Biotransformation of lindane (γ-hexachlorocyclohexane) to non-toxic end products by sequential treatment with three mixed anaerobic microbial cultures

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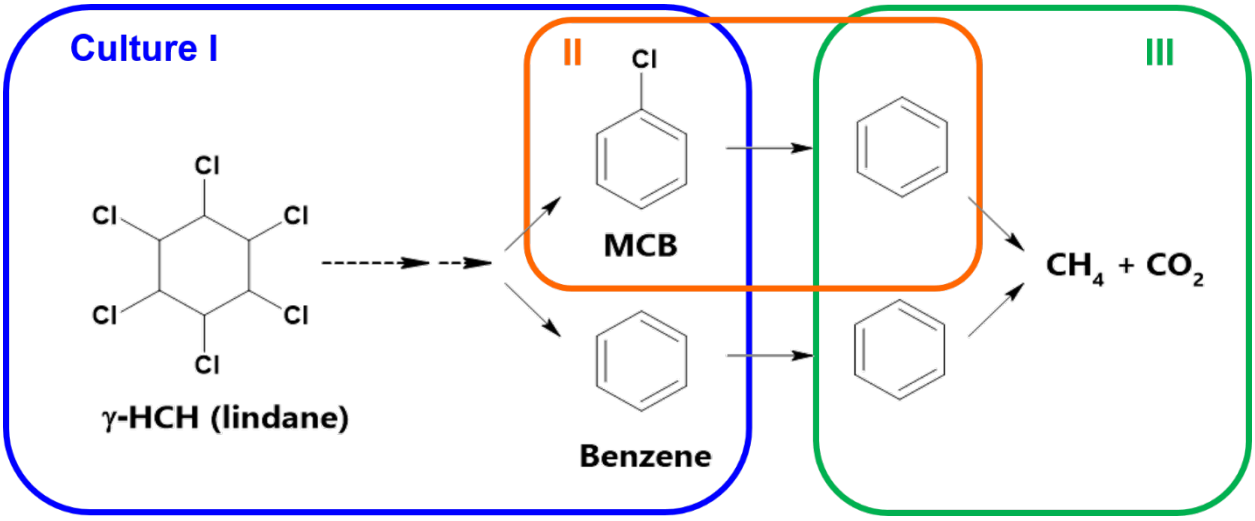


Abstract

The γ isomer of hexachlorocyclohexane (HCH), also known as lindane, is a carcinogenic persistent organic pollutant. Lindane was used worldwide as an agricultural insecticide. Legacy soil and groundwater contamination with lindane and other HCH isomers is still a big concern. The biotic reductive dechlorination of HCH to non-desirable and toxic lower chlorinated compounds such as monochlorobenzene (MCB) and benzene, among others, has been broadly documented. Here, we demonstrate for the first time that complete biotransformation of lindane to non-toxic end products is attainable using a sequential treatment approach with three mixed anaerobic microbial cultures referred to as culture I, II, and III. Biaugmentation with culture I achieved dechlorination of lindane to MCB and benzene. Culture II was able to dechlorinate MCB to benzene, and finally, culture III carried out methanogenic benzene degradation. Distinct *Dehalobacter* populations, corresponding to different 16S rRNA amplicon sequence variants in culture I and culture II, were responsible for lindane and MCB dechlorination, respectively. This study continues to highlight key roles of *Dehalobacter* spp. as chlorobenzene- and HCH-organohalide-respiring bacteria and demonstrates that sequential treatment with specialized anaerobic cultures may be explored at field sites in order to address legacy soil and groundwater contamination with HCH.

Introduction

Hexachlorocyclohexane (HCH) isomers are persistent organic pollutants. Lindane, the γ isomer of hexachloroyclohexane (γ-HCH), is the only HCH isomer exhibiting insecticidal properties, and it is a known carcinogen. During the manufacture of "pure" lindane, large amounts of HCH isomers were produced as waste by-products. Lindane (γ-HCH) and other HCH isomers (α-, β-, δ-, and ε-HCH) were components of the agriproduct known as "technical HCH". Technical HCH

35 and lindane were extensively applied to a variety of crops for pest control. In some instances,
36 HCH manufacture by-products were also improperly disposed of in landfills or waste dumps.
37 Although most countries have banned the use of HCH, groundwater, soil, and sediment
38 contaminated with HCH is of worldwide concern. There is extensive evidence of HCH
39 contamination at a variety of sites in Spain,[1, 2] Germany,[3] China,[4] and the French West Indies,[5]
40 among others. Legacy HCH contamination, estimated at ~ 4 to 7 million tons,[6-8] has been
41 difficult to address and manage, likely because of the extent of the contamination and the
42 physicochemical characteristics of each HCH isomer.

43 Hexachlorocyclohexane isomers are poorly soluble in water. Estimates of water solubility for γ-
44 HCH (lindane) vary; some sources report it to be around 1 to 2 mg/L[9] and others estimate it to be
45 as high as 7 to 10 mg/L[10, 11] at temperatures near 20◦C. HCH isomers partition into organic
46 compounds (log K_{ow}~3.3) and organic carbon (log K_{oc}~3.0).[12] Thus, HCHs sorb readily to
47 sediment and soil fractions. HCHs are persistent in the environment, yet the amount of detectable
48 HCH that remains in agricultural soils varies largely depending on the characteristics of the
49 contaminated site; for a review on the persistence of HCH isomers refer to Bhatt et al. (2009).[13]
50 Long-range global atmospheric transport and deposition of HCHs has also been demonstrated,[14,
51 15] resulting in pollution of pristine environments including the Canadian Artic. Natural
52 attenuation of HCH contamination over years or decades has been documented; a summary of
53 these studies is also included in Bhatt et al. (2009).[13] In light of the extent of HCH contamination
54 and the associated risk posed to human health and the environment, active remediation of
55 contaminated sites may be desirable. Bioremediation could be a suitable approach for HCH
56 transformation as demonstrated at a field study in the Netherlands.[16]

57 The aerobic biodegradation of HCH is well documented: pathways, microorganisms, and
58 enzymes are known.[17] The anaerobic biodegradation of HCH has been studied to a lesser extent,
59 yet anaerobic conditions often prevail in HCH-impacted sediments and groundwater. In such
60 environments, HCH isomers can undergo biologically-mediated dechlorination reactions.
61 Dechlorination of HCH isomers has been observed in sediment microcosms,[18] co-cultures,[19]
62 anaerobic sludge,[20] and enrichment cultures.[21] Known dechlorinating bacteria, such as
63 *Dehalobacter*, *Dehalococcoides*, and *Clostridium* have been linked to HCH dechlorination,[19, 20,
64 22, 23] *Dehalobacter* sp. E1 can metabolically dechlorinate β-HCH to MCB and benzene.[19] Partial
65 HCH dechlorination (in which only a fraction of the added HCH mass was dechlorinated) was
66 observed in pure cultures of *Dehalococcoides mccartyi* strains BTF08 and 195 after being pre-
67 grown with tetrachloroethene (PCE);[23, 24] it has been suggested that *Dehalococcoides mccartyi*
68 strain 195 may be able to respire γ-HCH.[23] Monochlorobenzene (MCB) and benzene are
69 frequently reported as stable end products of HCH dechlorination. However, since MCB is toxic
70 and benzene is a known carcinogen, these are undesirable biotransformation products. Although
71 MCB and benzene can be biodegraded aerobically, they are often found at contaminated sites
72 along with HCH.[1, 3, 16] HCH bioremediation could be feasible as long as any potentially toxic by-
73 products can be biodegraded as well. At some sites, anaerobic biodegradation of these
74 dechlorination by-products may be challenging. However, the sustained anaerobic conversion of
75 MCB and benzene was previously demonstrated in laboratory microcosm experiments[25] and
76 could be coupled with HCH-dechlorinating bioremediation technologies to achieve complete
77 detoxification.

