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

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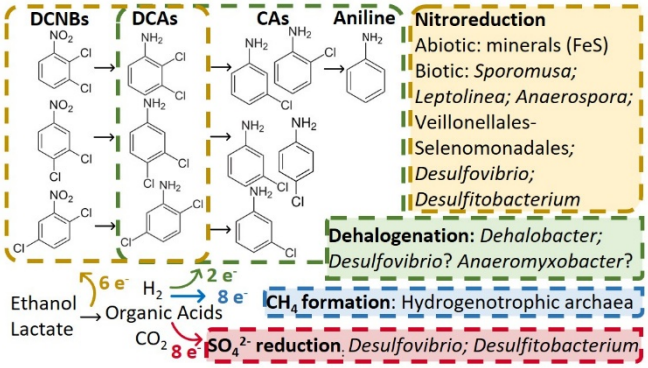
15

16 **ABSTRACT**

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

Graphical Abstract



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

Short synopsis statement

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

46 # 1 INTRODUCTION

47 Many historically-contaminated sites worldwide are a challenge to remediate owing to
48 complex mixtures of toxic contaminants and heterogeneous stratigraphy. For example, an
49 industrial facility located in Bahia in NE Brazil has been the subject of several remedial
50 investigations.[1,2] As a former manufacturing facility of the herbicide diuron, it is impacted by
51 high concentrations of multiple compounds including dichloronitrobenzenes (DCNB; ~3,000
52 mg/kg) and dichloroanilines (DCA; ~700 mg/kg) among many others.[1,2] Anoxic conditions
53 typically prevail in regions with low permeability, high organic matter, or high contaminant
54 concentrations, where the rate of oxygen depletion exceeds rates of replenishment by diffusion
55 or groundwater flow. Therefore, we sought to understand the fate of DCNB and DCA isomers
56 under anoxic conditions with particular attention to associated microbial populations to
57 elucidate microbial pathways and thus create a better conceptual model of the site and a more
58 effective remediation strategy.

59 In subsurface anoxic environments, nitroaromatic compounds tend to become reduced to
60 their corresponding aromatic amines. As early as 1975, the pesticide pentachloronitrobenzene
61 was shown to be reduced to pentachloroaniline.[3] Nitro group reduction to the corresponding
62 amine is now understood to involve broad specificity and oxygen-independent nitroreductases
63 and related enzymes that are widely distributed in bacteria and archaea.[4] These reactions
64 produce toxic transient nitroso and hydroxylamino intermediates, which are highly reactive
65 and unstable.[4–6] Nitroaromatic reduction may also be a respiratory process akin to nitrate

66 reduction.[7–9] A 1983 study reported the reduction of 3,4-DCNB to a variety of poorly quantified
67 products including 3,4-DCA, and condensation products.[5] Despite their environmental
68 relevance, few studies have investigated the anaerobic biodegradation of DCNB isomers.

69 Literature on the anaerobic biodegradation of chloroanilines is also limited. The reductive
70 dechlorination of 3,4-DCA to a mixture of monochloroaniline isomers (3-CA and 4-CA) under
71 anaerobic/methanogenic conditions was first reported in 1989 and 1990, but the organisms
72 responsible were not identified.[10–12] Under nitrate-reducing conditions, a novel reductive
73 deamination reaction was observed in a *Rhodococcus* strain where 3,4-DCA was deaminated
74 primarily to dichlorobenzene,[13] however, additional reports of this process could not be found.
75 More recently, *Dehalococcoides mccartyi* strain CBDB1 and *Dehalobacter sp*. strain 14DCB1,
76 both obligate organohalide-respiring-bacteria (OHRB) that dechlorinate chlorinated
77 benzenes, were shown to dechlorinate 2,3-DCA to 2- and 3-CA and 3,4-DCA to 3-CA with no
78 further transformation when acetate was provided as carbon source and hydrogen as electron
79 donor.[14]

80 Reports of further dechlorination of monochloroanilines to aniline are sparse and
81 inconclusive. Early reports (1997-98) described dechlorination of DCA to CA and then to small
82 amounts of aniline under sulfate-reducing conditions.[15,16] These studies did not characterize
83 the microbial community. A 2019 study showed that *Geobacter* sp. KT5 could transform
84 dichloroanilines under iron-reducing conditions; mineralization of the products via aniline
85 and 4-aminobenzoate was proposed.[17] Two years later, a mixed culture composed of *Geobacter*

86 sp., *Paracoccus denitrificans*, *Pseudomonas* sp., and *Rhodococcus* sp. strains was described to
87 transform propanil to 3,4-DCA, and further to 3-CA, chlorobenzene, and aniline, prior to
88 mineralization.[18] Another study found that 2-aminoanthraquinone-graphene oxide enhanced
89 dechlorination in an enriched anaerobic sludge community that was able to dechlorinate 2-
90 CA. In this work, some aniline and hexanoic acid were detected and organisms from
91 *Oscillospira*, *Lactobacillales*, *Veillonellaceae*, and *Ruminococcus* groups were positively
92 correlated with 2-CA transformation.[19]

93 Given the scarce literature on the biodegradation of dichloronitrobenzenes and
94 dichloroanilines, the goal of this study was to identify organisms responsible for
95 transformation reactions of such compounds and to use the information to design biomarkers
96 to track biodegradation and delineate bioactive zones across a contaminated site. We
97 determined that *Dehalobacter* plays a very active and critical role in the dechlorination of
98 dichloroanilines at a complex site in Brazil. This knowledge is crucial for developing cost-
99 effective *in situ* remediation strategies for many other complex contaminated sites.

100

101

102 2 MATERIALS AND METHODS

103 2.1 Chemicals and stock solutions

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

114 **2.2 Preparation of original microcosm study and enrichment cultures**

115 Cultures were enriched from microcosms originating from a large laboratory treatability
116 study done to evaluate the potential for in situ bioremediation of chlorinated and
117 nitroaromatic compounds at an industrial site in Brazil. The original microcosms were
118 constructed in 2015 with solid subsurface material and groundwater from the industrial site,
119 incubated under anaerobic conditions at site pH (4.6 to 6.5), and amended with various
120 electron donors and acceptors. The microcosms had mixtures of chloroanilines,
121 dichloroanilines, dichloronitrobenzenes and chlorobenzenes at concentrations typical of the
122 site, and biodegradation was monitored closely over a period of one year. After this, some of

123 the most active microcosms continued to be monitored and re-amended with contaminant
124 mixtures and electron donors (ethanol and lactate), and topped up with groundwater, as
125 needed, until March 2020. At this time, six of these most active microcosms (microcosms
126 Cam16, Cam17, Cam64, Cam65, Cam88, and Cam89), all established with electron donor to
127 stimulate methanogenic conditions, were selected as inoculum for the bottles described in this
128 study (Figure S1). The microcosm setup is detailed in Supporting Information Text S1 and in a
129 Master's Thesis.[20] Results are summarized in Table S1 and Figure S2.

