Dehalobacter dechlorinates dichloroanilines and contributes to the natural attenuation of dichloronitrobenzenes at a complex industrial site

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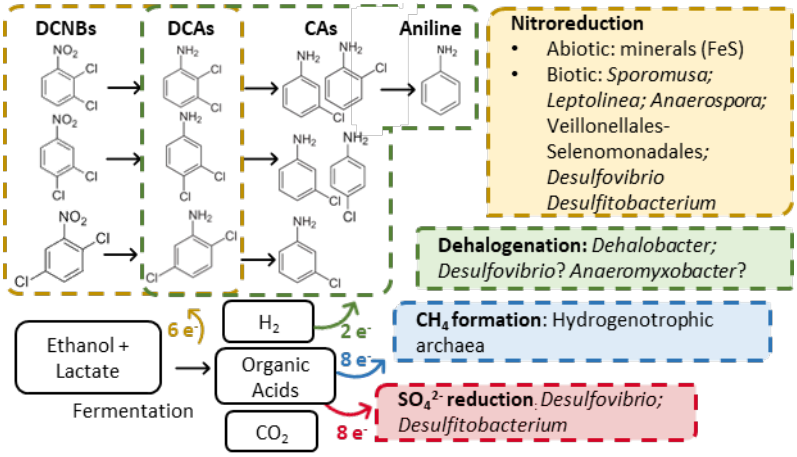
ABSTRACT

The potential for bioremediation of dichloroanilines (2,3- and 3,4-DCA) and dichloronitrobenzenes (3,4-, 2,5- and 2,3-DCNB) was investigated using inocula from an industrial site in northeast of Brazil. Anaerobic biotransformation of these chlorinated compounds was observed in microcosms simulating site conditions, particularly when electron donor was added. To disentangle specific transformation reactions, sub-cultures from active anaerobic microcosms were enriched with individual DCA or DCNB isomers. In these single-compound enrichment cultures, DCNB isomers were completely reduced to the corresponding DCA isomers. Each DCA isomer was stoichiometrically dechlorinated to the corresponding monochloroaniline (CA) isomers. Further dechlorination of CA to aniline was noted. The reduction of DCNB to DCA was sustained by known fermenters and possibly *Desulfitobacterium*, while *Dehalobacter* was clearly responsible for dechlorination of DCAs. These findings helped to attribute function to microbial community data retrieved from field samples to inform the conceptual site model and enable identification of bioactive zones and rate-limiting conditions at a complex site. More generally, these data broaden the understanding of the metabolic repertoire of *Dehalobacter* and provide a model for incorporating microbial community data into site remediation efforts.

38 **Graphical Abstract**



39

40 Keywords: Dichloronitrobenzene; Dichloroaniline; Monochloroaniline; anaerobic
41 biotransformation; bioremediation.

42 **Short synopsis statement**

43 A diverse microbial community contributes to natural attenuation at a complex contaminated
44 site. Notably, dichloroanilines produced by biologically-mediated nitroreduction of
45 dichloronitrobenzenes were dechlorinated by *Dehalobacter*.

46 **1 INTRODUCTION**

47 Many historically-contaminated sites worldwide are a challenge to remediate owing to complex
48 mixtures of toxic contaminants and heterogeneous stratigraphy. For example, an industrial facility
49 located in Bahia in NE Brazil has been the subject of several remedial investigations.[1,2] As a former
50 manufacturing facility of the herbicide diuron, it is impacted by high concentrations of multiple
51 compounds including dichloronitrobenzenes (DCNB; ~3,000 mg/kg) and dichloroanilines (DCA;
52 ~700 mg/kg) among many others.[1,2] Anoxic conditions typically prevail in regions with low

53 permeability, high organic matter, or high contaminant concentrations, where the rate of oxygen
54 depletion exceeds rates of replenishment by diffusion or groundwater flow. Therefore, we sought
55 to understand the fate of DCNB and DCA isomers under anoxic conditions with particular
56 attention to associated microbial populations to elucidate microbial pathways and thus create a
57 better conceptual model of the site and a more effective remediation strategy.

58 In subsurface anoxic environments, nitroaromatic compounds tend to become reduced to their
59 corresponding aromatic amines. As early as 1975, the pesticide pentachloronitrobenzene was
60 shown to be reduced to pentachloroaniline.[3] Nitro group reduction to the corresponding amine is
61 now understood to involve broad specificity and oxygen-independent nitroreductases and related
62 enzymes that are widely distributed in bacteria and archaea.[4] These reactions produce toxic
63 transient nitroso and hydroxylamino intermediates, which are highly reactive and unstable.[4–6]
64 Nitroaromatic reduction may also be a respiratory process akin to nitrate reduction.[7–9] A 1983
65 study reported the reduction of 3,4-DCNB to a variety of poorly quantified products including 3,4-
66 DCA, and condensation products.[5] Despite their environmental relevance, few studies have
67 investigated the anaerobic biodegradation of DCNB isomers.

68 Literature on the anaerobic biodegradation of chloroanilines is also limited. The reductive
69 dechlorination of 3,4-DCA to a mixture of monochloroaniline isomers (3-CA and 4-CA) under
70 anaerobic/methanogenic conditions was first reported in 1989 and 1990, but the organisms
71 responsible were not identified.[10–12] Under nitrate-reducing conditions, a novel reductive
72 deamination reaction was observed in a *Rhodococcus* strain where 3,4-DCA was deaminated
73 primarily to dichlorobenzene,[13] however, additional reports of this process could not be found.
74 More recently, *Dehalococcoides mccartyi* strain CBDB1 and *Dehalobacter sp*. strain 14DCB1,
75 both obligate organohalide-respiring-bacteria (OHRB) that dechlorinate chlorinated benzenes,

76 were shown to dechlorinate 2,3-DCA to 2- and 3-CA and 3,4-DCA to 3-CA with no further
77 transformation when acetate was provided as carbon source and hydrogen as electron donor.[14]
78 Reports of further dechlorination of monochloroanilines to aniline are sparse and inconclusive.
79 Early reports (1997-98) described dechlorination of DCA to CA and then to small amounts of
80 aniline under sulfate-reducing conditions.[15,16] These studies did not characterize the microbial
81 community. A 2019 study showed that *Geobacter* sp. KT5 could transform dichloroanilines under
82 iron-reducing conditions; mineralization of the products via aniline and 4-aminobenzoate was
83 proposed.[17] Two years later, a mixed culture composed of *Geobacter* sp., *Paracoccus*
84 *denitrificans*, *Pseudomonas* sp., and *Rhodococcus* sp. strains was described to transform propanil
85 to 3,4-DCA, and further to 3-CA, chlorobenzene, and aniline, prior to mineralization.[18] Another
86 study found that 2-aminoanthraquinone-graphene oxide enhanced dechlorination in an enriched
87 anaerobic sludge community that was able to dechlorinate 2-CA. In this work, some aniline and
88 hexanoic acid were detected and organisms from *Oscillospira*, *Lactobacillales*, *Veillonellaceae*,
89 and *Ruminococcus* groups were positively correlated with 2-CA transformation.[19]
90 Given the scarce literature on the biodegradation of dichloronitrobenzenes and dichloroanilines,
91 the goal of this study was to identify organisms responsible for transformation reactions of such
92 compounds and to use the information to design biomarkers to track biodegradation and delineate
93 bioactive zones across a contaminated site. We determined that *Dehalobacter* plays a very active
94 and critical role in the dechlorination of dichloroanilines at a complex site in Brazil. This
95 knowledge is crucial for developing cost-effective *in situ* remediation strategies for many other
96 complex contaminated sites.
97
98