78 MCB and benzene, the products of anaerobic HCH dechlorination, have been successfully
79 biotransformed anaerobically.[26-30] Independent long term investigations in our laboratory have

80 led to the development of three distinct sets of enrichment cultures of interest for HCH
81 bioremediation. The first set of cultures are HCH-dechlorinating enrichment cultures
82 (dechlorinating α-, β-, δ-, and γ-HCH), derived from contaminated sediments from Guadeloupe
83 (French West Indies), which completely transform HCH to MCB and benzene.[21] Here, the
84 culture that dechlorinates γ-HCH will be referred as culture I. The second culture, or culture II, is
85 a KB-1-derived enrichment culture that dechlorinates MCB to benzene.[26] The third culture, or
86 culture III, is a methanogenic enrichment culture in which benzene is converted to methane and
87 carbon dioxide[29] that has been enriched in the Edwards lab for over 20 years. We hypothesized
88 that a combined treatment using the three cultures would be able to completely transform HCH
89 to non-toxic products. Rather than simply mixing the 3 specific cultures together, we tested the
90 hypothesis via sequential addition of each culture. Sequential addition would allow us to
91 minimize any detrimental interactions between the different microbial populations, i.e. the
92 benzene-degrading culture (culture III) uses benzene as electron donor and its activity could be
93 impaired by the presence of alternative electron donors which are required for cultures I and II.
94 Using the sequential approach, we were able to demonstrate the anaerobic bioconversion of
95 lindane (γ-HCH) all the way to non-toxic end products. In addition, we demonstrate that distinct
96 native *Dehalobacter* populations, corresponding to different 16S rRNA amplicon sequence
97 variants in culture I and culture II, are responsible for the biotransformation of lindane to MCB
98 and benzene in culture I, and for the dechlorination of MCB to benzene in culture II. Here, we
99 also discuss the implications of our findings which together support the development of
100 anaerobic bioremediation technologies for HCH, MCB, and benzene.
101

102 **Materials and Methods**

103 **Chemicals**

104 Chemicals were purchased through Millipore Sigma (Canada) at the highest purity available.
105 Ethanol and methanol for the preparation of feedstocks were HPLC grade. Gases for anaerobic
106 culturing were purchased through Praxair (Canada).

107 **Enrichment cultures used in this study**

108 Culture I is an anaerobic mixed microbial culture, enriched from soil and river sediment
109 microcosms originating from lindane-contaminated sites in Guadeloupe (French West Indies). [21]
110 The microcosms were first set up in 2010, and details of the history of culture enrichment are
111 shown in the Supplementary Information (Figure S1). Culture I dechlorinates lindane (γ-HCH,
112 electron acceptor) stoichiometrically to a mixture of MCB and benzene (see **Error! Reference**
113 **source not found.**, panel A, and Figure S2). Ethanol is currently supplied as the electron donor
114 at a ratio of 5 to 1 electron equivalents of donor to acceptor.

115 Culture II is a KB-1-derived anaerobic enrichment culture that dechlorinates MCB (electron
116 acceptor) to benzene (see **Error! Reference source not found.**, panel B). The culture was
117 derived from an enrichment culture that dechlorinates 1,2,4-trichlorobenzene to a mixture of
118 dichlorobenzene, MCB, and benzene.[26] Culture II is supplied with methanol (electron donor) at a
119 ratio of 5 to 1 electron equivalents of donor to acceptor, but additional re-amendments of donor
120 are generally needed for complete dechlorination of MCB to benzene.

121 Culture III is an anaerobic methanogenic enrichment culture that transforms benzene (electron
122 donor) through a series of syntrophic microbial interactions to methane and carbon dioxide (see
123 Figure 1, panel C).[29] This culture is currently being scaled-up at the SiREM Lab

124 (https://www.siremlab.com/) as DGG-B® and pilot tests are underway to evaluate its
125 effectiveness at field scale.

126 **Sequential treatment with specialized anaerobic microbial enrichment cultures**

127 The biotransformation experiment was performed in three phases, each phase initiated by the
128 addition of one of the three distinct cultures described above (see illustration of experiment setup
129 in Figure S3). A bicarbonate-buffered mineral medium[26], pre-reduced with an amorphous iron
130 (II) sulfide (FeS) slurry (~0.8 g/L), was used to set up and top up the experimental bottles. All
131 the steps involving feeding, inoculation, and sampling were performed in an anaerobic chamber
132 (Coy Lab Products, USA). All the experiments were set up using 115 mL clear glass bottles with
133 screw cap Mininert® valves to a final liquid volume of 90 mL. During phase I (~150 days), nine
134 active bottles (labelled as experimental bottles 1 to 9 in the supplementary Excel file) containing
135 lindane (~4.3 µmol or 1.25 mg per bottle) were inoculated (on Day 0) with a 10% transfer of the
136 lindane-dechlorinating culture (culture I), resulting in a final concentration of 1 x 10^7 bacterial
137 cells/mL in each experimental bottle. Three additional bottles were inoculated with 10%
138 autoclaved inoculum (negative controls); the inoculum was autoclaved in a liquid cycle held at
139 121°C and 15 psi for 45 min. Glass bottles were pre-loaded with lindane prior to bringing them
140 into the anaerobic chamber as follows: lindane was added to autoclaved empty bottles as a
141 concentrated lindane/acetone solution which was completely evaporated under a stream of
142 nitrogen. The bottles were then transferred to the anaerobic chamber for a minimum of 2 days
143 prior to inoculation. During inoculation, sterile mineral medium and culture I were aliquoted
144 directly into each bottle using sterile pipettes. On Day 93, after all lindane had been converted to
145 MCB and benzene, cultures were transferred into new bottles with additional lindane (~8.6 µmol
146 or 2.25 mg per bottle, prepared as described above). Electron donor, ethanol, was added every

147 four weeks to experimental and control bottles (on Day 0, 27, 59, 93, and 123) at a concentration
148 of 0.3 mM. The total amount of donor added during phase I is equivalent to 20 times the total
149 electron equivalents of acceptor added (assuming 6 electron equivalents per mole of lindane).

150 During phase II, from Day 148 to 337 (~190 days), six of the nine active bottles from phase I
151 (labelled as experimental bottles 1 to 6 in the supplementary Excel file) were bioaugmented on
152 two separate dates (Day 148 and Day 275) with the MCB-dechlorinating culture (culture II). For
153 each bioaugmentation event of phase II, the experimental bottles were inoculated with 1 x 10^6
154 bacterial cells of culture II per mL. From phase II onwards, cultures were added to each bottle as
155 concentrated inoculum using sterile plastic syringes via the septum on the screw cap to minimize
156 the potential loss of MCB and benzene and to replenish the experimental working volume (90
157 mL). For the first and second phase-II bioaugmentation events, 5 mL of 10X-concentrated
158 culture II and 10 mL of 4X-concentrated culture II were added to each bottle, respectively (refer
159 to the SI for concentration protocol). Culture II-bioaugmented bottles received methanol as
160 electron donor. Methanol was fed at a concentration of 0.1 mM (Day 148 and Day 198) and 0.2
161 mM (Day 218, 258, 275). The total amount of methanol added to these bottles throughout phase
162 II was equivalent to 50 times the electron equivalents of acceptor (total MCB produced during
163 the experiment; 2 electron equivalents per mole of MCB). The three remaining active bottles
164 from phase I were not inoculated with culture II, but were monitored as controls. Ethanol was
165 added to these control bottles on Day 198, 218, and 296.