130 The first set of transfer cultures (34DCNB-T1, 25DCNB-T1, 23DCNB-T1, 34DCA-T1 and
131 23DCA-T1) and an autoclaved killed control (KC) were inoculated with cell pellets from one
132 of the microcosms mentioned above (Figure S1). The pellet used as inoculum was obtained
133 from a 40 ml sample from a shaken microcosm by centrifuging at 12,000 g for 15 min. These
134 transfer cultures (group T1) were repeatedly amended with single individual chlorinated
135 substrates: 3,4-DCNB, 2,5-DCNB, 2,3-DCNB, 3,4-DCA, and 2,3-DCA (Figure S1). The killed
136 control (KC) was autoclaved three times on three consecutive days prior to being amended
137 with a mix of six compounds: 3,4-DCNB, 2,5-DCNB, 3,4-DCA, 2,3-DCA, 2-CA, and aniline.
138 An abiotic control (AC1) was also prepared, containing only reduced mineral medium
139 supplemented with a slightly modified set of six compounds: 3,4-DCNB, 2,3-DCNB, 3,4-DCA,
140 2,3-DCA, 2-CA, and aniline. This modification was necessary because 2,5-DCNB and 2,3-
141 DCNB co-elute under the chromatographic conditions used to measure compound
142 concentrations.

143 A second generation of transfer cultures (34DCNB-T2, 25DCNB-T2, 23DCNB-T2, 34DCA-
144 T2, and 23DCA-T2) was prepared from each T1 culture after ~4 months. These were amended
145 with the same individual compounds as the first generation transfers (3,4-DCNB, 2,5-DCNB,
146 2,3-DCNB, 3,4-DCA, and 2,3-DCA, respectively) (Figure S1). The DCNB-T2 cultures were
147 inoculated with a 50% transfer from the corresponding T1 culture (50 mL into 50 mL fresh
148 medium) while DCA-T2 cultures were inoculated with a 10% transfer from T1 cultures (10
149 mL of T1 into 90 mL). At the time we initiated the T2 cultures, the mechanism of DCNB
150 reduction was unclear (discussed later). Therefore, we opted for a more conservative transfer
151 approach, using a 50% inoculum instead of the 10% used for DCA cultures. Additional mineral
152 medium supernatant was added to a T2 culture bottle when its volume was depleted to below
153 50 mL due to sampling, and the cultures were topped up to at least 80 mL. Occasionally, a little
154 more medium was added to increase the volume of culture up to 250 mL. A second abiotic
155 control (AC2) was prepared with mineral medium devoid of FeS and amended with 3,4-DCNB,
156 2,3-DCNB, 3,4-DCA, and 2,3-DCA. The data presented herein focus on the second transfer
157 (T2) enrichment cultures.

158 All transfer cultures as well as controls were prepared in autoclaved 250-mL Boston round
159 bottles sealed with Mininert caps, containing 100 mL of anaerobic mineral medium (pH 7)
160 reduced with iron sulfide (FeS).[21] Only medium supernatant was used to avoid adding
161 precipitated FeS. To simulate site groundwater conditions and to promote mineralization of
162 nitrogen-containing compounds, ammonium was omitted from the medium recipe. All bottles

163 were incubated statically in the dark at room temperature in an anaerobic glovebox (Coy)
164 supplied with a gas mix of N_2:CO_2:H_2 (80:10:10%). No replicates were conducted.

165 The semi-volatile substrates (electron acceptors) were added as evaporated acetone stocks as
166 described in Text S2. The target concentration was 10 mg/L (52 μM [DCNB] and 62 μM [DCA].
167 Second transfer enrichments had the concentration increased up to ~27 mg/L (140 μM [DCNB]
168 and 170 μM [DCA]) over time, to expose the cultures to a higher concentration of the
169 corresponding compound for selection purposes. As electron donors, ethanol and lactate were
170 added to the active cultures and the two controls (AC1 and KC) initially and were re-amended
171 to active cultures when degradation rates slowed. Electron donors were added in ~17-fold
172 excess in electron equivalents (eeq) considering both nitroreduction and complete
173 dechlorination of DCNB to aniline (Text S2). The initial pH was between 6.6 and 7.1 in all
174 active enrichments.

175 **2.3 Analytical procedures**

176 Concentrations of DCNB, DCA and CA isomers and aniline were measured using a Hewlett-
177 Packard 1050 series high-performance liquid chromatography (HPLC) system (Acclaim™ 120
178 C18 column, Thermo Scientific) equipped with a UV detector (set at 254 nm). The mobile
179 phase was an isocratic 50:50 mixture of Milli-Q water and acetonitrile (Millipore Sigma) at 1
180 mL/min with a runtime of 25 min. Samples (2 mL) were taken from cultures using a 2 mL
181 gastight glass syringe and filtered through a 0.22 μm (13 mm) Chromspec PTFE syringe filter

182 (pre-wetted with methanol) prior to being injected into the HPLC. Lactate, acetate,
183 propionate, formate, butyrate, pyruvate, chloride, nitrate, nitrite, sulfate, and phosphate were
184 measured by ion chromatography (IC) using a Dionex™ Integrion™ IC system (Thermo Fisher
185 Scientific). Methane was measured in headspace samples by gas chromatography (GC) using
186 an Agilent 7890A gas chromatograph with headspace autosampler G1888 (Agilent
187 Technologies). Method details are provided in Text S3.

