in the undegraded compounds to demonstrate that significant fractionation occurs and ensure reliable interpretation of biodegradation (U. S. EPA, 2008). According to the determined ϵ_{bulk} values for aerobic biodegradation, a shift of $\delta^{13}\text{C}$ above 2‰ (i.e., $\Delta\delta^{13}\text{C} > 2.0\text{‰}$) would be obtained after a degradation extent of approximately 94% and 86% of MCB and 1,4-DCB, respectively. This result indicates that qualitative and quantitative assessments of MCB and 1,4-DCB transformation by aerobic biodegradation would only be possible in the aquifer parts with very large contaminant degradation. Previous studies showed negligible enrichment of ^{13}C associated to aerobic degradation of MCB (Kaschl et al., 2009; Marchesi et al., 2018). However, the assess-

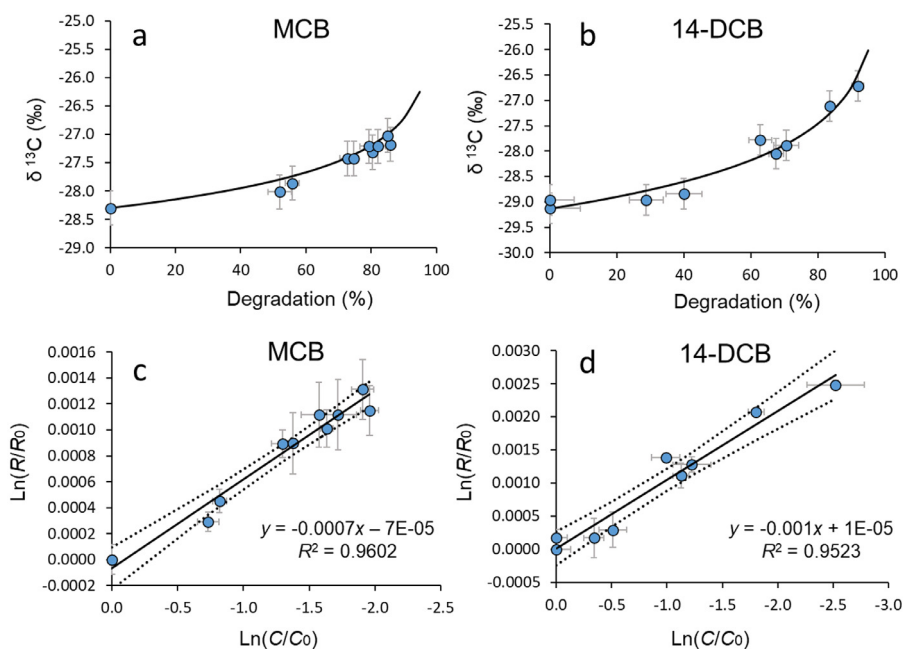


Fig. 3 – Percentage of degradation and carbon isotopic composition of MCB and 1,4-DCB in aerobic microcosms containing a mixture of groundwater collected from wells P1 and P2 (Panel a and b) and double logarithmic plot according to the Rayleigh equation of the carbon isotope ratio versus the residual concentration of MCB and 1,4-DCB (Panel c and d). The error bars show the one standard deviation ($\pm 1\sigma$) for duplicate measurements and dotted lines represent the 95% confidence intervals of the linear regression.

ment of aerobic degradation of MCB might be monitored by chlorine isotope fractionation in the light of a recent study highlighting enrichments of $\delta^{37}\text{Cl}$ higher than 2.5 ‰ from aerobic microcosms established with groundwater contaminated with MCB (Marchesi et al., 2018).