166 During phase III (Day 148 to 337), three of the six active bottles that were bioaugmented in
167 phase II (labelled as experimental bottles 4, 5, and 6 in the supplementary Excel file) were
168 inoculated with 4 x 10^8 bacterial cells of the benzene-degrading culture (culture III) per mL,
169 while the remaining three bottles were monitored as controls. Additionally, two positive controls,

170 inoculated with culture III in the same fashion as bottles 4, 5, and 6, were set up in parallel to
171 monitor the activity of the benzene-degrading culture; neat benzene was added directly to fresh
172 mineral medium. Similarly to phase II, culture III was added to each bioaugmented bottle as
173 concentrated inoculum (6 mL at 6X) using sterile plastic syringes via the septum on the screw
174 cap (refer to the SI for concentration protocol).

175 **Analytical methods**

176 MCB, benzene, and methane were quantified using GC-FID (gas chromatography with flame
177 ionization detection). For the analysis, aqueous samples (1 mL) were added to 5 mL of acidified
178 (using 6N HCl) deionized water (pH < 2). Calibration standards for MCB and benzene were
179 prepared gravimetrically by adding a known mass of a combined MCB/benzene stock prepared
180 in methanol to bottles containing known volumes of water and headspace. Methane standards
181 were prepared by adding known volumes of pure methane gas, using gas-tight syringes, to
182 bottles containing known volumes of water and headspace. External calibrations were prepared
183 to convert area counts into concentrations (refer to the SI for details on GC method). Analysis of
184 γ-hexachlorocyclohexane (lindane) was not performed for the sequential biotransformation
185 experiment since that would have required removal of more than 1 mL of culture volume per
186 bottle per sampling point during phase I. However, lindane was measured in a separate
187 experiment shown in Figure S2, in which it was verified that culture I biotransforms lindane to
188 MCB and benzene stoichiometrically (moles of lindane degraded = moles of MCB + benzene
189 produced). This stoichiometric relationship between lindane fed and MCB and benzene produced
190 has consistently been observed in this culture throughout its enrichment process.

191 **DNA sampling and quantitative PCR (qPCR)**

192 DNA was extracted from 2 mL culture samples. Cells were harvested by centrifugation at
193 10,000x g for 15 min at 4°C. Cell pellets were resuspended in 100 µl of supernatant and DNA
194 was extracted using the DNeasy PowerSoil kit following the manufacturer's recommendations
195 (during steps 9 and 12 of the protocol, all supernatant was transferred). Real-time polymerase
196 chain reaction (qPCR) assays were performed to track the gene copies of total Bacteria,
197 *Dehalobacter* and Desulforomonadales using specific 16S rRNA gene primers (see Table
198 S1 and Table S2 in the SI for qPCR primers and calibration information, respectively). For this
199 study, a new primer set that targets representative sequences classified as either
200 Desulforomonadales and/or *Geobacter* (a genus of the order Desulforomonadales) was designed;
201 the target sequences were obtained from previous 16S rRNA gene amplicon sequencing studies
202 of culture I[21] (refer to the SI for the qPCR prep and quantification protocol).

203 **16S rRNA gene amplicon sequencing**

204 DNA was extracted as described above and sent for Illumina amplicon sequencing at the
205 Genome Quebec Innovation Centre (McGill University). DNA was sequenced using the Illumina
206 MiSeq PE300 platform with modified versions of primers 926f and 1392r (926f-modified:
207 AAACTYAAAKGAATWGRCGG; 1392r-modified: ACGGGCGGTGWGTRC) which target
208 the V6−V8 variable region of the 16S rRNA gene from bacteria and archaea, as well as the 18S
209 rRNA gene in eukarya. After sequencing, raw data was processed using the QIIME 2[31] Core
210 2020.2 distribution. Primers were removed using the cutadapt[32] plugin. Sequences were denoised
211 and merged into amplicon sequence variants (ASVs) using DADA2[33] (forward sequence
212 truncated at 260 bp, reverse sequence truncated at 240 bp). Taxonomy was assigned to ASVs
213 using the feature-classifier plugin[34] with the classify-sklearn method using a pre-trained Naïve

214 Bayes Classifier with the SILVA 132 99% OTUs full-length sequences database (available at
215 https://docs.qiime2.org/2020.2/data-resources/). Data was taken to MS Excel and RStudio for
216 further processing. An Excel supplementary file is included with the resulting taxonomy and
217 summary of ASVs of interest. Raw 16S rRNA amplicon sequencing data has been deposited to
218 the NCBI Sequence Read Archive under bioproject PRJNA661282.

219 **Estimation of absolute abundance of amplicon sequence variants (ASVs)**

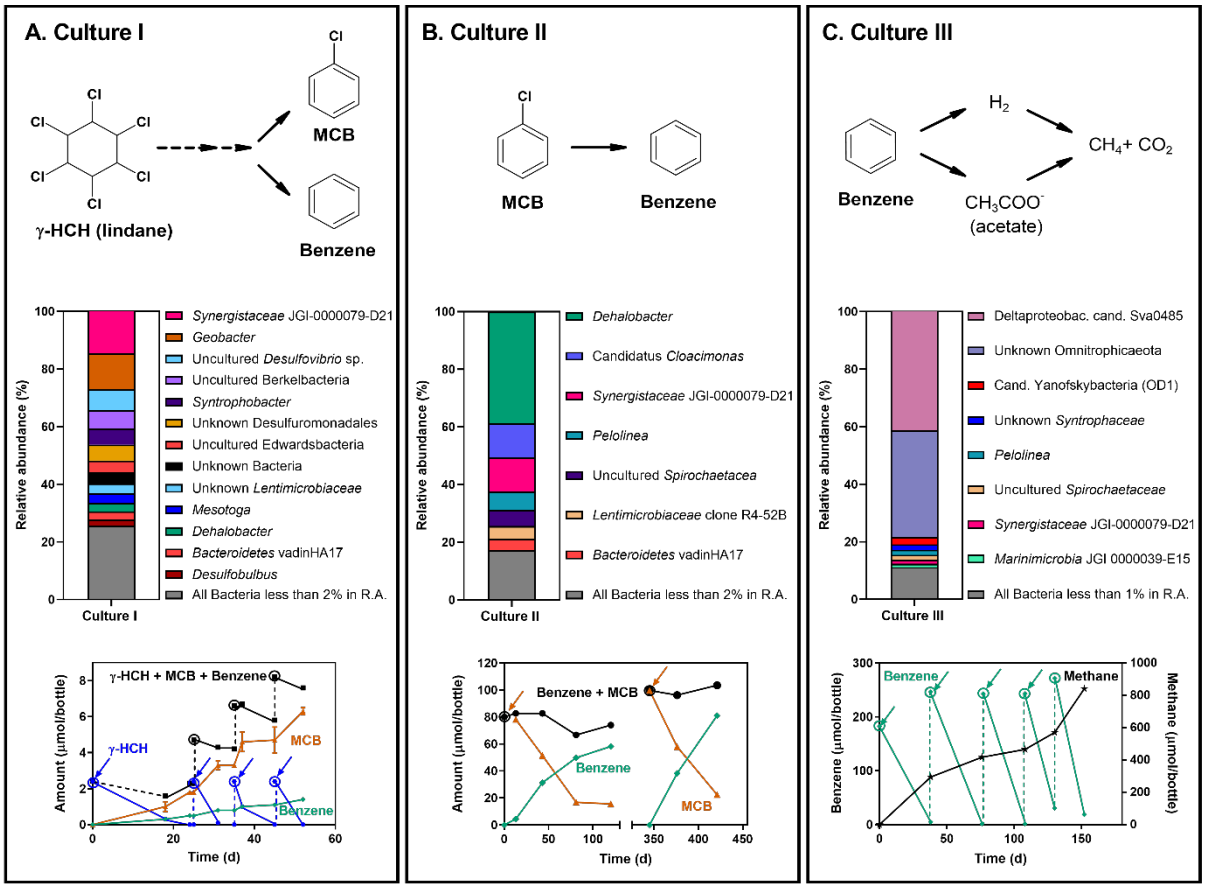
220 Absolute abundance of ASVs of interest was calculated as the product of the qPCR-determined
221 Bacteria 16S rRNA gene copy numbers (determined using the general Bacteria primer shown in
222 Table S1) and the relative abundance of each ASV (included in the Supplementary Excel file)
223 determined by 16S rRNA gene amplicon sequencing via the QIIME 2 pipeline (described
224 above). This approach was used to obtain the absolute abundance of bacterial groups of interest
225 during phase III and for the different *Dehalobacter* ASVs.