188 **2.4 Microbial community analysis and qPCR**

189 Well-mixed samples (4 mL) from enrichment cultures were collected periodically and
190 centrifuged at 10,000 rpm for 20 minutes. The supernatant was removed and cell pellets stored
191 at -80°C. DNA was extracted using the DNeasy PowerSoil Kit (Qiagen) and quantified using a
192 Qubit® 3.0 Fluorometer (Thermo Fisher Scientific). DNA samples were sent for Illumina
193 MiSeq PE300 amplicon sequencing at an external laboratory (Genome Quebec Innovation
194 Centre, Montreal, Canada), using modified "staggered end"[22] versions of primers 926f-modified
195 (5'-AAACTYAAAKGAATWGRCGG-3') and 1392r-modified (5'-ACGGGCGGTGWGTRC-
196 3'), which target the V6–V8 region of the 16S rRNA gene from both Bacteria and Archaea, as
197 well as the 18S rRNA gene in Eukarya.[23,24] Sequencing data was processed using QIIME 2
198 version 2021.2.[25] After trimming the primer region with the cutadapt plug-in, amplicon sequence
199 variants (ASVs) were generated using the DADA2 plugin,[26] and taxonomy was assigned to the
200 ASVs through the feature-classifier plugin using the SSU SILVA v.138 database.[27] Data were

201 further processed in Excel. Sequences <400 bp in length were removed and ASVs were ranked
202 by maximum sequence abundance across all DNA samples. ASVs with abundance greater than
203 1% in at least one sample were kept for graphing. The Basic Local Alignment Search Tool
204 (BLAST) available at the National Center for Biotechnology Information (NCBI) was used to
205 compare ASV sequences of interest. To investigate the presence of the organisms of interest
206 identified in the cultures, key genera were subsequently searched in a database containing 16S
207 rRNA gene sequences retrieved from field samples collected by our collaborating partner
208 during various remediation pilot projects conducted at the site. This database also contains all
209 raw DNA sequences from microcosms and enrichments and was uploaded to the NCBI
210 (Accession PRJNA1040917 as described in Table S2).

211 DNA samples were also analyzed by quantitative polymerase chain reaction (qPCR) using a
212 CFX96TM real-time PCR detection system (Bio-Rad Laboratories Inc.). Primers GenBac1055f
213 (5'-ATGGYTGTCGTCAGCT-3') and GenBac1392r (5'-ACGGGCGGTGTGTAC-3') targeting
214 most bacteria[24,28,29] and primers GenArch787f (5'-ATTAGATACCCGBGTAGTCC-3') and
215 GenArch1059r (5'-GCCATGCACCWCCTCT-3') targeting most archaea[30] were used. A no-
216 template control was also included in each run. All qPCR samples were run in triplicate.
217 Details on the qPCR method are provided in Text S4. Absolute total bacterial and archaeal
218 abundance obtained by qPCR (copies 16S rRNA gene per ml) was used to convert the relative
219 abundances obtained from amplicon sequencing to absolute abundance for each ASV.

220 A total of forty-eight DNA samples were sequenced and analyzed by qPCR, including
221 samples from the original subsurface solid material, microcosms, and enrichments. On average
222 66,000 16S rRNA gene amplicon reads were retrieved per sample (Table S3a). Reads were
223 grouped by bacteria and archaea, then sequences with relative abundance >1% in any sample
224 (Table S3b for bacteria and Table S3c for archaea) were used to calculate absolute abundances
225 (copies/mL) of each ASV using total abundances of bacteria and archaea determined by qPCR
226 (Table S4). Final absolute abundances for each ASV (copies/mL) are reported in Tables S5a to
227 S5f for bacteria; Tables S6a to S6f for archaea. Finally, Non-metric Multi-dimensional Scaling
228 (NMDS) and bubble plots were constructed as explained in Text S5.

229 **3 RESULTS AND DISCUSSION**

230 **3.1 Abiotic transformation of DCNB was not sustained**

231 Contaminant profiles in three controls (AC1, AC2 and KC) were assessed for possible abiotic
232 reactions. Chloroanilines and aniline were not transformed in any of these controls.
233 Dichloronitrobenzenes were not transformed in medium control (AC2) without FeS (Figure
234 S3; Table S7). In the medium control (AC1) with FeS, 3,4-DCNB was not transformed, but 2,3-
235 DCNB was reduced to 2,3-DCA (Figure S4; Table S7); however, this activity was not sustained
236 upon reamending with 2,3-DCNB. These data are consistent with reports that reduced
237 minerals, such as FeS or perhaps reduced resazurin, can sometimes mediate reduction of the
238 nitro group. [31–38] Curiously, DCNB isomers were repeatedly transformed to DCA in the

239 autoclaved control KC (Figure S5; Table S7); however later analyses (to be discussed) revealed
240 that KC was in fact not sterile.

241 **3.2 Biologically-mediated nitroreduction and dechlorination**

242 Each transfer culture established on single substrates remained active, sustained by refeeding
243 chlorinated substrates (DCNB or DCA) and electron donors as needed for over 500 days.
244 Historical data are provided in Table S8 (T1) and Table S9 (T2). Focusing on T2 transfers from
245 day 280 to 520 and on the three DCNB-amended cultures first, we observed consistent and
246 complete transformation of each DCNB isomer to the corresponding dichloroaniline (Figure
247 1a-c). In the culture amended with 3,4-DCNB the product, 3,4-DCA, was further
248 dechlorinated to 3- and 4-CA (Figure 1a), while in the cultures amended with 2,3- and 2,5-
249 DCNB, the transformation stopped at the corresponding dichloroaniline (Figures 1b and 1c).
250 The sum of measured chlorinated products agreed well with the cumulative DCNB added.
251 Reduction of 2,3-DCNB (<~1 μM per day) was ~5 times slower than the reduction of either
252 3,4- or 2,5-DCNB (5-6 μM per day).