The weak carbon isotope fractionation during aerobic biodegradation of MCB contrasts with the significant ^{13}C enrichment factor of -5.0 ‰ observed during anaerobic reductive dechlorination of MCB (Liang et al., 2011). Accordingly, some studies have correlated the occurrence of anaerobic reductive dechlorination of MCB to benzene at field sites with a progressive enrichment of the $\delta^{13}\text{C}_{\text{MCB}}$ (Alberti et al., 2017; Kaschl et al., 2009; Stelzer et al., 2009). Similarly, for 1,4-DCB a much higher ϵ_{bulk} value ($-6.3\text{‰} \pm 0.2\text{‰}$) compared to the one determined in this study ($-1.0\text{‰} \pm 0.2\text{‰}$) was determined during anaerobic reductive dechlorination in methanogenic laboratory microcosms (Liang et al., 2014). Although the weak carbon isotope fractionation in the aerobic pathway would only allow tracing of *in situ* degradation in aquifer parts with high extent of degradation, relatively high carbon isotope shifts (i.e., $\Delta\delta^{13}\text{C} > 4.0$ ‰) of MCB or 1,4-DCB in contaminated groundwaters would indicate that their degradation is controlled by anaerobic reductive dechlorination.

In order to characterize the isotope effect at the reactive position of MCB and 1,4-DCB, apparent kinetic isotopic effect values (AKIE) were calculated. In the presence of oxygen, chlorinated benzenes are generally transformed by a ring dioxygenase (stereospecific ring deoxygenation) that introduces two OH-groups in neighboring positions in the aromatic ring van der Meer et al., 1991) (Fig. 4). For both MCB and 1,4-

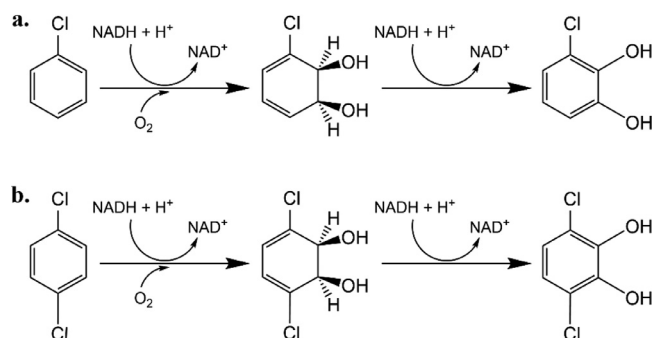


Fig. 4 – Aerobic biodegradation pathways and initial enzymatic dioxygenase reaction for (a) monochlorobenzene and (b) 1,4-dichlorobenzene.

DCB, the initial attack on the aromatic ring takes place via the formation of two C-O bonds, leading to the formation of 3-chloro-cis-1,2-dihydroxycyclohexa-3,5-diene (Werlen et al., 1996) and 3,6-dichloro-cis-1,2-dihydroxycyclohexa-3,5-diene (Spiess et al., 1995), respectively. For MCB, an AKIE of 1.0021 ± 0.0003 was calculated using $n = 6$, $x = 4$ and $z = 2$ in Eqs. (3) and (4), assuming that the two carbon atoms involved during deoxygenation of the aromatic ring are in ortho- and meta-position. The AKIE of 1,4-DCB was calculated in the same way and a value of 1.0030 ± 0.0006 was obtained. The AKIEs of MCB and 1,2-DCB are similar and very small, reflecting the absence of bond breakage during the first (rate-limiting) reaction step. Liang et al. (2011) suggested that the weak carbon iso-

topic effect during aerobic degradation of MCB is associated to the initial enzymatic reaction catalyzed by dioxygenases (rate-limiting C-O bond formation) whereas the larger carbon isotope effect observed for anaerobic degradation is likely the result from rate-limiting C-Cl bond cleavage.

The AKIE of MCB (1.0021 ± 0.0003) is slightly higher than those obtained by Kaschl et al. (2009), from 1.0003 ± 0.0003 to 1.0012 ± 0.0003 . These values were calculated in this study using the same parameters (n , x and z) indicated above. The AKIEs of MCB and 1,4-DCB obtained here are also consistent with those obtained for ring dioxygenation reaction of benzene (from 1.002 ± 0.0003 to 1.004 ± 0.0006 , Fischer et al., 2008), toluene (1.001 ± 0.001 to 1.006 ± 0.001 , Vogt et al., 2008) and ethylbenzene (1.002 , Dorer et al., 2014).