226 **Results and Discussion**

227 **Inoculum samples from enrichment cultures used during the biotransformation**
228 **experiment**

229 Samples from cultures I, II, and III were used as inoculum for each respective phase of the
230 biotransformation experiment. **Error! Reference source not found.** shows the observed
231 biotransformation step (top diagram), the bacterial community composition determined by 16S
232 rRNA amplicon sequencing at the time of inoculation (middle), and culture maintenance data
233 (bottom) for each culture; cultures I, II, and III, are shown in panels A, B, and C, respectively.
234 For culture I (**Error! Reference source not found.**A), lindane is dechlorinated to MCB and
235 benzene. The proposed series of reactions that leads to the formation of MCB and benzene in

236 culture I are discussed in Qiao et al.[21] Dechlorination rates for this culture range from ~ 6.0 to 13
237 µmol/L/day (1.8 – 3.8 mg/L/day). Detected abundant bacterial genera (> 2% relative abundance)
238 that are known organohalide-respirers include *Geobacter*, and *Dehalobacter.* Other abundant
239 bacterial genera include representatives of the fermentative and sulfate-reducing genus
240 *Desulfovibrio,* members of the recently revealed candidate phyla Berkelbacteria, as well as
241 members of the *Syntrophobacter*, and the Desulforomonadales family. Culture II, which
242 dechlorinates MCB to benzene, contains abundant bacteria classified as *Dehalobacter,*
243 *Cloacimonas*, *Synergistaceae, Pelolinea,* and *Spirochaetacea* (**Error! Reference source not**
244 **found.**B). MCB dechlorination to benzene is observed a rate of ~ 2.0 ± 0.3 µmol/L/day (0.16 ±
245 0.02 mg/L/day). Culture III, degrades benzene to methane and carbon dioxide (**Error!**
246 **Reference source not found.**C). Culture III is highly enriched in bacteria classified as
247 Deltaproteobacterial candidate Sva0485 which represents the key microorganism, historically
248 termed "ORM2" for Oil Refinery Methanogenic Clone 2, responsible for the initial attack on the
249 benzene ring.[30, 35] Other abundant bacteria in this culture belong to the phyla Omnitrophicaeota
250 and Yanofskybacteria. The ASVs classified as Yanofskybacteria correspond to another
251 previously described abundant bacterium in the benzene-degrading cultures, referred to as OD1.
252 [29] The benzene degradation rate for culture III used in this study was ~ 6.4 µmol/L/day (~ 0.5
253 mg/L/d).



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Phase I: dechlorination of lindane (γ-HCH) to MCB and benzene

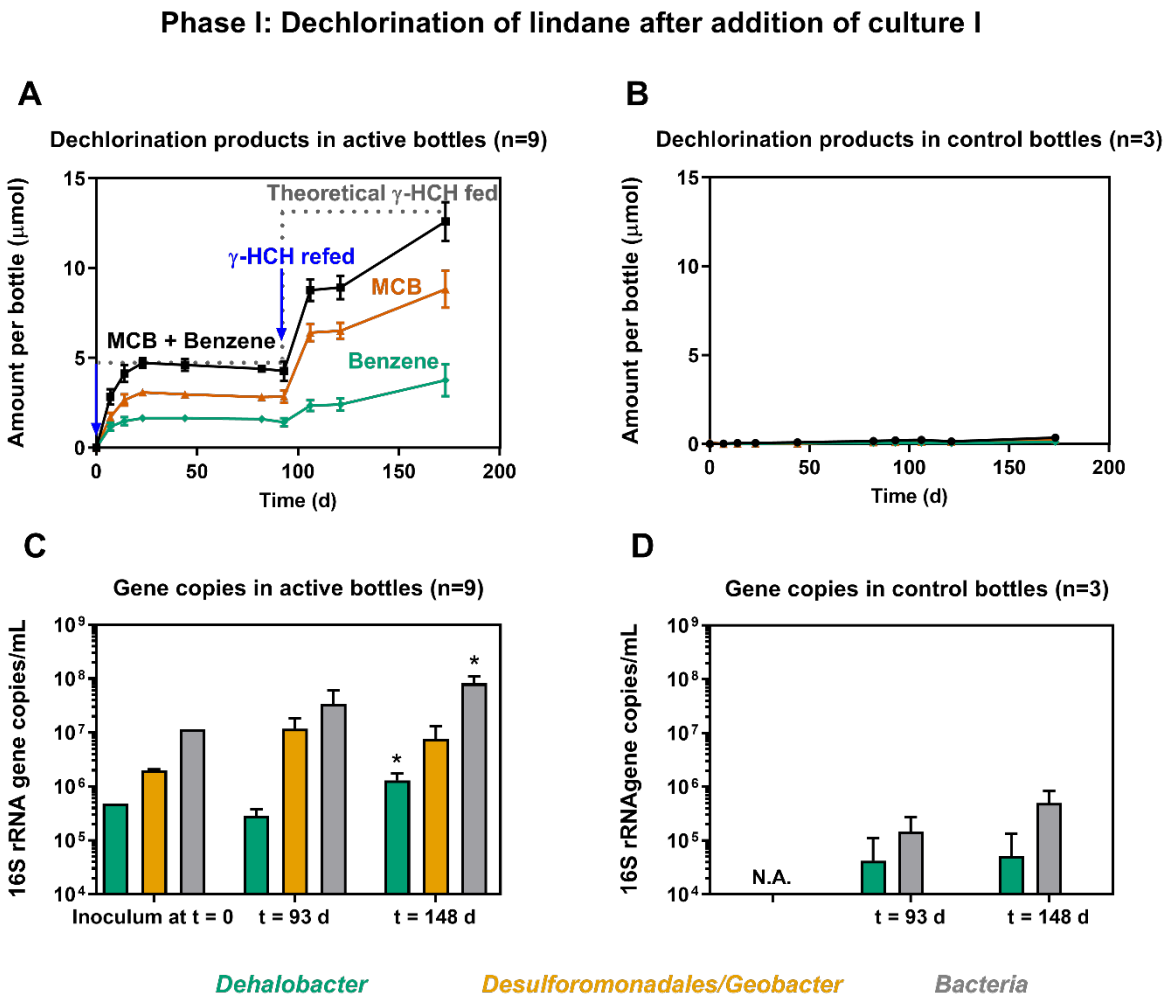
Error! Reference source not found.A shows the dechlorination of lindane from Day 0 to Day 173, in the 9 experimental bottles inoculated with culture I. Within 14 days, about 96% of the theoretical mass of lindane added to the bottles was dechlorinated at an average rate of 3.3 ± 0.4 µmol/L/d (1.0 ± 0.1 mg/L/d). By Day 23, all lindane was transformed to MCB and benzene. Even though electron donor (ethanol) was re-amended (Day 27 and Day 59), further dechlorination of MCB to benzene did not occur. This is consistent with the observations gathered for this culture during years of sequential transfers and maintenance (Figure S1, Supplementary Information). For the second feeding event (Day 93), the added mass of lindane