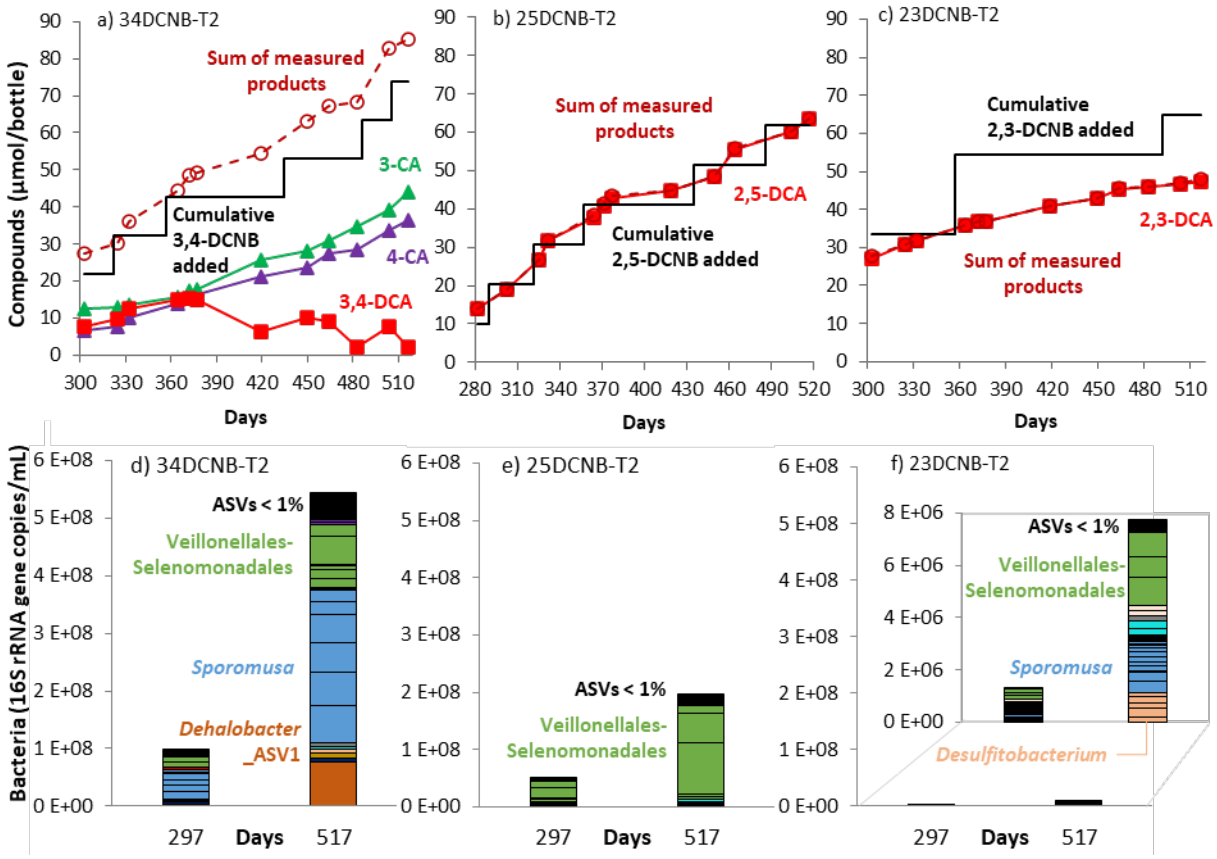


Figure 1. Degradation profiles (upper panels a), b) and c); µmole/bottle) and corresponding major microbial taxa (lower panels d), e) and f); copies/mL) in DCNB-amended T2 transfer cultures from day 280 to 520. Cumulative DCNB added (———) is shown for each amendment (step) of 70 to 140 µM (10 to 20 µmol per bottle). The sum of products (– –○– –), including DCA (—■—), 4-CA (—▲—), 3-CA (—▲—), 2-CA and aniline (not shown, mostly below detection), illustrates a reasonable mole balance. Measured DCNB concentrations are not shown for clarity but can be found in Table S9. In lower panels amplicon sequence variants (ASVs) from the same taxonomic designation are assigned the same colour.

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

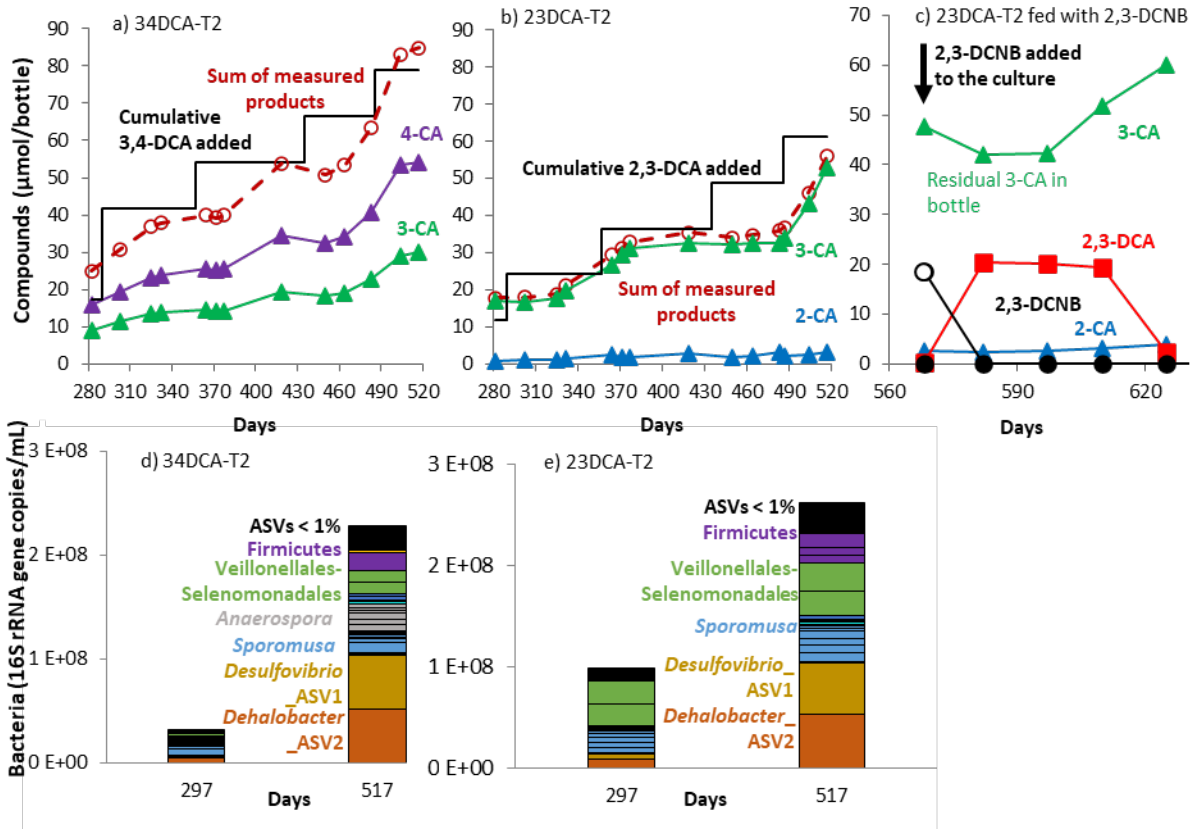


Figure 2. Degradation profiles (upper panels a), b) and c); μmole/bottle) and corresponding major microbial taxa (lower panels, d) and e); copies/mL) in two DCA-amended T2 transfer cultures from day 280 to 520 (panels a), b), d), and e)) and from day 560 to 625 (panel c)). In panels a) and b), cumulative DCA added (———) is shown for each amendment (step) of ~80 μM (~12 μmol per bottle). The sum of products (- -o- -), including DCA (—■—), 4-CA (—▲—), 3-CA (—▲—), 2-CA (—▲—), and aniline (not shown, mostly below detection),