99 # 2 MATERIALS AND METHODS

100 ## 2.1 Chemicals and stock solutions

101 Substrates added to enrichment cultures in this study were 3,4-dichloronitrobenzene (3,4-
102 DCNB), 2,5-dichloronitrobenzene (2,5-DCNB), 2,3-dichloronitrobenzene (2,3-DCNB), 3,4-
103 dichloroaniline (3,4-DCA), and 2,3-dichloroaniline (2,3-DCA) (Sigma Aldrich, purity > 99%,
104 (Figure S1). Stock solutions of each compound were prepared in acetone (Sigma Aldrich) at a
105 concentration of 5 g/L. Ethanol (100%: Sigma Aldrich) and 60% sodium lactate (TCI America)
106 were used as electron donors. The mentioned DCNB and DCA isomers, aniline, 2-, 3-, and 4-
107 chloroaniline (CA), 2,5-DCA, DCB isomers (Sigma Aldrich, purity > 99%), and methane (Scott)
108 were also used as analytical standards. Methanol (Sigma Aldrich) was used as the solvent for the
109 chlorinated standards and acetonitrile (Fisher Scientific) was used for the mobile phase for liquid
110 chromatography (described below).

111 ## 2.2 Preparation of original microcosm study and enrichment cultures

112 The original microcosms were constructed in 2015 with solid subsurface material and
113 groundwater from the industrial facility in Brazil, incubated under anaerobic conditions at site pH
114 (4.6 to 6.5), and amended with various electron donors and acceptors. The microcosms were
115 amended with various mixtures of chloroanilines, dichloroanilines, dichloronitrobenzenes and
116 chlorobenzenes at concentrations typical of the site, and biodegradation was monitored for over
117 one year. The microcosm setup is detailed in Supporting Information Text S1 and in a Master's
118 Thesis.[20] Results are summarized in Table S1 and Figure S2.

119 Five microcosms established with electron donors to stimulate methanogenic conditions
120 (Cam16, Cam17, Cam64, Cam88, and Cam89) showed the most activity and were therefore
121 selected for enrichment (Figure S1). All transfer cultures as well as controls were prepared in
122 autoclaved 250-mL Boston round bottles sealed with Mininert caps, containing 100 mL of
123 anaerobic mineral medium (pH 7) reduced with iron sulfide (FeS).[21] Only medium supernatant
124 was used to avoid adding precipitated FeS. To simulate site groundwater conditions and to promote
125 mineralization of nitrogen-containing compounds, ammonium was omitted from the medium
126 recipe. All cultures were incubated statically in the dark at room temperature in an anaerobic
127 glovebox (Coy) supplied with a gas mix of N_2:CO_2:H_2 (80:10:10%).

128 The first transfer (T1) cultures were inoculated in March 2020 with the cell pellet from the
129 respective parent microcosm. The pellet was obtained from a 40 ml sample from a shaken
130 microcosm by centrifuging at 12,000 g for 15 min. These T1 enrichment cultures were amended
131 with individual target compounds and ethanol and lactate as electron donors (Figure S1). An
132 autoclaved killed control (KC) was also prepared with a cell pellet from an original microcosm
133 (Cam 65). This control was autoclaved three times on three consecutive days prior to being
134 amended with a mix of six compounds (3,4-DCNB, 2,5-DCNB, 3,4-DCA, 2,3-DCA, 2-CA, and
135 aniline). An abiotic control (AC1) was also established containing only reduced mineral medium
136 amended with a slightly different set of six compounds (3,4-DCNB, 2,3-DCNB, 3,4-DCA, 2,3-
137 DCA, 2-CA, and aniline).

138 The 5 active T1 transfer cultures, labelled 34DCNB-T1, 25DCNB-T1, 23DCNB-T1, 34DCA-
139 T1 and 23DCA-T1, were repeatedly amended with individual chlorinated substrates (Figure S1).
140 These semi-volatile substrates (electron acceptors) were added as evaporated acetone stocks as
141 described in Text S2. The target concentration was initially 10 mg/L, which was increased to ~27

142 mg/L over time. Electron donors were also added to the active cultures and the two controls (AC1
143 and KC) initially and were re-amended to active cultures when degradation rates slowed. Electron
144 donor was added in ~17-fold excess in electron equivalents (eeq) considering both nitroreduction
145 and complete dechlorination of DCNB to aniline (Text S2). The initial pH was between 6.6 and
146 7.1 in all active enrichments.

147 A second set of transfer cultures (T2) were prepared from T1 cultures after ~ 4 months (Figure
148 S1). The DCNB T2 cultures were inoculated with a 50% transfer from T1 cultures (50 mL into 50
149 mL fresh medium) while DCA T2 cultures were inoculated with a 10% transfer from T1 cultures
150 (10 mL of T1 into 90 mL). Additional mineral medium supernatant was added to a culture bottle
151 when its volume was depleted to below 50 mL due to sampling. Occasionally, a little more medium
152 was added to gradually increase the volume of culture. A second abiotic control (AC2) was
153 prepared containing 3,4-DCNB, 2,3-DCNB, 3,4-DCA, and 2,3-DCA, and mineral medium devoid
154 of FeS. The data presented herein focus on the second transfer (T2) enrichment cultures.

155 **2.3 Analytical procedures**

156 Concentrations of DCNB, DCA and CA isomers and aniline were measured using a Hewlett-
157 Packard 1050 series high-performance liquid chromatography (HPLC) system (Acclaim™ 120
158 C18 column, Thermo Scientific) equipped with a UV detector (set at 254 nm). The mobile phase
159 was an isocratic 50:50 mixture of Milli-Q water and acetonitrile (Millipore Sigma) at 1 mL/min
160 with a runtime of 25 min. Samples (2 mL) were taken from cultures using a 2 mL gastight glass
161 syringe and filtered through a 0.22 μm (13 mm) Chromspec PTFE syringe filter (pre-wetted with
162 methanol) prior to being injected into the HPLC. Lactate, acetate, propionate, formate, butyrate,
163 pyruvate, chloride, nitrate, nitrite, sulfate, and phosphate were measured by ion chromatography

164 (IC) using a Dionex™ Integrion™ IC system (Thermo Fisher Scientific). Methane was measured
165 in headspace samples by gas chromatography (GC) using an Agilent 7890A gas chromatograph
166 with headspace autosampler G1888 (Agilent Technologies). Method details are provided in Text
167 S3.