265 was 1.8 times greater than the first feeding. As expected, corresponding stoichiometric amounts
266 of MCB and benzene were produced. After 13 days (Day 106), 53% of the re-amended
267 theoretical mass of lindane was dechlorinated at an average dechlorination rate of 3.9 ± 0.3
268 μmol/L/d (1.1 ± 0.1 mg/L/d). Electron donor limitation became evident after sampling on Day
269 121, so ethanol was re-amended (Day 123). All lindane was dechlorinated afterwards. At the end
270 of phase I, the average molar ratio of benzene to MCB was 0.32 ± 0.06. In the control bottles
271 with autoclaved inoculum, only trace amounts of benzene and MCB were detected (**Error!**
272 **Reference source not found.**B), likely due to abiotic reductive dechlorination.

273 The qPCR-determined 16S rRNA gene copies per mL of culture of *Dehalobacter*,
274 Desulfuromomadales/*Geobacter*, and total Bacteria are shown in **Error! Reference source not**
275 **found.**C and D. There was a significant increase in *Dehalobacter* and total Bacteria copy
276 numbers from Day 93 (prior to the second lindane feeding) to Day 148. *Dehalobacter* copy
277 numbers increased by 4.3-fold. Desulfuromomadales/*Geobacter* copy numbers did not increase.
278 This indicates that the growth of *Dehalobacter* is linked to the dechlorination of lindane to MCB
279 and benzene, and that bacteria belonging to the Desulfuromomadales/*Geobacter* are likely not
280 directly implicated. These findings are consistent with observations reported earlier for culture
281 I.[21] Since some *Geobacter* spp. are known to dechlorinate organochlorines[36-38], we had
282 hypothesized that they could be involved in lindane dechlorination. *Geobacter* in culture I are
283 potentially using ethanol (supplied during culture maintenance) or other bacterial metabolites,
284 e.g. acetate or H_2 as electron donor. The use of ethanol as electron donor is a known feature of
285 some *Geobacter* spp. such as *G. chapellei* strain 172T, *G. metallireducens* GS-15T, *G.*
286 *hydrogenophilus* H-2T, and *G. grbiciae* TACP-2T[37, 39]. In addition, *G. hydrogenophilus* H-2T, *G.*
287 *grbiciae* TACP-2 T, and *G. sulfurreducens* can use use H_2 as electron donor[39] and require acetate

288 as a carbon source for growth with H_2. *Geobacter* spp. can use a wide range of electron
289 acceptors, e.g. elemental sulfur, nitrate, malate, Mn (IV), Fe (III), and some organochlorines, and
290 can also transfer electrons directly to other microbes[40]. Bacteria belonging to the
291 Desulfuromomadales could also be fermenting organic acids.

292 Interestingly, *Dehalobacter* but not Desulfuromomadales/*Geobacter* copy numbers were
293 quantifiable via qPCR in the bottles with autoclaved inoculum. *Dehalobacter* gene copies were
294 also detected in heat-treated experimental control bottles from a previous study with
295 *Dehalobacter*-enriched cultures.[26] *Dehalobacter* spp. are members of the Firmicutes which are
296 known to produce heat-resistant endospores.[41] Endospores may survive the autoclaving process
297 and thus result in cell viability and DNA preservation. *Dehalobacter* genomes do contain
298 sporulation genes[42], yet the *Dehalobacter restrictus* type strain, DSM 9455, formerly known as
299 PER-K23, was reported as not spore-forming.[43] Additional studies on *Dehalobacter* spp. are
300 required to determine if spore formation is a feature of select strains.



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Phase II: dechlorination of MCB to benzene

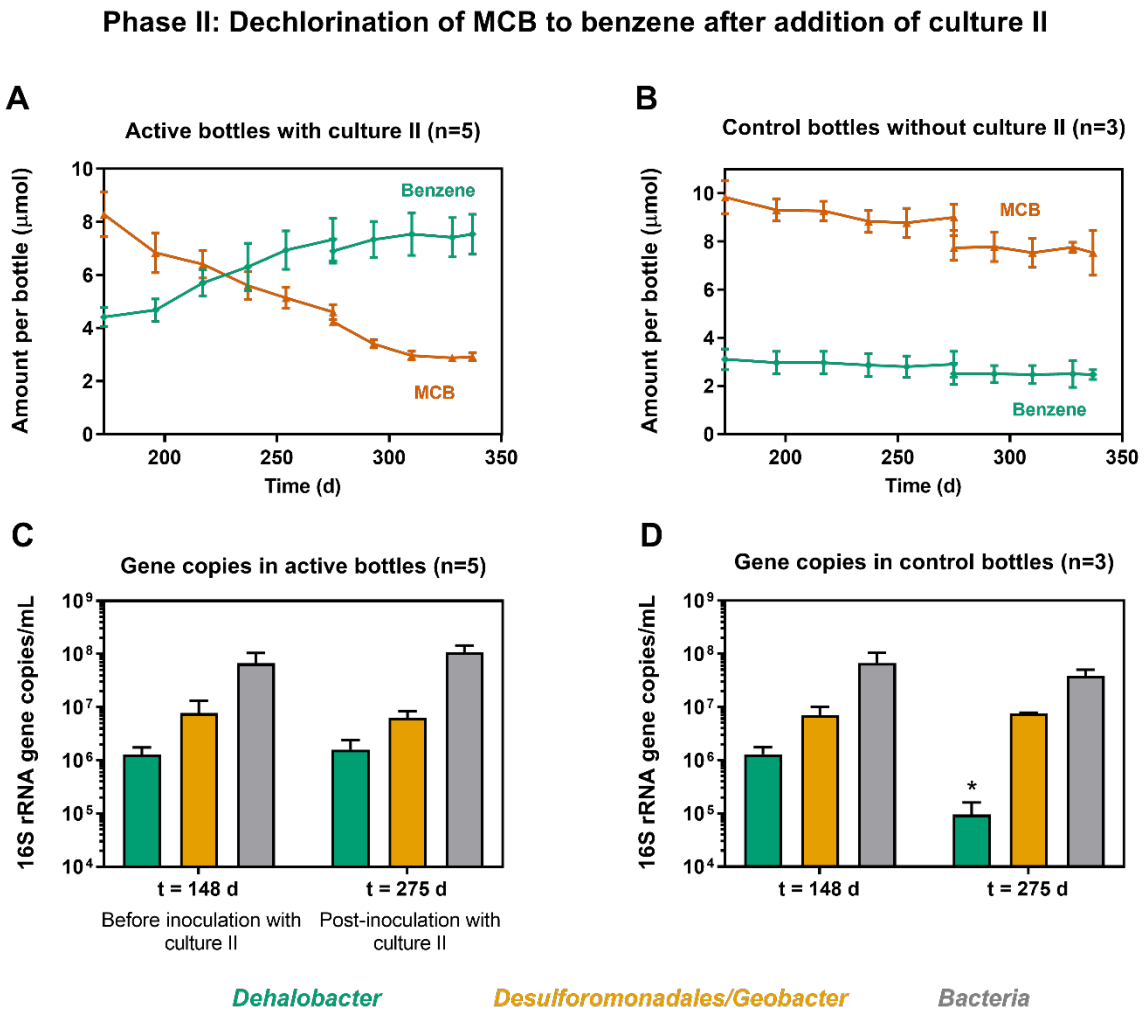
On Day 148, six of the active bottles (experimental bottles 1 to 6) from phase I were bioaugmented with culture II. Experimental bottles 7, 8, 9 were not bioaugmented with culture II, and remained as controls. **Error! Reference source not found.** shows the steady dechlorination of MCB to benzene in five out of the six bioaugmented bottles; one of the bioaugmented bottles did not initially exhibit significant MCB dechlorination, and is not included in the graph. A slight reduction in dechlorination rates was observed for the time period