272 illustrates a reasonable mole balance. Measured DCA concentrations are not shown for clarity
273 but can be found in Table S9. Panel c) shows degradation profile after 2,3-DCA-T2 was fed
274 with 2,3-DCNB (—●—) instead of 2,3-DCA on Day 568. The open circle shows the amount of
275 2,3-DCNB fed to 23DCA-T2 (—○—). In lower panels amplicon sequence variants (ASVs) from
276 the same taxonomic designation are assigned the same color.

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

284 **3.3 Microbial community composition reflects electron balance**

285 To relate the microbial community to activity, we first identified abundant bacterial and
286 archaeal ASVs (>1%) and then calculated their absolute abundance in active cultures focusing
287 on 2-time points for each sample. Figure 3 provides a summary of the most relevant taxa
288 identified. Most of the enriched bacterial ASVs belong to the phylum Bacillota (Firmicutes)
289 including *Sporomusa*, *Anaerospora*, and unassigned members of the Veillonellales-
290 Selenomonadales. These organisms clearly grew on the ample electron donor amendments to

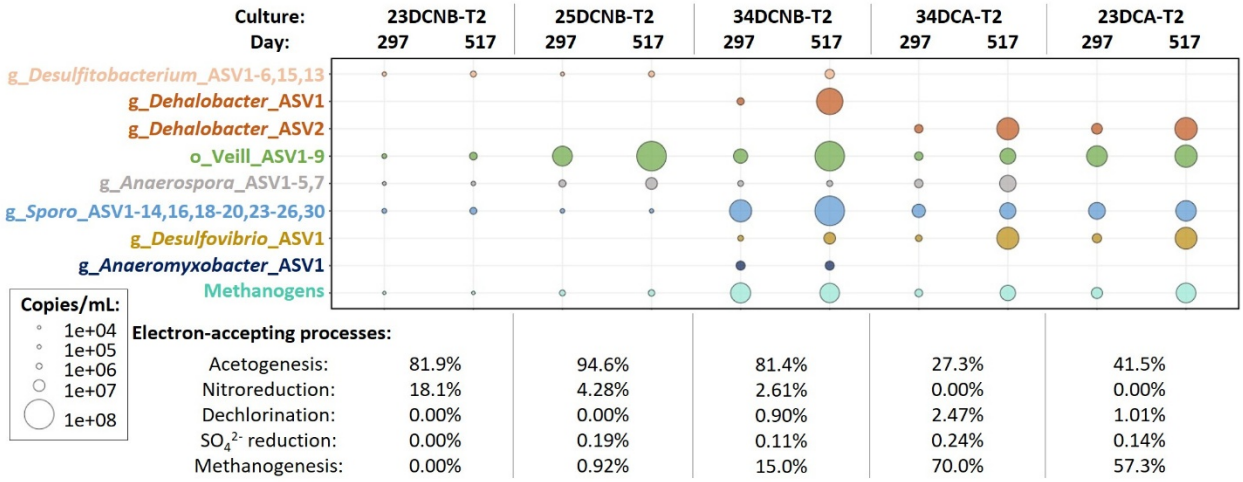
291 the cultures. ASVs for *Anaeromyxobacter*, *Desulfitobacterium* and *Desulfovibrio* were also

292 retrieved. These genera are also often implicated in nitrate or sulfate reduction though some

293 members may be facultative dechlorinators. ASVs for obligate dechlorinators of the genus

294 *Dehalobacter* were also identified (Figure 3). A complete overview of all major taxa (>1%) are

295 shown in Figure S6, for bacteria, and Figure S7, for archaea.



296

297 **Figure 3**. Absolute abundance of major organisms (copies/mL) and proportion of electron

298 equivalents from amended donors channeled to each electron-accepting processes (% electron

299 equivalents) for days 297 and 517. Veillonellales-Selenomonadales order is shortened to

300 "Veill"; *Sporomusa* genus is shortened to "*Sporo*". Calculated absolute abundances for all ASVs

301 are available in Table S5 (bacteria) and Table S6 (archaea).

302 To confirm the extent of dechlorination, an electron balance of possible donors and acceptors

303 was completed using measured concentrations of chlorinated organics, methane (Table S9) and

304 anions (Table S10). As expected from stoichiometry (e.g., $C_6H_5Cl_2N + H_2 \rightarrow C_6H_6ClN + HCl$),

305 chloride concentrations increased proportionally in cultures that exhibited dechlorination
306 (34DCNB-T2, 34DCA-T2, and 23DCA-T2). The measured increase in chloride was slightly
307 higher than expected in almost all cases (Table S11), likely because of the error associated with
308 measuring small changes against a high background of ~1900 μM chloride.

309 Potential electron-accepting processes include acetogenesis, methanogenesis,
310 nitroreduction, dechlorination, and sulfate reduction. For T2 cultures between days 280 and
311 520, we calculated the proportion of electron equivalents from added donors channeled to
312 each of the different electron-accepting processes (Figure 3, bottom table). In DCNB-amended
313 cultures, acetogenesis from lactate and ethanol was the dominant process, accounting for over
314 80% of electrons from donors. Methanogenesis was severely limited. By contrast, in DCA-
315 amended cultures, methanogenesis accounted for 60-70% of electron equivalents from donors,
316 which combined with acetogenesis accounted for over 98% of electron equivalents. A buildup
317 of acetate in all cultures (Tables S10 and S12) suggested possible inhibition of acetoclastic
318 methanogens specifically, particularly by DCNBs. Culture 34DCNB-T2 produced the most
319 methane among DCNB-amended cultures but also exhibited the most rapid and extensive
320 nitroreduction and dechlorination, thus relieving inhibition associated with DCNBs and
321 DCAs.