3. Conclusions

The laboratory microcosms established in this study were a valuable tool to assess biodegradation strategies to remediate an aquifer contaminated with multiple chlorinated aromatic hydrocarbons. Our laboratory tests provided a positive indication that enhanced *in situ* aerobic bioremediation is a feasible strategy to degrade MCB and DCBs in this site. Notwithstanding, there is a need for additional laboratory studies to assess the potential inhibitory effect of some chlorinated compounds on the biodegradability of low chlorinated benzenes in the source area. The enrichment factor for 1,4-DCB offers an important contribution to the use of ^{13}C -CSIA in future studies and it could be used to assess aerobic biodegradation of 1,4-DCA in aquifer parts with high extent of biodegradation. In addition, on the basis of previous studies, the occurrence of significant changes in carbon isotope ratios of MCB and 1,4-DCB in the field (i.e., $\Delta\delta^{13}\text{C} > 4.0$ ‰) might suggest the predominance of anaerobic reductive dechlorination pathways.

Acknowledgments

This work was supported by the Catalan Water Agency (No. CTN1900901). Jordi Palau was also supported by the projects CGL2017–82331-R (Spanish Ministry of Economy and Competitiveness) and 2017SGR 1733 (Catalan Government). We thank Emilio Orejudo and Joan Sanchez (Catalan Agency of Water) for their help on field work.

Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.jes.2021.12.025](https://doi.org/10.1016/j.jes.2021.12.025).

REFERENCES

Adrian, L., Görisch, H., 2002. Microbial transformation of chlorinated benzenes under anaerobic conditions. *Res. Microbiol.* 153, 131–137. doi:10.1016/s0923-2508(02)01298-6.

Adrian, L., Szewzyk, U., Wecke, J., Gorisch, H., 2000. Bacterial dehalorespiration with chlorinated benzenes. *Nature* 408, 580–583. doi:10.1038/35046063.

Alberti, L., Marchesi, M., Trefiletti, P., Aravena, R., 2017. Compound-specific isotope analysis (CSIA) application for source apportionment and natural attenuation Assessment of chlorinated benzenes. *Water*. 9, 872 DOI:10.3390/w9110872.

Anderson, R.T., Rooney-Varga, J.N., Gaw, C.V., Lovely, D.R., 1998. Anaerobic benzene oxidation in the Fe(III) reduction zone of petroleum-contaminated aquifers. *Environ. Sci. Technol.* 32 (9), 1222–1229. doi:10.1021/es9704949.

ATSDR (Agency for Toxic Substances and Disease Registry), 2019. Priority list of hazardous substances. Available: <http://www.atsdr.cdc.gov/SPL/index>. (accessed on 09.07.2021).

Badin, A., Buttet, G., Maillard, J., Holliger, C., Hunkeler, D., 2014. Multiple dual C-Cl isotope patterns associated with reductive dechlorination of tetrachloroethene. *Environ. Sci. Technol.* 48, 9179–9186. doi:10.1021/es500822d.

Blázquez-Pallí, Rosell, M., Varias, J., Bosch, M., Soler, A., Vicent, T., et al., 2019a. Integrative isotopic and molecular approach for the diagnosis and implementation of an efficient *in-situ* enhanced biological reductive dechlorination of chlorinated ethenes. *Water Res* 167, 115106. doi:10.1016/j.watres.2019.115106.

Blázquez-Pallí, N., Shouakar-Stash, O., Palau, J., Trueba-Santiso, A., Varias, J., Bosch, M., et al., 2019b. Use of dual element isotope analysis and microcosm studies to determine the origin and potential anaerobic biodegradation of dichloromethane in two multi-contaminated aquifers. *Sci. Total Environ.* 696, 134066. doi:10.1016/j.scitotenv.2019.134066.

Capozzi, S.L., Rodenburg, L.A., Krumins, V., Fennell, D.E., Mack, E.E., 2018. Using positive matrix factorization to investigate microbial dehalogenation of chlorinated benzenes in groundwater at a historically contaminated site. *Chemosphere* 211, 515–523. doi:10.1016/j.chemosphere.2018.07.180.