168 **2.4 Microbial community analysis and qPCR**

169 Well-mixed samples (4 mL) from enrichment cultures were collected periodically, centrifuged
170 at 10,000 rpm for 20 minutes, the supernatant removed, and cell pellets stored at -80ºC. DNA was
171 extracted using the DNeasy PowerSoil Kit (Qiagen) and quantified using a Qubit® 3.0
172 Fluorometer (Thermo Fisher Scientific). DNA samples were sent for Illumina MiSeq PE300
173 amplicon sequencing at an external laboratory (Genome Quebec Innovation Centre, Montreal,
174 Canada), using modified "staggered end"[22] versions of primers 926f-modified (5'-
175 AAACTYAAAKGAATWGRCGG-3') and 1392r-modified (5'-ACGGGCGGTGWGTRC-3'),
176 which target the V6−V8 region of the 16S rRNA gene from both Bacteria and Archaea, as well as
177 the 18S rRNA gene in Eukarya.[23,24] Sequencing data was processed using QIIME 2 version
178 2021.2.[25] After trimming the primer region with the cutadapt plug-in, amplicon sequence variants
179 (ASVs) were generated using the DADA2 plugin,[26] and taxonomy was assigned to the ASVs
180 through the feature-classifier plugin using the SSU SILVA v.138 database.[27] Data were further
181 processed in Excel. Sequences <400 bp in length were removed and ASVs were ranked by
182 maximum sequence abundance across all DNA samples. ASVs with abundance greater than 1%
183 in at least one sample were kept for graphing. The Basic Local Alignment Search Tool (BLAST)
184 available at the National Center for Biotechnology Information (NCBI) was used to compare ASV

185 sequences of interest. Raw DNA sequences were uploaded to the NCBI (Accession:
186 PRJNA1040917; Table S2).

187 DNA samples were also analyzed by quantitative polymerase chain reaction (qPCR) using a
188 CFX96TM real-time PCR detection system (Bio-Rad Laboratories Inc.). Primers GenBac1055f
189 (5'-ATGGYTGTCGTCAGCT-3') and GenBac1392r (5'-ACGGGCGGTGTGTAC-3') targeting
190 most bacteria[24,28,29] and primers GenArch787f (5'-ATTAGATACCCGBGTAGTCC-3') and
191 GenArch1059r (5'-GCCATGCACCWCCTCT-3') targeting most archaea[30] were used. A no-
192 template control was also included in each run. All qPCR samples were run in triplicate. Details
193 on the qPCR method are provided in Text S4. Absolute total bacterial and archaeal abundance
194 obtained by qPCR (copies 16S rRNA gene per ml) was used to convert the relative abundances
195 obtained from amplicon sequencing to absolute abundance for each ASV.

196 A total of forty-eight DNA samples were sequenced and analyzed by qPCR, including samples
197 from the original subsurface solid material, microcosms, and enrichments. On average 66,000 16S
198 rRNA gene amplicon reads were retrieved per sample (Table S3a). Reads were grouped by bacteria
199 and archaea, then sequences with relative abundance >1% in any sample (Table S3b for bacteria
200 and Table S3c for archaea) were used to calculate absolute abundances (copies/mL) of each ASV
201 using total abundances of bacteria and archaea determined by qPCR (Table S4). Final absolute
202 abundances for each ASV (copies/mL) are reported in Tables S5a to S5f for bacteria; Tables S6a
203 to S6f for archaea. Finally, Non-metric Multi-dimensional Scaling (NMDS) and bubble plots were
204 constructed as explained in Text S5.

205

206

207 # 3 RESULTS AND DISCUSSION

208 ## 3.1 Abiotic transformation of DCNB was not sustained

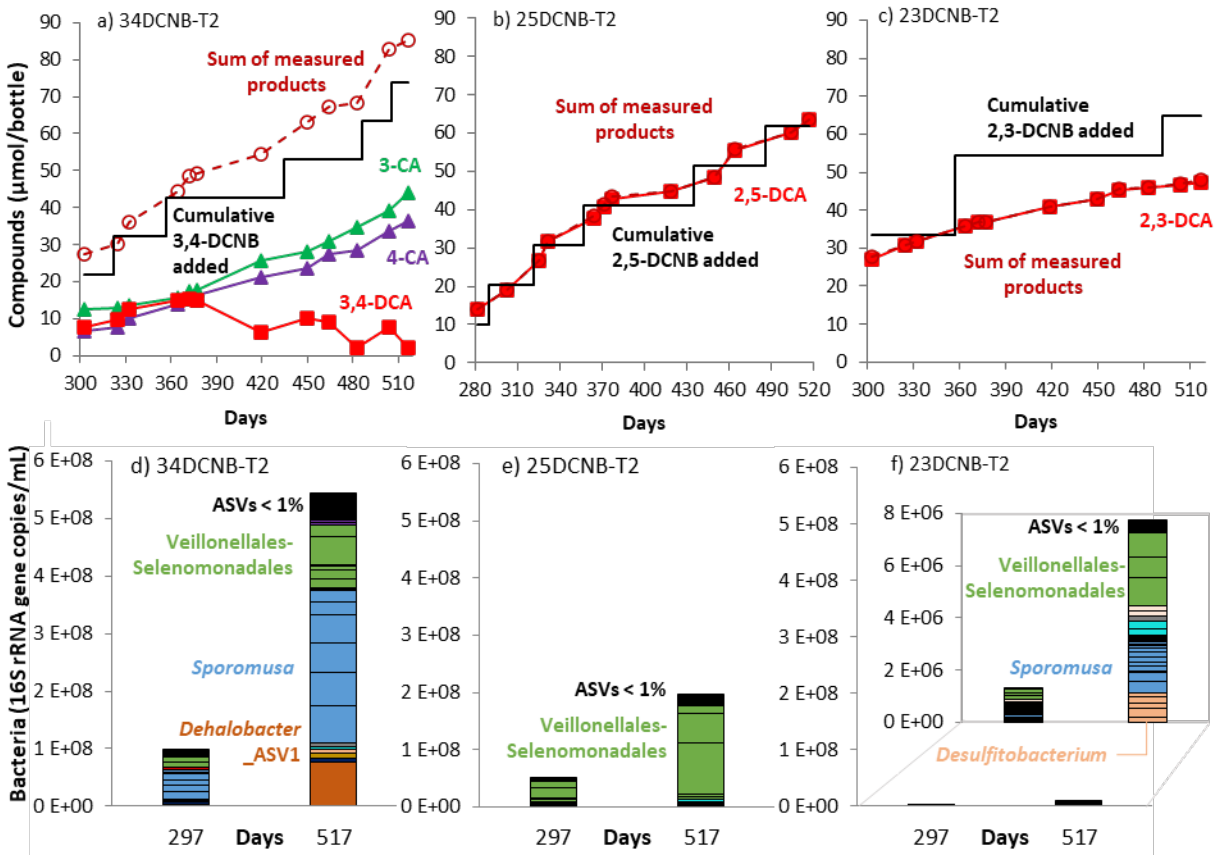
209 Contaminant profiles in three controls (AC1, AC2 and KC) were assessed for possible abiotic
210 reactions. Chloroanilines and aniline were not transformed in any of these controls.
211 Dichloronitrobenzenes were not transformed in medium control (AC2) without FeS (Figure S3;
212 Table S7). In the medium control (AC1) with FeS, 3,4-DCNB was not transformed, but 2,3-DCNB
213 was reduced to 2,3-DCA (Figure S4; Table S7); however, this activity was not sustained upon
214 reamending with 2,3-DCNB. These data are consistent with reports that reduced minerals, such as
215 FeS or perhaps reduced resazurin, can sometimes mediate reduction of the nitro group. [31–38]
216 Curiously, DCNB isomers were repeatedly transformed to DCA in the autoclaved control KC
217 (Figure S5; Table S7); however later analyses (to be discussed) revealed that KC was in fact not
218 sterile.