310 between Day 237 and 254 with some bottles exhibiting significant deviations from the mean
311 dechlorination rate. As a result, a second bioaugmentation event, in which culture II was again
312 added to experimental bottles 1 to 6, was performed on Day 275. Interestingly, after this
313 bioaugmentation event, MCB dechlorination was kick-started in the single bottle in which no
314 significant MCB dechlorination had been observed resulting in detectable MCB to benzene
315 transformation in all the bioaugmented bottles. The mean MCB to benzene dechlorination rate
316 for phase III was 0.3 ± 0.2 μmol/L/day (0.03 ± 0.02 mg/L/day, n = 5). This rate is about 5 times
317 lower than the average rate measured during routine cultivation of culture II (0.03 vs 0.16
318 mg/L/day). One of the factors that may have contributed to the rate reduction is the increased
319 competition for electron donor that the MCB-dechlorinating population likely faced in these
320 experimental bottles. As shown in **Error! Reference source not found.**, culture I (lindane
321 enrichment) is a mixed enrichment culture with organisms known to be able to use methanol, e.g.
322 for methane production in certain members of the *Methanosarcinales*, or potentially as electron
323 donor. Several amplicon sequence variants in culture I were classified to the genus
324 *Desulfosporosinus*. Some *Desulfosporosinus* spp. such as *D. meridie*, a homoacetogenic
325 bacterium, use methanol as electron donor during sulfate reduction.[44]

326 Benzene and MCB amounts in the control bottles (non-bioaugmented with culture II) are shown
327 in **Error! Reference source not found.**B. As expected, benzene did not increase in these bottles.
328 As phase II progressed, there was a slight decline in the amount of MCB and benzene measured
329 in the control and active bottles. This was due to addition of inoculum (10 mL) to the active
330 bottles during the second bioaugmentation event, and to the fact that all the experimental bottles
331 were topped up to 90 mL on Day 275. As shown in **Error! Reference source not found.**C, the
332 qPCR-measured Bacteria and *Dehalobacter* gene copies per mL of culture did not increase in the

333 active bottles over the 127-day period following inoculation. However, there was a measurable
334 decline (14-fold) of *Dehalobacter* gene copies per mL of culture in the control (non-
335 bioaugmented) bottles (**Error! Reference source not found.**D). This finding provides additional
336 evidence for the role of the *Dehalobacter* population in culture I as the lindane-dechlorinating
337 population. In the active bottles, the *Dehalobacter* gene copies did not decline because the
338 lindane-dechlorinating *Dehalobacter* population was being replaced by the MCB-dechlorinating
339 *Dehalobacter* population native to culture II. This will be further discussed later. In the controls
340 (non-bioaugmented with culture II, Desulfuromomadales/*Geobacter* gene copies did not decline
341 suggesting that these organisms were metabolizing some of the available exogenous or
342 endogenous electron donor (e.g. acetate) that likely was still present in the bottles; ethanol was
343 added to these controls in two occasions (Day 198 and 218).

344 On Day 337, before proceeding with the addition of culture III, some MCB still remained in the
345 culture II-bioaugmented bottles. For complete MCB dechlorination to have occurred, additional
346 bioaugmentation events, likely with a higher inoculum density, would have been required. As
347 will be discussed later, the *Dehalobacter* population in culture II is usually in the order of 10^7
348 cells/mL of culture, not very dense compared to other dechlorinating enrichment cultures, and its
349 calculated yield is lower than that of other *Dehalobacter* populations respiring different
350 chlorinated substrates.



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Phase III: benzene biodegradation

After 190 days into phase II (Day 337), we proceeded with phase III, i.e. the bioaugmentation with the benzene-degrading culture (culture III). At this point, 67 ± 7% of the mass of MCB had been converted to benzene following the bioaugmentation with culture II. Culture III was added to three of the active bottles from phase II (experimental bottles 4, 5, and 6); the remaining three bottles from phase II served as controls. As shown in **Error! Reference source not found.**, benzene was biodegraded at a rate of 1.3 ± 0.1 µmol/L/day (0.1 ± 0.01 mg/L/day) in two out of