Chau, A.T.T., Lee, M., Adrian, L., Manefield, M.J., 2018. Syntrophic partners enhance growth and respiratory dehalogenation of hexachlorobenzene by *Dehalococcoides mccartyi* strain CBDB1. *Front. Microbiol.* 9, 1927. doi:10.3389/fmicb.2018.01927.

Dinglasan-Panlilio, M.J., Dworatzek, S., Mabury, S., Edwards, E., 2006. Microbial oxidation of 1,2-dichloroethane under anoxic conditions with nitrate as electron acceptor in mixed and pure cultures. *FEMS. Microbiol. Ecol.* 56 (3), 355–364. doi:10.1111/j.1574-6941.2006.00077.x.

Dorer, C., Vogt, C., Kleinstueber, S., Stams, A.J.M., Richnow, H.H., 2014. Compound-specific isotope analysis as a tool to characterize biodegradation of ethylbenzene. *Environ. Sci. Technol.* 48, 9122–9132. doi:10.1021/es500282t.

Elsner, M., Zwank, L., Hunkeler, D., Schwarzenbach, R.P., 2005. A new concept linking observable stable isotope fractionation to transformation pathways of organic pollutants. *Environ. Sci. Technol.* 39, 6896–6916. doi:10.1021/es0504587.

Elsner, M., 2010. Stable isotope fractionation to investigate natural transformation mechanisms of organic contaminants: principles, prospects and limitations. *J. Environ. Monit.* 12, 2005–2031. doi:10.1039/c0em00277a.

Fennell, D.E., Nijenhuis, I., Wilson, S.F., Zinder, S.H., Häggblom, M.M., 2004. *Dehalococcoides ethenogenes* strain 195 reductively dechlorinates diverse chlorinated aromatic pollutants. *Environ. Sci. Technol.* 38, 2075–2081. doi:10.1021/es034989b.

Field, J.A., Sierra-Alvarez, R., 2008. Microbial degradation of chlorinated benzenes. *Biodegradation* 19 (4), 463–480. doi:10.1007/s10532-007-9155-1.

Fischer, A., Herklotz, I., Herrmann, S., Thullner, M., Weelink, S.A.B., Stams, A.J.M., et al., 2008. Combined carbon and hydrogen isotope fractionation investigations for elucidating benzene biodegradation pathways. *Environ. Sci. Technol.* 42, 4356–4363. doi:10.1021/es702468f.