219 ## 3.2 Biologically-mediated nitroreduction and dechlorination

220 Each transfer culture established on single substrates remained active, sustained by refeeding
221 chlorinated substrate and electron donors as needed for over 500 days. Historical data are provided
222 in Table S8 (T1) and Table S9 (T2). Focusing on T2 transfers from day 280 to 520 and on the three
223 DCNB-amended cultures first, we observed consistent and complete transformation of each
224 DCNB isomer to the corresponding dichloroaniline (Figure 1a-c). In the culture amended with 3,4-
225 DCNB the product, 3,4-DCA, was further dechlorinated to 3- and 4-CA (Figure 1a), while in the
226 cultures amended with 2,3- and 2,5-DCNB, the transformation stopped at the corresponding
227 dichloroaniline (Figures 1b and 1c). The sum of measured chlorinated products agreed well with

228 the cumulative DCNB added. Reduction of 2,3-DCNB (<~1 µM per day) was ~5 times slower

229 than the reduction of either 3,4- or 2,5-DCNB (5-6 µM per day).



230

231 **Figure 1.** Degradation profiles (upper panels a), b) and c); µmole/bottle) and corresponding major

232 microbial taxa (lower panels d), e) and f); copies/mL) in DCNB-amended T2 transfer cultures from

233 day 280 to 520. Cumulative DCNB added (———) is shown for each amendment (step) of 70 to

234 140 µM (10 to 20 µmol per bottle). The sum of products (- -o- -), including DCA (—■—), 4-

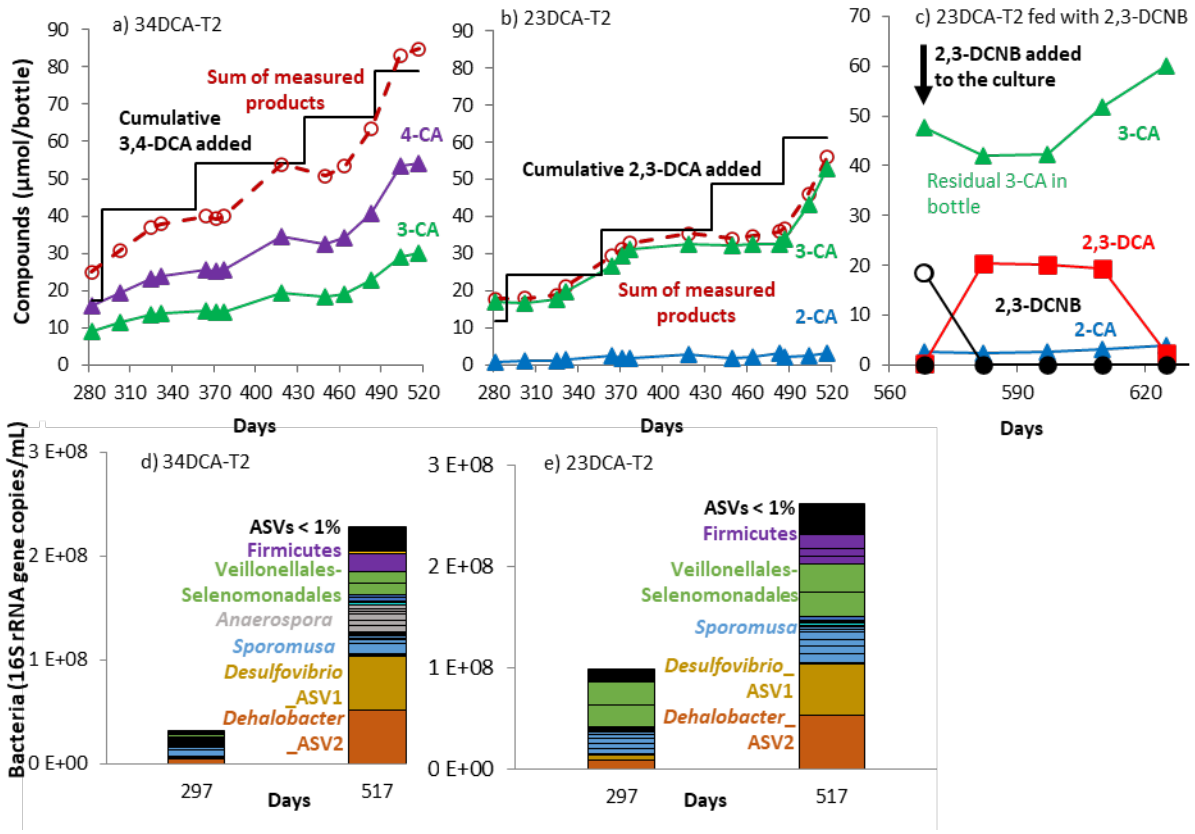
235 CA (—▲—), 3-CA (—▲—), 2-CA and aniline (not shown, mostly below detection), illustrates a

236 reasonable mole balance. Measured DCNB concentrations are not shown for clarity but can be

237 found in Table S9. In lower panels amplicon sequence variants (ASVs) from the same taxonomic

238 designation are assigned the same colour.

239 Focusing next on the two DCA-amended transfers, 3,4-DCA was dechlorinated to a mix of 3-
240 and 4-CA (Figure 2a), while 2,3-DCA was dechlorinated primarily to 3-CA, with traces of 2-CA
241 (Figure 2b). These cultures dechlorinated about ~5 to 6 µM DCA to CA per day.