322 Other electron accepting processes accounted for a much lower proportion of activity
323 (Figure 3). Nitroreduction consumed 5% of electron equivalents in cultures fed with 3,4- and
324 2,5-DCNB isomers and up to 20% of electron equivalents in culture 23DCNB-T2, although

325 activity in this culture was very low and no lactate was consumed (Tables S10 and S12). In all
326 cases, dechlorination accounted for less than 3% of all electron equivalents provided. The small
327 amount of sulfate (~30 μM) present in the medium was rapidly depleted in the 4 highly active
328 cultures (34DCNB-T2, 25DCNB-T2, 34DCA-T2, and 23DCA-T2). However, less than 1% of
329 electron equivalents were channeled to sulfate reduction. In culture 23DCNB-T2, sulfate was
330 not consumed, consistent with low activity observed in this enrichment (Table S11).

331 We compared the proportions of electrons consumed by each electron-accepting process
332 with corresponding microbial proportions (Figure 3) to infer function (Table S12). Methane
333 production was greatest in cultures 23DCA-T2 and 34DCA-T2, lower in 34DCNB-T2, very
334 low in 25DCNB-T2 and absent in 23DCNB-T2. Methanogens were correspondingly most
335 abundant in 23DCA-T2, 34DCA-T2, and 34DCNB-T2. ASVs corresponding to
336 hydrogenotrophic *Methanoregula* dominated the archaeal population, with a much lower
337 abundance of *Methanocella* and other hydrogenotrophic genera (Figure S7). The presence of
338 only hydrogenotrophic methanogens combined with substantial accumulation of acetate
339 strongly indicates that DCNB isomers and to a lesser extent DCA isomers inhibited acetoclastic
340 methanogens.

341 **3.4 Nitro group reduction can be mediated by many different microbes**

342 We had hoped to observe enrichment of specific microbes in DCNB-amended cultures to
343 enable functional attribution. Rather, it seems that many different genera can likely reduce

344 the nitro group. Biologically regenerated FeS is unlikely to play a role as a reductant, as there
345 was no evidence of sulfate or iron cycling in the cultures, and the rates of DCNB reduction
346 were similar regardless of FeS concentrations. In all cultures, there was significant
347 representation of fermenting or acetogenic microbes, particularly Veillonellales-
348 Selenomonadales, *Sporomusaceae*, *Sporomusa*, and *Anaerospora* (Figure S6). This is consistent
349 with the ample supply of ethanol and lactate to all cultures.

350 We wondered if these fermenting and acetogenic organisms could also be reducing DCNB
351 to DCA as a growth-associated electron-accepting process akin to nitrate reduction. In
352 25DCNB-T2, where only nitroreduction was observed without dechlorination, 6 ASVs (ASV1
353 to 5 and 8) (>90% identity) classified only to the order level as Veillonellales-Selenomonadales
354 were predominant (Table S5b). They represented more than 80% of the bacterial community
355 in this culture on day 517 (Figure 1e). The closest matches to these sequences are affiliated
356 with the genera *Pelosinus*, *Anaerospora*, *Sporomusa*, *Dendrosporobacter*, among other
357 organisms; interestingly, one of the closely matching sequences (KF460373.1) comes from a
358 study on the impact of nitrate on pentachlorophenol dechlorination[39] (Figure S8).

359 In fact, ASVs from Veillonellales-Selenomonadales were abundant in all 5 active cultures
360 (Figures 1d, e, and f, Figures 2d and e and Figure 3), whether fed DCNB or DCA, making
361 functional attribution difficult. Finding that DCNB isomers could be reduced in all cultures
362 regardless of enrichment substrate is perhaps then not so surprising. An autoclaved control
363 (KC) amended with a mixture of compounds (3,4-DCNB, 2,5-DCNB, 3,4-DCA, 2,3-DCA, 2-

364 CA, aniline) was prepared to try to distinguish between biotic and abiotic reactions.
365 Unfortunately, the bottle did not achieve sterility, despite autoclaving it on three consecutive
366 days. In this "killed" control, both 3,4-DCNB and 2,5-DCNB were reduced to the
367 corresponding DCA isomers (Figure S5). DNA was readily extracted from this control, and
368 although the total microbial abundance was an order of magnitude lower than other cultures,
369 the KC culture was dominated by a unique Butyricoccaceaea ASV as well as other spore-
370 forming and presumably heat-resistant microbes. Among the microbes detected in KC after
371 refeeding 3,4- and 2,5-DCNB 4 times, *Desulfitobacterium* and *Anaerospora* were also detected
372 in other active nitroreducing cultures (i.e., 23DCNB-T2 and 25DCNB-T2) (Figure S6),
373 suggesting these genera are also candidates for nitroreduction. Ultimately it became clear that
374 nitroreduction is a widespread microbial activity that was easy to stimulate with electron
375 donor in the cultures, however, the contribution of biotic nitroreduction to microbial growth
376 is yet to be investigated.

377 **3.5 Dechlorination of 2,3- and 3,4-DCA supports growth of *Dehalobacter***

378 In actively dechlorinating cultures, *Dehalobacter* was the only microbe to systematically
379 show a substantial increase in abundance. *Dehalobacter* was not detected in cultures that were
380 only catalyzing nitroreduction (25DCNB-T2 and 23DCNB-T2). Given that it is an obligate
381 organohalide respiring bacterium, we conclude that *Dehalobacter* was responsible for the

382 DCA-dechlorinating activity in enrichment cultures amended with 3,4-DCNB, 3,4-DCA, and
383 2,3-DCA (Figures 1d and 2d and e).

384 Two distinct *Dehalobacter* ASVs were identified: ASV1 in 34DCNB-T2 and ASV2 in 34DCA-
385 T2 and 23DCA-T2 (Text S6), with maximum bacterial abundances of 14, 25, and 31% in each
386 culture, respectively (Table S3b). ASV1 (468 bp) is 100% identical to several previously
387 published 16S rRNA gene sequences of *Dehalobacter restrictus* strains, including strain SAD
388 (CP148032.1), strain DSM 9455 (CP007033.1), and strain 12DCA (CP046996.1), as well as to
389 three *Dehalobacter* strains classified only to the genus level, including strain DCA
390 (CP003869.1), strain DAD (CP148031.1), and strain CF (1CP003870.1). The known substrates
391 for these *Dehalobacter* strains include tetra- and trichloroethene,[40] 1,2-dichloroethane,[41] tri-
392 and dichloromethane,[42] and 1,1,1-trichloroethane and 1,1-dichloroethane.[43,44]