- Fung, J.M., Weisenstein, B.P., Mack, E.E., Vidumsky, J.E., Ei, T.A., Zinder, S.H., 2009. Reductive dehalogenation of dichlorobenzenes and monochlorobenzene to benzene in microcosms. *Environ. Sci. Technol.* 43, 2302–2307. doi:10.1021/es802131d.
- Gholami, F., Mosmeri, H., Shavandi, M., Mehdi Dastgheib, S.M., Ali Amoozegar, M., 2019. Application of encapsulated magnesium peroxide (MgO₂) nanoparticles in permeable reactive barrier (PBR) for naphthalene and toluene bioremediation from groundwater. *Sci. Total. Environ.* 655, 633–640. doi:10.1016/j.scitotenv.2018.11.253.
- Griebler, C., Adrian, L., Meckenstock, R.U., Richnow, H.H., 2004. Stable carbon isotope fractionation during aerobic and anaerobic transformation of trichlorobenzene. *FEMS Microbiol. Ecol.* 48, 313–321. doi:10.1016/j.femsec.2004.02.005.
- Gossett, J.M., 2010. Sustained aerobic oxidation of vinyl chloride at low oxygen concentrations. *Environ. Sci. Technol.* 44 (4), 1405–1411. doi:10.1021/es9033974.
- Hölscher, T., Lisek, J., Baani, M., Duan, T.H., Adrian, L., 2010. Bacterial cultures preferentially removing singly flanked chlorine substituents from chlorobenzenes. *Environ. Sci. Technol.* 44, 8936–8942. doi:10.1021/es101971m.
- Kaschl, A., Vogt, C., Uhlig, S., Nijenhuis, I., Weiss, H., Kästner, M., et al., 2009. Isotopic fractionation indicates anaerobic monochlorobenzene biodegradation. *Environ. Toxicol. Chem.* 24, 1315–1324. doi:10.1897/04-321R.1.
- Kranzioch-Seipel, I., Beckert, U., Shen, C., Yin, D., Tiehm, A., 2016. Microbial dechlorination of HCB, PCP, PCB180, HCH and PCE in a Yangtze Three Gorges Reservoir enrichment culture. *China. Environ. Earth Sci.* 10, 928. doi:10.1007/s12665-016-5653-y.
- Lawrence, S.J., 2006. *Description, Properties, and Degradation of Selected Volatile Organic Compounds Detected In Ground Water — A Review of Selected Literature: Atlanta, Georgia, U. S. Geological Survey. Open-File Report 2006-1338. a Web-only publication, p. 62* at <http://pubs.usgs.gov/ofr/2006/1338/>.
- Liang, X., Scott, O.C., Mundle, S.C.P., Nelson, J.L., Passeport, E., Chan, C.C.H., et al., 2014. Distinct carbon isotope fractionation during anaerobic degradation of dichlorobenzene isomers. *Environ. Sci. Technol.* 48, 4844–4851. doi:10.1021/es4054384.
- Liang, X., Devine, C.E., Nelson, J., Sherwood Lollar, B., Zinder, S., Edwards, E.A., 2013. Anaerobic conversion of chlorobenzene and benzene to CH₄ and CO₂ in bioaugmented microcosms. *Environ. Sci. Technol.* 47, 2378–2385. doi:10.1021/es3043092.
- Liang, X., Howlett, M.R., Nelson, J.L., Grant, G., Dworatzek, S., Lacrampe-Couloume, 2011. Pathway-dependent isotope fractionation during aerobic and anaerobic degradation of monochlorobenzene and 1,2,4-trichlorobenzene. *Environ. Sci. Technol.* 45 (19), 8321–8327. doi:10.1021/es201224x.
- Marchesi, M., Alberti, L., Shouakar-Stash, O., Pietrini, I., de Ferra, F., Carpani, G., et al., 2018. ³⁷Cl-compound specific isotope analysis and assessment of functional genes for monitoring monochlorobenzene (MCB) biodegradation under aerobic conditions. *Sci. Total Environ.* 619–620, 784–793. doi:10.1016/j.scitotenv.2017.11.150.
- Nelson, J.L., Jiang, J., Zinder, S.H., 2014. Dehalogenation of chlorobenzenes, dichlorotoluenes, and tetrachloroethene by three *Dehalobacter* spp. *Environ. Sci. Technol.* 48, 3776–3782. doi:10.1021/es4044769.
- Nijenhuis, I., Richnow, H.H., 2016. Stable isotope fractionation concepts for characterizing biotransformation of organohalides. *Curr. Opin. Biotechnol.* 41, 108–113. doi:10.1016/j.copbio.2016.06.002.