242

243 **Figure 2.** Degradation profiles (upper panels a), b) and c); µmole/bottle) and corresponding major
244 microbial taxa (lower panels, d) and e); copies/mL) in two DCA-amended T2 transfer cultures
245 from day 280 to 520 (panels a), b), d), and e)) and from day 560 to 625 (panel c)). In panels a) and
246 b), cumulative DCA added (———) is shown for each amendment (step) of ~80 µM (~12 µmol
247 per bottle). The sum of products (- -o- -), including DCA (—■—), 4-CA (—▲—), 3-CA (—▲—
248), 2-CA (—▲—), and aniline (not shown, mostly below detection), illustrates a reasonable mole
249 balance. Measured DCA concentrations are not shown for clarity but can be found in Table S9.
250 Panel c) shows degradation profile after 2,3-DCA-T2 was fed with 2,3-DCNB (—●—) instead of

251 2,3-DCA on Day 568. The open circle shows the amount of 2,3-DCNB fed to 23DCA-T2 (—○—
252). In lower panels amplicon sequence variants (ASVs) from the same taxonomic designation are
253 assigned the same color.

254 Given the very slow transformation rate in 23DCNB-T2, we wondered if the enrichment culture
255 dechlorinating 2,3-DCA (23DCA-T2; Figure 2b) could also reduce 2,3-DCNB, even though this
256 culture - and the microcosm (Cam16) from which it was derived - had never been exposed to any
257 DCNB isomer. Surprisingly, 2,3-DCNB was reduced without a lag to 2,3-DCA which was further
258 dechlorinated to 3-CA (Figure 2c). We then tested all active DCA-degrading cultures with a mix
259 of the three DCNB isomers; all the cultures were able to reduce DCNBs to their respective DCA
260 isomer (data not shown).

261 **3.3 Microbial community composition reflects electron balance**

262 To relate the microbial community to activity, we first identified abundant bacterial and archaeal
263 ASVs (>1%) and then calculated their absolute abundance in active cultures focusing on 2-time
264 points for each sample. Figure 3 provides a summary of the most relevant taxa identified. Most of
265 the enriched bacterial ASVs belong to the phylum Bacillota (Firmicutes) including *Sporomusa*,
266 *Anaerospora*, and unassigned members of the Veillonellales-Selenomonadales. These organisms
267 clearly grew on the ample electron donor amendments to the cultures. ASVs for
268 *Anaeromyxobacter*, *Desulfitobacterium* and *Desulfovibrio* were also retrieved. These genera are
269 also often implicated in nitrate or sulfate reduction though some members may be facultative
270 dechlorinators. ASVs for obligate dechlorinators of the genus *Dehalobacter* were also identified
271 (Figure 3). A complete overview of all major taxa (>1%) are shown in Figure S6, for bacteria, and
272 Figure S7, for archaea.

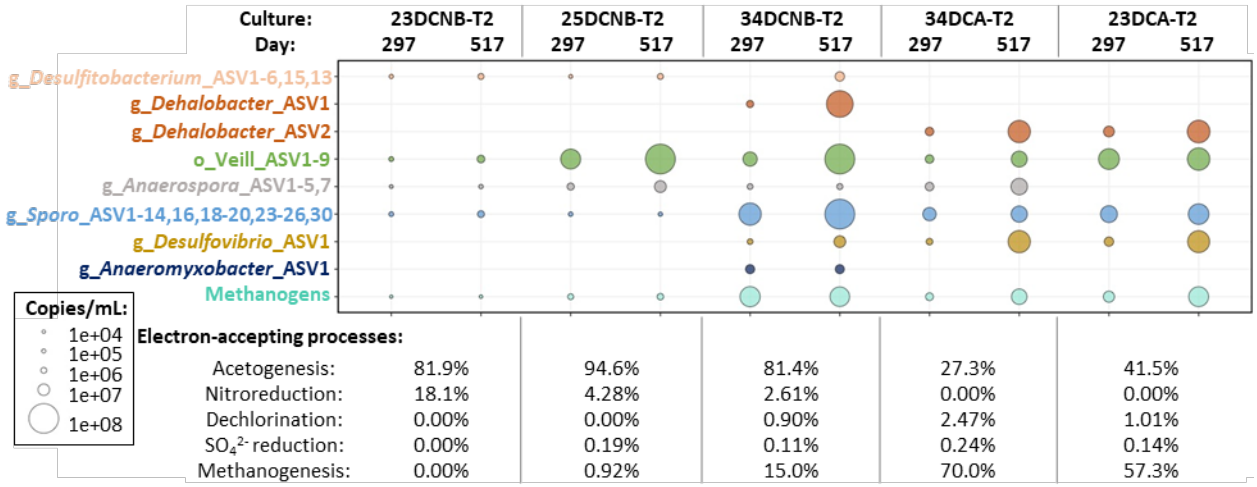


Figure 3. Absolute abundance of major organisms (copies/mL) and proportion of electron equivalents from amended donors channeled to each electron-accepting processes (% electron equivalents) for days 297 and 517. Veillonellales-Selenomonadales order is shortened to "Veill"; *Sporomusa* genus is shortened to "*Sporo*". Calculated absolute abundances for all ASVs are available in Table S5 (bacteria) and Table S6 (archaea).

To confirm the extent of dechlorination, an electron balance of possible donors and acceptors was completed using concentrations of chlorinated organics (Table S9) and anions (Table S10). As expected from stoichiometry (e.g., $C_6H_5Cl_2N + H_2 \rightarrow C_6H_6ClN + HCl$), chloride concentrations increased proportionally in cultures that exhibited dechlorination (34DCNB-T2, 34DCA-T2, and 23DCA-T2). The measured increase in chloride was slightly higher than expected in almost all cases (Table S11), likely because of the error associated with measuring small changes against a high background of ~1900 μM chloride.

Potential electron-accepting processes include acetogenesis, methanogenesis, nitroreduction, dechlorination, and sulfate reduction. For T2 cultures between days 280 and 520, we calculated the proportion of electron equivalents from added donors channeled to each of the different

289 electron-accepting processes (Figure 3, bottom table). In DCNB-amended cultures, acetogenesis
290 from lactate and ethanol was the dominant process, accounting for over 80% of electrons from
291 donors. Methanogenesis was severely limited. By contrast, in DCA-amended cultures,
292 methanogenesis accounted for 60-70% of electron equivalents from donors, which combined with
293 acetogenesis accounted for over 98% of electron equivalents. A buildup of acetate in all cultures
294 (Tables S10 and S12) suggested possible inhibition of acetoclastic methanogens specifically,
295 particularly by DCNBs. Culture 34DCNB-T2 produced the most methane among DCNB-amended
296 cultures but also exhibited the most rapid and extensive nitroreduction and dechlorination, thus
297 relieving inhibition associated with DCNBs and DCAs.