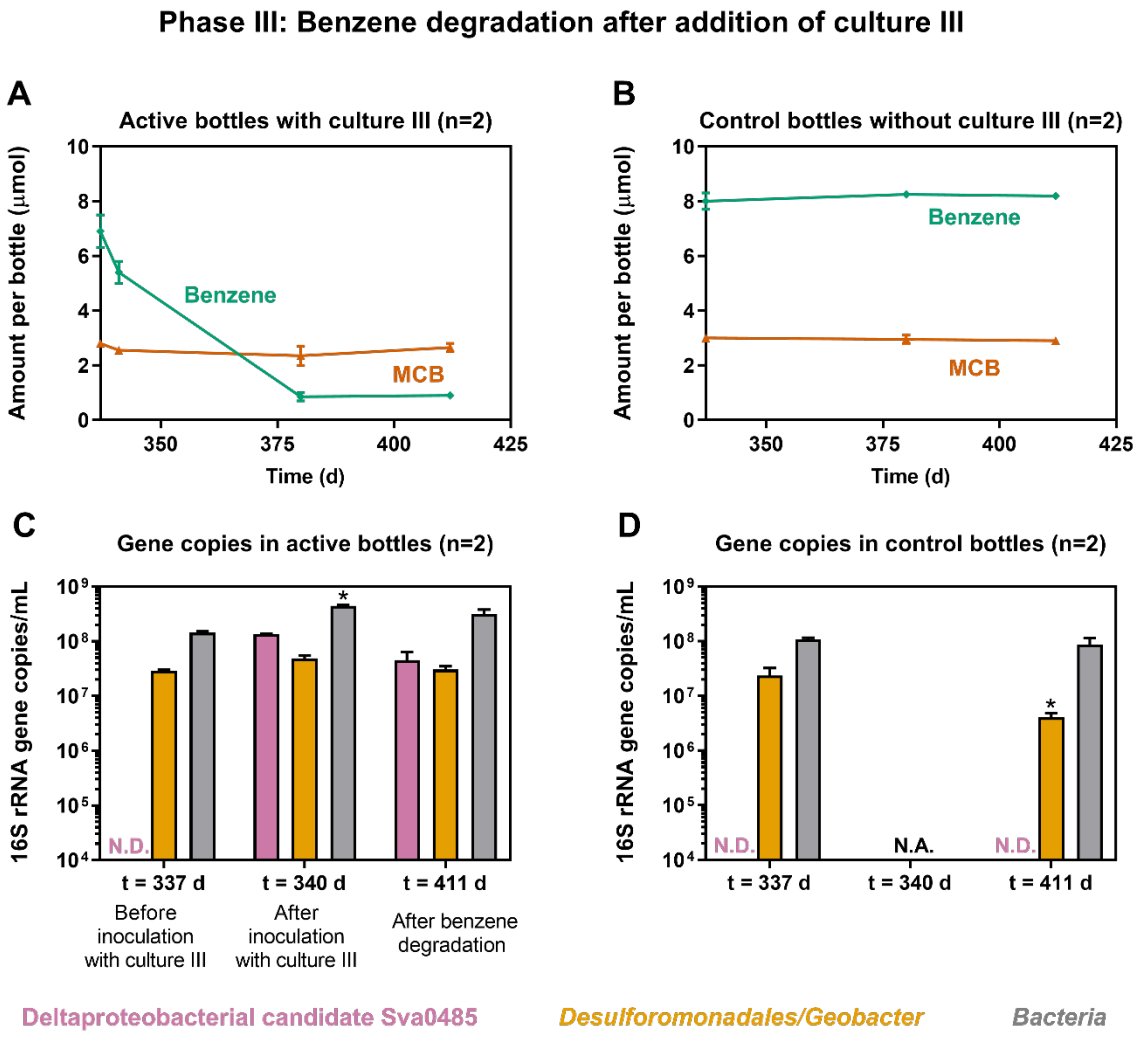
360 the three bioaugmented bottles. In the remaining bioaugmented bottle, benzene degradation
361 occurred as well but at a lower rate. In the controls (not bioaugmented with culture III), benzene
362 degradation did not occur (**Error! Reference source not found.**B).

363 After the bioaugmentation with culture III, the resulting gene copies per mL of
364 Deltaproteobacterial candidate Sva0485 (previously referred to as ORM2), were in the order of
365 10^8 and remained at 10^7 after benzene degradation (**Error! Reference source not found.**C).
366 Recall that ORM2 is the bacterium responsible for the initial attack on the benzene ring. The
367 average amount of benzene produced in the experimental bottles (6.5 mg/L) was likely not
368 enough to maintain the population of ORM2 at such high density (10^8 ORM2 gene copies per
369 mL); culture III is generally maintained at higher concentrations, ranging from 30 to 150 mg/L,
370 in order to support high cell density. The gene copies per mL for
371 Desulfuromomadales/*Geobacter* remained at 10^7 for the active bottles and declined to 10^6 in the
372 control bottles (**Error! Reference source not found.**D). In the active bottles, endogenous
373 electron donors may have been available to sustain the population of
374 Desulfuromomadales/*Geobacter*. In the control bottles, electron donor was not added during
375 phase III, and thus the microbial community derived from phase I likely faced electron donor
376 shortage. The decline in Desulfuromomadales/*Geobacter* copy numbers in these control bottles
377 strongly suggests that these organisms were metabolizing some of the available electron donor
378 which was likely exhausted during the transition from phase II to phase III (methanol was last
379 supplied on Day 296).

380 Neat benzene (not produced from lindane dechlorination) was subsequently added to the three
381 experimental bottles on Day 441 to confirm that the benzene-degrading microbial community
382 remained active. As shown in Figure S4, benzene was indeed biodegraded afterwards. The

383 bottles that were not bioaugmented with culture II or culture III did not exhibit further

384 dechlorination or benzene degradation.



385

386 **Error! Reference source not found.**

387 **MCB is dechlorinated to benzene in culture II by a native KB-1-derived *Dehalobacter***

388 **population distinct from the native *Dehalobacter* population in culture I**

389 As mentioned earlier, culture II originated from a KB-1-derived enrichment culture (referred to

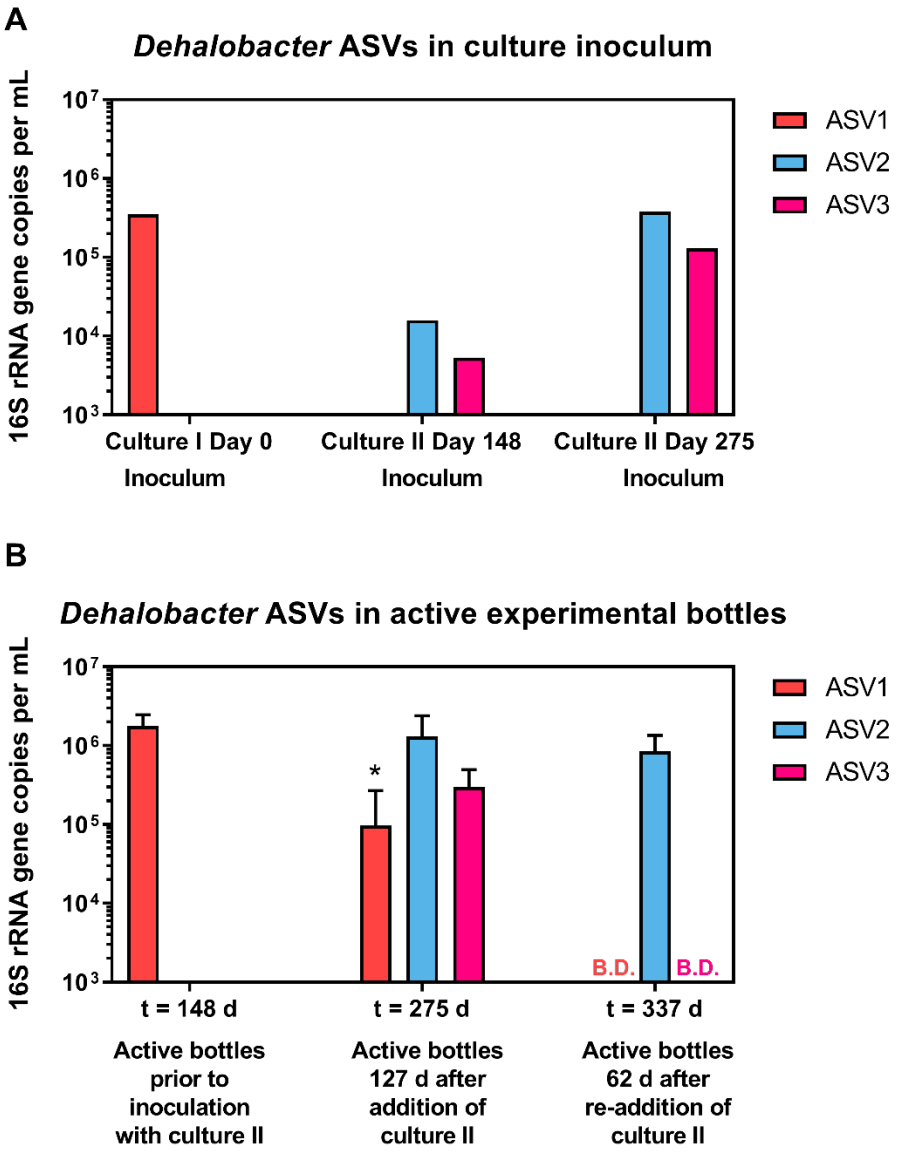
390 in the lab as 1,2,4TCB/M_2014) that dechlorinates 1,2,4-trichlorobenzene (1,2,4-TCB) to a