393 *Dehalobacter* ASV2 (468 bp) was not a perfect match to any 16S rRNA gene sequence in the
394 NCBI database. It shares only 97.01% identity to ASV1 and 98.93% to *Dehalobacter* strain
395 DCM (CP092282.1) described as "dichloromethane-degrading bacterium from Xi river" (Direct
396 Submission by Yang, Y. *et al.*, 13-Jan-2022). Neither *Dehalobacter* ASV1 nor ASV2 sequences
397 matched (<95% identity) to the 16S rRNA gene fragment (937 bp) from *Dehalobacter* sp. strain
398 14DCB1 (JN051267.1), a strain enriched on dichlorobenzene[45] but reported to dechlorinate
399 dichloroaniline.[14]

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

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

413 A shift in activity in culture 23DCA-T2 was noted as monitoring continued for over 1000
414 days (Figure S10). As time progressed, 2-CA rather than 3-CA became the major product from
415 2,3-DCA. Thereafter, 2-CA was slowly yet consistently transformed to aniline. On day 881,
416 *Dehalobacter*_ASV2 constituted 16% of the bacterial community (Table S5e). 2-CA was
417 dechlorinated to aniline in subsequent transfers from this culture as well (data not shown). A
418 subsample of 23DCA-T2 was taken on day 653 for additional growth experiments to ultimately
419 build up enough culture to measure compound specific isotopic enrichment factors. This

420 ongoing research implicates *Dehalobacter* in aniline formation as described in a companion
421 publication.[46]

422 **3.7 Other organisms identified in enriched cultures**

423 Several other ASVs belonging to known facultative OHRB were identified, including
424 *Anaeromyxobacter*, *Desulfovibrio*, and *Desulfitobacterium* (Figure 3). While some members
425 of these genera can dechlorinate, most are known to be quite versatile and may reduce sulfate,
426 nitrate or even grow fermentatively in the absence of a terminal electron acceptor. An
427 *Anaeromyxobacter* ASV found in culture 34DCNB-T2 is 99.79% similar over ~450 bp to the
428 16S rRNA gene of *Anaeromyxobacter* sp. strain RF71 (MZ254878.1), 99.57% similar to *A.*
429 *dehalogenans* 2CP-C (CP000251.1) and 99.36% similar to *A. dehalogenans* 2CP-1
430 (CP001359.1). Both strains 2CP-C and 2CP-1 can gain energy from the reductive
431 dechlorination of chlorophenol and contain putative dehalogenases.[47–49] However, the
432 abundance of *Anaeromyxobacter* in the culture was far less than *Dehalobacter* and is therefore
433 unlikely to be primarily responsible for dechlorination.

434 A single ASV of *Desulfovibrio* increased in abundance over time in the three cultures
435 dechlorinating DCA isomers (Figure 3). *Desulfovibrio* are sulfate-reducing bacteria that can
436 also ferment substrates such as lactate; some can respire organohalides.[50,51] In cultures 23DCA-
437 T2 and 34DCA-T2, *Desulfovibrio* increased between days 280 and 520 to similar extent as
438 *Dehalobacter* (Figures 2d and 2e). However, a similar increase was not seen in dechlorinating

439 culture 34DCNB-T2 (Figure 1d). The amount of sulfate in the medium (around 30 μM) is too
440 small to explain this growth, therefore *Desulfovibrio* was more likely metabolizing lactate to
441 acetate, possibly participating in dechlorination alongside *Dehalobacter*. The *Desulfovibrio*
442 ASV in our cultures is 100% identical to a non-dechlorinating *Desulfovibrio* sp. (JQ599691.1)
443 detected in a dichloroethane dechlorinating enrichment.[52] The sequence was also 100%
444 identical to an uncultured *Desulfovibrio* clone (AB854345.1) found in a non-dechlorinating
445 environment.[53] Therefore, the more likely role of this organism is fermentation and
446 acetogenesis, in support of dechlorinating *Dehalobacter*.

447 Several ASVs (sharing > 94% identity) were assigned to *Desulfitobacterium*. Interestingly,
448 these ASVs were only found in the cultures reducing DCNBs and in the KC control that
449 exhibited nitroreduction; they were not detected in the DCA-amended cultures (Figure 3).
450 This seems to implicate *Desulfitobacterium* in nitroreduction, however the ASVs were in low
451 abundance. Furthermore, DCA-amended cultures could also rapidly reduce the nitro group,
452 therefore *Desulfitobacterium* was not solely responsible for nitroreduction, if at all. Moreover,
453 the 8 most abundant *Desulfitobacterium* ASV sequences are > 95% similar to a
454 *Desulfitobacterium* sequence found in *Dehalococcoide*s-containing dechlorinating cultures
455 (AB596883.1) (Figure S11), where they were not implicated in dechlorination,[54] a likely
456 scenario in our study as well.

457 **3.8 Data integration and application to improving remediation outcomes**

458 Non-metric multidimensional scaling (NMDS) was used to evaluate relationships among
459 cultures as they were enriched from subsurface material to initial microcosms and finally to
460 enrichment cultures (Figure 4). First, there is clearly an impact of the original source material,
461 shown in red, blue and green for three original sediment sources used to construct the
462 microcosms. Second, there was a convergence of community depending on substrate.
463 Enrichment cultures that dechlorinate DCAs are distinct from those that only carry out
464 nitroreduction. Samples from 34DCNB-T2 culture, in which both nitroreduction and
465 dehalogenation were observed, are located between the dechlorinating-exclusive culture
466 samples (34DCA-T2, and 23DCA-T2) and the nitroreducing-exclusive cultures (25DCNB-T2,
467 23DCNB-T2 and KC). *Dehalobacter* in the top groupings and *Desulfitobacterium* in the bottom
468 two cultures (23DCNB-T2 and KC) are major genera driving the vertical spread of samples in
469 the NMDS plot. Relationships between original microcosms and transfers are also illustrated
470 in Figure S1.