298 Other electron accepting processes accounted for a much lower proportion of activity (Figure
299 3). Nitroreduction consumed 5% of electron equivalents in cultures fed with 3,4- and 2,5-DCNB
300 isomers and up to 20% of electron equivalents in culture 23DCNB-T2, although activity in this
301 culture was very low and no lactate was consumed (Tables S10 and S12). In all cases,
302 dechlorination accounted for less than 3% of all electron equivalents provided. The small amount
303 of sulfate (~30 μM) present in the medium was rapidly depleted in the 4 highly active cultures
304 (34DCNB-T2, 25DCNB-T2, 34DCA-T2, and 23DCA-T2). However, less than 1% of electron
305 equivalents were channeled to sulfate reduction. In culture 23DCNB-T2, sulfate was not
306 consumed, consistent with low activity observed in this enrichment (Table S11).

307 We compared the proportions of electrons consumed by each electron-accepting process with
308 corresponding microbial proportions (Figure 3) to infer function (Table S12). Methane production
309 was greatest in cultures 23DCA-T2 and 34DCA-T2, lower in 34DCNB-T2, very low in 25DCNB-
310 T2 and absent in 23DCNB-T2. Methanogens were correspondingly most abundant in 23DCA-T2,
311 34DCA-T2, and 34DCNB-T2. ASVs corresponding to hydrogenotrophic *Methanoregula*

312 dominated the archaeal population, with a much lower abundance of *Methanocella* and other
313 hydrogenotrophic genera (Figure S7). The presence of only hydrogenotrophic methanogens
314 combined with substantial accumulation of acetate strongly indicates that DCNB isomers and to a
315 lesser extent DCA isomers inhibited acetoclastic methanogens.

316 **3.4 Nitro group reduction can be mediated by many different microbes**

317 We had hoped to observe enrichment of specific microbes in DCNB-amended cultures to enable
318 functional attribution. Rather, it seems that many different genera can likely reduce the nitro group.
319 Biologically regenerated FeS is unlikely to play a role as a reductant, as there was no evidence of
320 sulfate or iron cycling in the cultures, and the rates of DCNB reduction were similar regardless of
321 FeS concentrations. In all cultures, there was significant representation of fermenting or acetogenic
322 microbes, particularly Veillonellales-Selenomonadales, *Sporomusaceae*, *Sporomusa*, and
323 *Anaerospora* (Figure S6). This is consistent with the ample supply of ethanol and lactate to all
324 cultures.

325 We wondered if these fermenting and acetogenic organisms could also be reducing DCNB to
326 DCA as a growth-associated electron-accepting process akin to nitrate reduction. In 25DCNB-T2,
327 where only nitroreduction was observed without dechlorination, 6 ASVs (ASV1 to 5 and 8) (>90%
328 identity) classified only to the order level as Veillonellales-Selenomonadales were predominant
329 (Table S5b). They represented more than 80% of the bacterial community in this culture on day
330 517 (Figure 1e). The closest matches to these sequences are affiliated with the genera *Pelosinus*,
331 *Anaerospora*, *Sporomusa*, *Dendrosporobacter*, among other organisms; interestingly, one of the
332 closely matching sequences (KF460373.1) comes from a study on the impact of nitrate on
333 pentachlorophenol dechlorination[39] (Figure S8).

334 In fact, ASVs from Veillonellales-Selenomonadales were abundant in all 5 active cultures
335 (Figures 1d, e, and f, Figures 2d and e and Figure 3), whether fed DCNB or DCA, making
336 functional attribution difficult. Finding that DCNB isomers could be reduced in all cultures
337 regardless of enrichment substrate is perhaps then not so surprizing. In the autoclaved "killed"
338 control (KC) that was amended with a mixture of compounds (3,4-DCNB, 2,5-DCNB, 3,4-DCA,
339 2,3-DCA, 2-CA, aniline) both 3,4-DCNB and 2,5-DCNB were reduced to the corresponding DCA
340 isomers (Figure S5) but we suspected that it was not sterile. DNA was readily extracted from this
341 control, and although the total microbial abundance was an order of magnitude lower than other
342 cultures, the KC culture was dominated by a unique Butyricoccaceaea ASV as well as other spore-
343 forming and presumably heat-resistant microbes. Among the microbes detected in KC after
344 refeeding 3,4- and 2,5-DCNB 4 times, *Desulfitobacterium* and *Anaerospora* were also detected in
345 other active nitroreducing cultures (i.e., 23DCNB-T2 and 25DCNB-T2) (Figure S6), suggesting
346 these genera are also candidates for nitroreduction. Ultimately it became clear that nitroreduction
347 is a widespread microbial activity that was easy to stimulate with electron donor in the cultures.

348 **3.5 Dechlorination of 2,3- and 3,4-DCA supports growth of *Dehalobacter***

349 In actively dechlorinating cultures, *Dehalobacter* was the only microbe to systematically show
350 a substantial increase in abundance. *Dehalobacter* was not detected in cultures that were only
351 catalyzing nitroreduction. Given that it is an obligate organohalide respiring bacterium, we
352 conclude that *Dehalobacter* was responsible for the DCA-dechlorinating activity in enrichment
353 cultures amended with 3,4-DCNB, 3,4-DCA, and 2,3-DCA (Figures 1d and 2d and e).

354 Two distinct *Dehalobacter* ASVs were identified: ASV1 in 34DCNB-T2 and ASV2 in 34DCA-
355 T2 and 23DCA-T2 (Text S6), with maximum bacterial abundances of 14, 25, and 31% in each

356 culture, respectively (Table S3b). ASV1 (468 bp) is 100% identical to several previously published
357 16S rRNA gene sequences of *Dehalobacter restrictus* strains, including strain SAD (CP148032.1),
358 strain DSM 9455 (CP007033.1), and strain 12DCA (CP046996.1), as well as to three
359 *Dehalobacter* strains classified only to the genus level, including strain DCA (CP003869.1), strain
360 DAD (CP148031.1), and strain CF (1CP003870.1). The known substrates for these *Dehalobacter*
361 strains include tetra- and trichloroethene,[40] 1,2-dichloroethane,[41] tri- and dichloromethane,[42] and
362 1,1,1-trichloroethane and 1,1-dichloroethane.[43,44]