391 mixture of dichlorobenzene, MCB, and benzene.[26] Culture II (500 mL scale-up) had been
392 maintained on MCB as the sole electron acceptor for about 2 years. To clearly demonstrate
393 *Dehalobacter* population growth during MCB dechlorination to benzene, the culture was
394 sampled frequently over a period of 127 days and MCB/Benzene and *Dehalobacter* abundances
395 were analyzed. As shown in Figure S5, during the dechlorination of ~ 100 μmol of MCB, the
396 relative abundance of *Dehalobacter* increased from 2 to 17% which corresponds to a 12.5-fold
397 change in its population. Assuming one 16S rRNA gene copy per *Dehalobacter* cell, the
398 resulting *Dehalobacter* yield is $2.8 \pm 0.05 \times 10^{10}$ cells per mole of Cl^- released. This yield is two
399 orders of magnitude lower than that reported for the population grown using 1,2,4-TCB as
400 electron acceptor (4.6×10^{12} to 1.8×10^{13} cells per mole Cl^- released).[26] The lower yield may be
401 related to the fact that only two electron equivalents can be transferred per mole of MCB in
402 contrast to four or six electron equivalents per mole of TCB depending on the resulting
403 dechlorination products.

404 As mentioned earlier, in the bottles bioaugmented with culture II, the qPCR-determined
405 *Dehalobacter* gene copies (**Error! Reference source not found.**C) did not decline because the
406 lindane-dechlorinating *Dehalobacter* population was replaced by the MCB-dechlorinating
407 *Dehalobacter* population native to culture II. We were able to demonstrate this after a close
408 examination of the 16S rRNA gene amplicon sequencing data. The *Dehalobacter* in culture I are
409 represented by a single amplicon sequence variant (ASV), while *Dehalobacter* in culture II are
410 represented by two dominant ASVs. The absolute abundance of *Dehalobacter* ASVs in cultures I
411 and II, as well as in the active experimental bottles before and after inoculation with culture II
412 are shown in **Error! Reference source not found.**. The sum of the absolute abundance of all
413 *Dehalobacter* ASVs, which were estimated from the product of the relative ASV abundance and

414 the qPCR-determined Bacteria gene copies, closely matches the qPCR-determined abundance of
415 *Dehalobacter* (Figure S6); this result adds confidence to our approach to estimate the absolute
416 abundance of each ASV of interest.

417 *Dehalobacter* ASV1 belongs to culture I, while *Dehalobacter* ASV2 and ASV3 belong to culture
418 II (**Error! Reference source not found.**A). There are thirteen nucleotide differences between
419 ASV1 and ASV2, twelve differences between ASV1 and ASV3, and only one nucleotide
420 difference between ASV2 and ASV3 (see Figure S7; ASVs can be found in the Supplementary
421 Excel file). After addition of culture II, the population represented by ASV1 declined and was
422 replaced by that of ASV2 and ASV3 which further confirms that the dechlorination of MCB to
423 benzene was carried out by *Dehalobacter* populations originating from culture II. ASV1, the
424 single dominant ASV in culture I is a 100% match to one of the *Dehalobacter* operational
425 taxonomic units (OTU 1134628) reported in Qiao et al.[21] for the HCH-dechlorinating culture set
426 that includes culture I.



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After the second bioaugmentation with culture II, ASV2 remained in the biaugmented bottles and ASV3 was found below detection. The differences observed in the absolute abundance of ASV2 and ASV3 in the different samples indicates that these ASVs represent different *Dehalobacter* populations in culture II. After an examination of historical 16S rRNA gene amplicon sequencing data, we were able to determine that ASV2 is in fact the dominant

434 *Dehalobacter* ASV in the KB-1-derived enrichment cultures that dechlorinate chlorinated
435 benzenes, i.e. this ASV was the most abundant *Dehalobacter* sequence, and representative
436 operational taxonomic unit, in previous 16S rRNA gene amplicon analyses of the KB-1-derived
437 chlorinated benzene-fed cultures. The 468 bp sequence of ASV2 is a 100% match to the
438 corresponding region of the partial 16S rRNA gene (1508 bp, GI: 1221108851) of *Dehalobacter*
439 sp. TCB1 (referred to as KB1_124TCB1 in Puentes Jácome and Edwards [26]). Two additional
440 ASVs, at a relative abundance lower than 0.1%, were also detected in the bottles inoculated with
441 culture II, yet these ASVs were not consistently found among all inoculated bottles. These ASVs
442 might represent other *Dehalobacter* populations that exist in culture II at very low abundances.
443 The mother culture of culture II contains multiple *Dehalobacter* strains,[26, 45] so it is not unusual
444 to detect other ASVs that likely belong to low-abundance populations.

445 **Implications for HCH, MCB, and benzene contamination at field sites**

446 In this study, we have demonstrated for the first time that lindane (γ-hexachlorocyclohexane) can
447 be anaerobically biotransformed by sequential bioaugmentation using specialized microbial
448 enrichment cultures. The sequential treatment resulted in the biotransformation of 73 ± 3% of the
449 added lindane all the way to the non-toxic end products. Not only lindane, but also toxic MCB
450 and carcinogenic benzene were biodegraded by the anaerobic enrichment cultures. Although this
451 study focused on the γ isomer of HCH, this approach is applicable to other HCH isomers since
452 enrichment cultures that dechlorinate α-, β-, and δ-HCH to MCB and benzene are also currently
453 studied in the lab.[21] The success of the different biotransformation steps in this lab scale setting
454 was aided by the sequential nature of the treatment. Keeping the three transformation steps
455 separate could possibly have minimized any detrimental microbial interactions between the
456 different cultures, such as competition for the different electron donors or acceptors present. The

457 enrichment cultures described here have been studied extensively in the lab and in-house
458 techniques for culture growth and maintenance have improved during several years of
459 cultivation. Specifically, benzene-degrading culture III, has recently been evaluated as an
460 inoculum with different field site materials (soil and groundwater) and is undergoing field scale
461 pilot tests. At the field scale, HCH remediation could be tested sequentially, analogous to the lab
462 scale, taking into account the spatial and temporal gradients at the contaminated site and
463 different rates of transformation. This study further establishes the key role of *Dehalobacter* spp.
464 as chlorobenzene- and HCH-organohalide-respiring bacteria and demonstrates that sequential
465 treatment with specialized anaerobic cultures may be explored at field sites in order to address
466 legacy soil and groundwater contamination with HCH.

467

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477

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