- Palau, J., Yu, R., Mortan, S.H., Shouakar-Stash, O., Rosell, M., Freedman, D.L., et al., 2017. Distinct dual C-Cl isotope fractionation patterns during anaerobic biodegradation of 1,2-dichloroethane: potential to characterize microbial degradation in the field. *Environ. Sci. Technol.* 51, 2685–2694. doi:10.1021/acs.est.6b04998.
- Scott, K.M., Lu, X., Cavanaugh, C.M., Liu, J.S., 2004. Optimal methods for estimating kinetic isotope effects from different forms of the Rayleigh distillation equation. *Geochim. Cosmochim. Ac.* 68, 433–442. doi:10.1016/S0016-7037(03)00459-9.
- Sohn, S.Y., Kuntze, K., Nijenhuis, I., Häggblom, M.M., 2018. Evaluation of carbon isotope fractionation during anaerobic reductive dehalogenation of chlorinated and brominated benzenes. *Chemosphere* 193, 785–792. doi:10.1016/j.chemosphere.2017.11.089.
- Sohn, S.Y., Häggblom, M.M., 2016. Reductive dehalogenation activity of indigenous microorganism in sediments of the Hackensack River, New Jersey. *Environ. Pollut.* 214, 374–383. doi:10.1016/j.envpol.2016.04.022.
- Spieß, E., Sommer, C., Görisch, H., 1995. Degradation of 1,4-dichlorobenzene by *Xanthobacter flavus* 14p1. *Appl. Environ. Microbiol.* 61, 3884–3888.
- Stelzer, N., Imfeld, G., Thullner, M., Lehmann, J., Poser, A., Richnow, H.H., et al., 2009. Integrative approach to delineate natural attenuation of chlorinated benzenes in anoxic aquifers. *Environ. Pollut.* 6, 1800–1806. doi:10.1016/j.envpol.2009.01.029.
- Tas, N., van Eekert, M.H.A., Wagner, A., Schraa, G., de Vos, W.M., Smidt, H., 2011. Role of “*Dehalococcoides*” spp. in the anaerobic transformation of hexachlorobenzene in European rivers. *Appl. Environ. Microbiol.* 77, 4437–4445. doi:10.1128/AEM.01940-10.
- Trueba-Santiso, A., Fernández-Verdejo, D., Marco-Rius, I., Soder-Walz, J.M., Casabella, O., Vicent, T., et al., 2020. Interspecies interaction and effect of co-contaminants in an anaerobic dichloromethane-degrading culture. *Chemosphere* 240, 124877. doi:10.1016/j.chemosphere.2019.124877.
- U.S. Geological Survey, 2008. *A Guide for Assessing Biodegradation and Source Identification of Organic Ground Water Contaminants Using Compound Specific Isotope Analysis (CSIA). Office of Research and Development National Risk Management Research Laboratory, Ada, Oklahoma, p. 74820.*
- van der Meer, J.R., van Neerven, A.R.W., De Vries, E.J., De Vos, W.M., Zehnder, A.J., 1991. Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51. *J. Bacteriol.* 173, 6–15. doi:10.1128/jb.173.1.6-15.1991.
- van Breukelen, B.M., Thouement, H.A.A., Stack, P.E.M., Vanderford, Philp, P., Kuder, T., 2017. Modeling 3D-CSIA data: Carbon, chlorine, and hydrogen isotope fractionation during reductive dechlorination of TCE to ethene. *J. Cont. Hydrol.* 204, 79–89. doi:10.1016/j.jconhyd.2017.07.003.
- Vogt, C., Cyrus, E., Herklotz, I., Schlosser, D., Bahr, A., Herrmann, S., et al., 2008. Evaluation of toluene degradation pathways by two-dimensional stable isotope fractionation. *Environ. Sci. Technol.* 42, 7793–7800. doi:10.1021/es8003415.
- Werlen, C., Kohler, H.P., van der Meer, J.R., 1996. The broad substrate chlorobenzene dioxygenase and cischlorobenzene dihydrodiol dehydrogenase of *Pseudomonas* sp. strain P51 are linked evolutionarily to the enzymes for benzene and toluene degradation. *J. Biol. Chem.* 271, 4009–4016. doi:10.1074/jbc.271.8.4009.
- Wu, Q., Milliken, C.E., Meier, G.P., Watts, J.E.M., Sowers, K.R., May, H.D., 2002. Dechlorination of chlorobenzenes by a culture containing bacterium DF-1, a PCB dechlorinating microorganism. *Environ. Sci. Technol.* 36, 3290–3294. doi:10.1021/es0158612.
- Zhang, W., Lin, Z., Pang, S., Bhatt, P., Chen, S., 2020. Insights into the biodegradation of lindane (γ -Hexachlorocyclohexane) using a microbial system. *Front. Microbiol.* 11, 522. doi:10.3389/fmicb.2020.00522.