363 *Dehalobacter* ASV2 (468 bp) was not a perfect match to any 16S rRNA gene sequence in the
364 NCBI database. It shares only 97.01% identity to ASV1 and 98.93% to *Dehalobacter* strain DCM
365 (CP092282.1) described as "dichloromethane-degrading bacterium from Xi river" (Direct
366 Submission by Yang, Y. *et al*., 13-Jan-2022). Neither *Dehalobacter* ASV1 nor ASV2 sequences
367 matched (<95% identity) to the 16S rRNA gene fragment (937 bp) from *Dehalobacter* sp. strain
368 14DCB1 (JN051267.1), a strain enriched on dichlorobenzene[45] but reported to dechlorinate
369 dichloroaniline.[14]

370 To further confirm the role of *Dehalobacter* in chloroaniline dechlorination, we estimated
371 growth yields from qPCR and concentration data to compare with previously published yields.
372 The net growth yields ranged from 1.4×10^{13} to 6.5×10^{13} cells per mol of chloride released
373 assuming 3 copies of the 16S rRNA gene per cell (Table S13). The yields calculated in this study
374 are consistent with many previously reported yields for other *Dehalobacter* strains using various
375 chloroethanes, chlorobenzenes, and chloromethanes as electron acceptors for growth (Table S14).
376 In fact the yields align with the highest yields previously reported, solidifying chloroaniline
377 dechlorination as a growth-related process.

378 Additional evidence for *Dehalobacter* being responsible for DCA dichlorination was obtained
379 when non-dechlorinating culture 25DCNB-T2 was inoculated with 3 mL of dechlorinating culture
380 34DCNB-T2 (on day 653). After inoculation, residual 2,5-DCA previously formed from 2,5-
381 DCNB was dechlorinated to 3-CA and the relative abundance of *Dehalobacter*_ASV1 increased
382 from zero on day 517 to ~9% on day 653 (Figure S9).

383 A shift in activity in culture 23DCA-T2 was noted as monitoring continued for over 1000 days
384 (Figure S10). As time progressed, 2-CA rather than 3-CA became the major product from 2,3-
385 DCA. Thereafter, 2-CA was slowly yet consistently transformed to aniline. On day 881,
386 *Dehalobacter*_ASV2 constituted 16% of the bacterial community (Table S5e). 2-CA was
387 dechlorinated to aniline in subsequent transfers from this culture as well (data not shown). A
388 subsample of 23DCA-T2 was taken on day 653 for additional growth experiments to ultimately
389 build up enough culture to measure compound specific isotopic enrichment factors. This ongoing
390 research implicates *Dehalobacter* in aniline formation as described in a companion publication.[46]

391 **3.7 Other organisms identified in enriched cultures**

392 Several other ASVs belonging to known facultative OHRB were identified, including
393 *Anaeromyxobacter*, *Desulfovibrio*, and *Desulfitobacterium* (Figure 3). While some members of
394 these genera can dechlorinate, most are known to be quite versatile and may reduce sulfate, nitrate
395 or even grow fermentatively in the absence of a terminal electron acceptor. An *Anaeromyxobacter*
396 ASV found in culture 34DCNB-T2 is 99.79% similar over ~450 bp to the 16S rRNA gene of
397 *Anaeromyxobacter* sp. strain RF71 (MZ254878.1), 99.57% similar to *A. dehalogenans* 2CP-C
398 (CP000251.1) and 99.36% similar to *A. dehalogenans* 2CP-1 (CP001359.1). Both strains 2CP-C
399 and 2CP-1 can gain energy from the reductive dechlorination of chlorophenol and contain putative

400 dehalogenases.[47–49] However, the abundance of *Anaeromyxobacter* in the culture is far less than
401 *Dehalobacter* and is therefore unlikely to be primarily responsible for dechlorination.

402 A single ASV of *Desulfovibrio* increased in abundance over time in the three cultures
403 dechlorinating DCA isomers (Figure 3). *Desulfovibrio* are sulfate-reducing bacteria that can also
404 ferment substrates such as lactate; some can respire organohalides.[50,51] In cultures 23DCA-T2 and
405 34DCA-T2, *Desulfovibrio* increased between days 280 and 520 to similar extent as *Dehalobacter*
406 (Figures 2d and 2e). However, a similar increase was not seen in dechlorinating culture 34DCNB-
407 T2 (Figure 1d). The amount of sulfate in the medium (around 30 μM) is too small to explain this
408 growth, therefore *Desulfovibrio* is more likely metabolizing lactate to acetate, possibly
409 participating in dechlorination alongside *Dehalobacter*. The *Desulfovibrio* ASV in our cultures is
410 100% identical to a non-dechlorinating *Desulfovibrio* sp. (JQ599691.1) detected in a
411 dichloroethane dechlorinating enrichment. [52] The sequence was also 100% identical to an
412 uncultured *Desulfovibrio* clone (AB854345.1) found in a non-dechlorinating environment.[53]
413 Therefore, the more likely role of this organism is fermentation and acetogenesis, in support of
414 dechlorinating *Dehalobacter*.

415 Several ASVs (sharing > 94% identity) were assigned to *Desulfitobacterium*. Interestingly, these
416 ASVs were only found in the cultures reducing DCNBs and in the KC control that exhibited
417 nitroreduction; they were not detected in the DCA-amended cultures (Figure 3). This seems to
418 implicate *Desulfitobacterium* in nitroreduction, however the ASVs were in low abundance.
419 Furthermore, DCA-amended cultures could also rapidly reduce the nitro group, therefore
420 *Desulfitobacterium* is not solely responsible for nitroreduction, if at all. Moreover, the 8 most
421 abundant *Desulfitobacterium* ASV sequences are > 95% similar to a *Desulfitobacterium* sequence

422 found in *Dehalococcoide*s-containing dechlorinating cultures (AB596883.1) (Figure S11), where
423 they were not implicated in dechlorination,[54] a likely scenario in our study as well.

424 **3.8 Data integration and application to improving remediation outcomes**

425 Non-metric multidimensional scaling (NMDS) was used to evaluate relationships among
426 cultures as they were enriched from subsurface material to initial microcosms and finally to
427 enrichment cultures (Figure 4). First, there is clearly an impact of the original source material,
428 shown in red, blue and green for three original sediment sources used to construct the microcosms.
429 Second, there was a convergence of community depending on substrate. Enrichment cultures that
430 dechlorinate DCAs are distinct from those that only carry out nitroreduction. Samples from
431 34DCNB-T2 culture, in which both nitroreduction and dehalogenation are observed, are located
432 between the dechlorinating-exclusive culture samples (34DCA-T2, and 23DCA-T2) and the
433 nitroreducing-exclusive cultures (25DCNB-T2, 23DCNB-T2 and KC). *Dehalobacter* in the top
434 groupings and *Desulfitobacterium* in the bottom two cultures (23DCNB-T2 and KC) are major
435 genera driving the vertical spread of samples in the NMDS plot.

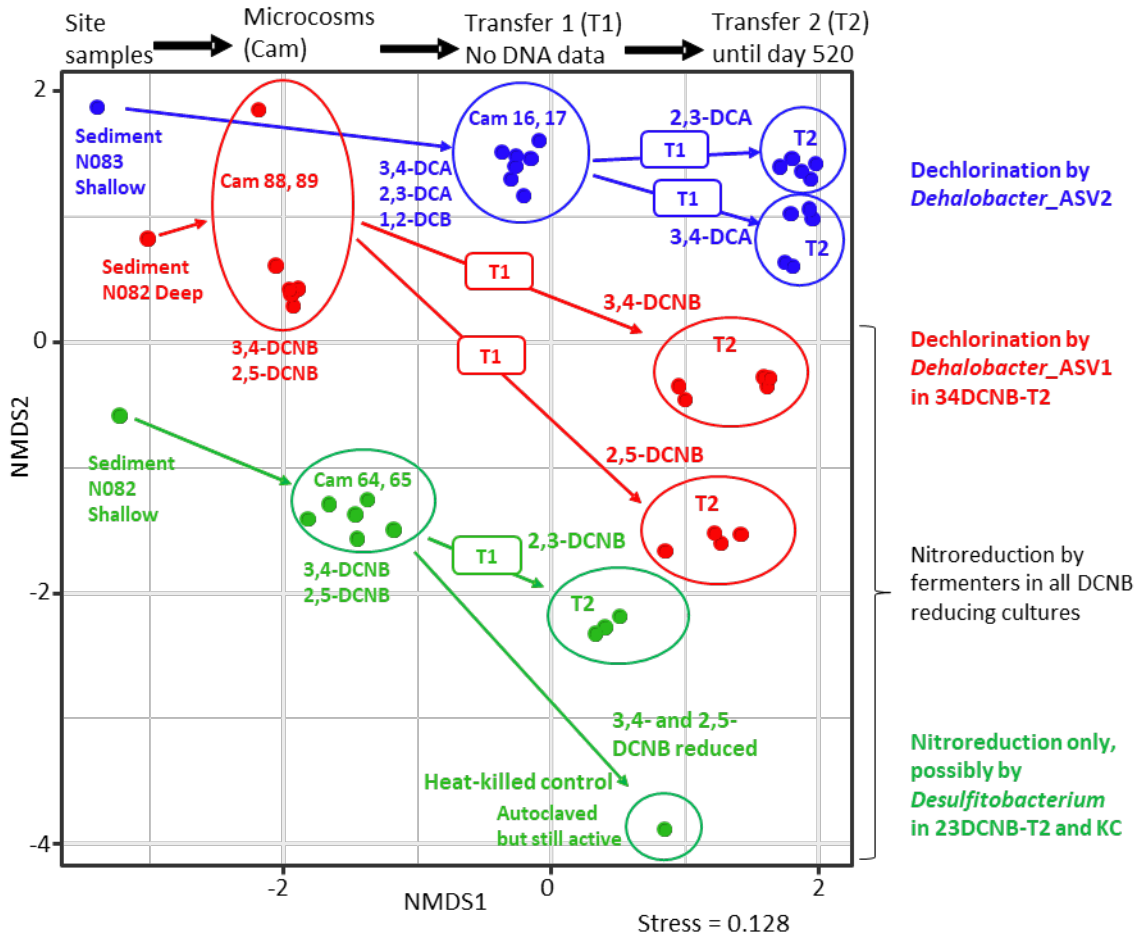


Figure 4. Non-metric multidimensional scaling plot based on relative abundance of 16S rRNA amplicons in 45 samples from the original site material used for microcosms, microcosms and enrichment cultures.

In a survey of over 60 groundwater samples from around the site in Brazil that were analyzed by amplicon sequencing, approximately 14% returned *Dehalobacter*. Of the ~400 samples that have been sequenced from various remediation trials – including samples from pilot wetlands and pre and post biosparging units – many different *Dehalobacter* ASVs (including those enriched in this study) were detected in 78 samples (~20%) clustered around more impacted zones with lower redox potential. Relative abundances of *Dehalobacter* reached 2% of the bacterial population in some locations, which is high considering the complex mixture of

447 chemicals and conditions at the site.[1,2] These abundances are being used to delineate biologically
448 active zones and to predict attenuation rates to most effectively remediate the site.

449 Other ASVs detected in the enrichment cultures are detected in site samples, including the
450 ones affiliated with *Anaeromyxobacter* and *Desulfitobacterium*, which were detected in ~53%
451 and ~18% of the total ~460 samples, respectively. The ASVs classified as Veillonellales-
452 Selenomonadales were detected in ~11% of the same group of samples, while the ones classified
453 further to *Sporomusa* and *Anaerospora* genera in ~3% and ~6%. The high relative abundance of
454 the Veillonellales-Selenomonadales order in our enriched cultures indicates those organisms
455 were favoured by the ample availability of donor (ethanol and lactate) added to the cultures,
456 which is not the case in the field.

457 The fact that the microbes identified in enrichment cultures are also present at the site attests to
458 the utility of such enrichments in site characterization and provides very strong evidence that these
459 processes are contributing to active natural attenuation in anaerobic zones. At the site,
460 concentrations of dichloroanilines are generally higher than dichloronitrobenzenes, consistent with
461 our finding that biologically-mediated nitroreduction occurs readily and that there are many genera
462 capable of catalyzing this reaction. It is highly likely that some of the other contaminants at the
463 site could be electron donors, including petroleum hydrocarbons, solvents and plasticizers. In
464 addition, abiotic reactions may also occur driven by reduced minerals or by reduced natural organic
465 matter, which can donate electrons for nitroreduction.[37,55–58] Further studies will seek to better
466 understand the players and nitroreduction mechanisms in DCNB cultures.

467 Two distinct *Dehalobacter* ASVs were unequivocally linked to dechlorination, and their
468 presence along with other ASVs in site samples is indicative of dechlorinating activity in situ. The

469 two ASVs originated from geographically distinct locations at the site. Traces of aniline were
470 found and continue to accumulate in 23DCA-T2, suggesting further dechlorination of CA is
471 possible in situ. Inhibition of acetoclastic methanogens was noted, which is also consistent with
472 site data and provides a means to evaluate site recovery: as DCNB and DCA concentrations
473 decrease with active remediation efforts, methanogenic populations in anoxic zones should
474 rebound. Biomarkers based on 16S rRNA genes are already being deployed at the site to track
475 population changes as remediation alternatives are evaluated. The robust enrichment cultures
476 generated herein will be invaluable for future studies of biotransformation mechanisms,
477 development of isotopic enrichment factors and of additional biomarkers, and possibly even for
478 use as bioaugmentation cultures.

479 **SUPPORTING INFORMATION**

480 The following files are available free of charge:

481 Additional details on experiments, materials and methods, and data obtained, including texts and
482 figures mentioned in this paper (PDF); Tables including data obtained and calculations mentioned
483 in this paper (.xls).

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