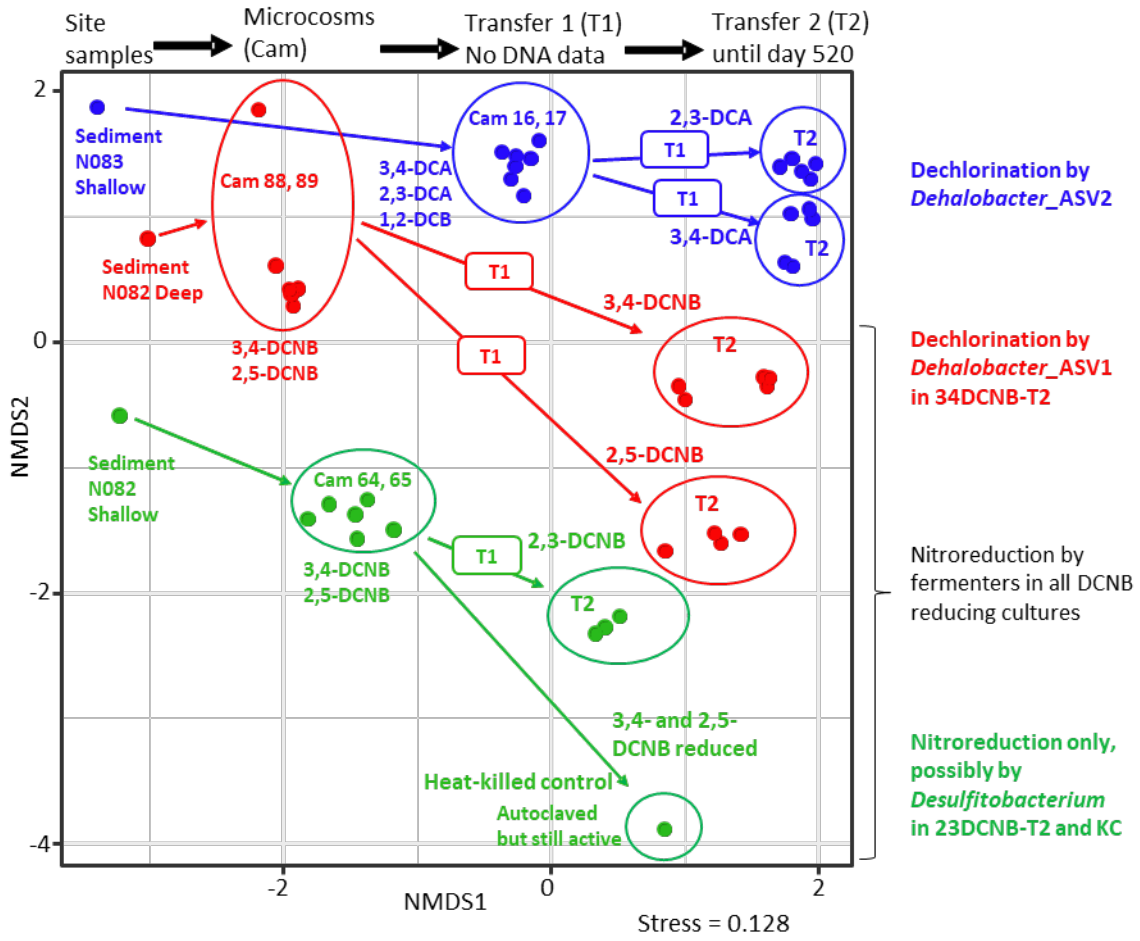


Figure 4. Non-metric multidimensional scaling plot based on relative abundance of 16S rRNA amplicons in 45 samples from the original site material used for microcosms and enrichment cultures.

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 implemented at the industrial site – 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

480 (~20%) clustered around more impacted zones with lower redox potential. Relative
481 abundances of *Dehalobacter* reached 2% of the bacterial population in some locations, which
482 is high considering the complex mixture of chemicals and conditions at the site.[1,2] These
483 abundances are being used to delineate biologically active zones and to predict attenuation
484 rates to most effectively remediate the site.

485 Other ASVs detected in the enrichment cultures were detected in site samples, including the
486 ones affiliated with *Anaeromyxobacter* and *Desulfitobacterium*, which were detected in ~53%
487 and ~18% of the total ~460 samples collected by the partner company, respectively. The ASVs
488 classified as Veillonellales-Selenomonadales were detected in ~11% of the same group of
489 samples, while the ones classified further to *Sporomusa* and *Anaerospora* genera in ~3% and
490 ~6%. The high relative abundance of the Veillonellales-Selenomonadales order in our
491 enriched cultures indicates those organisms were favoured by the ample availability of donor
492 (ethanol and lactate) added to the cultures, which is not the case in the field.

493 The fact that the microbes identified in enrichment cultures are also present at the site attests
494 to the utility of such enrichments in site characterization and provides very strong evidence
495 that these processes are contributing to active natural attenuation in anaerobic zones. At the
496 site, concentrations of dichloroanilines are generally higher than dichloronitrobenzenes,
497 consistent with our finding that biologically-mediated nitroreduction occurs readily and that
498 there are many genera capable of catalyzing this reaction. It is highly likely that some of the
499 other contaminants at the site could be electron donors, including petroleum hydrocarbons,

500 solvents and plasticizers. In addition, abiotic reactions may also occur driven by reduced
501 minerals or by reduced natural organic matter, which can donate electrons for
502 nitroreduction.[37,55–58] Further studies will seek to better understand the players and
503 nitroreduction mechanisms in DCNB cultures.

504 Two distinct *Dehalobacter* ASVs were unequivocally linked to dechlorination, and their
505 presence along with other ASVs in site samples is indicative of dechlorinating activity in situ.
506 The two ASVs originated from geographically distinct locations at the site. Traces of aniline
507 were found and continue to accumulate in 23DCA-T2, suggesting further dechlorination of
508 CA is possible in situ. Inhibition of acetoclastic methanogens was noted, which is also
509 consistent with site data and provides a means to evaluate site recovery: as DCNB and DCA
510 concentrations decrease with active remediation efforts, methanogenic populations in anoxic
511 zones should rebound. Biomarkers based on 16S rRNA genes are already being deployed at the
512 site to track population changes as remediation alternatives are evaluated. The robust
513 enrichment cultures generated herein will be invaluable for future studies of
514 biotransformation mechanisms, development of isotopic enrichment factors and of additional
515 biomarkers, and possibly even for use as bioaugmentation cultures.

516 **SUPPORTING INFORMATION**

517 The following files are available free of charge:

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

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and has no commercial intention between the parties. This is a collaboration project between the parties involved. Access Registration No. A3D9F11.

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REFERENCES

(1) Melo, N.; Araújo, S. P.; de Paula Queiroz Kraus, S.; Lomheim, L.; Quintero, P. B.; Mack, E. E.; Edwards, E. A.; Spain, J.; Gavazza, S. Strategies for Bioremediation of Soil from an Industrial Site Exposed to Chlorinated and Nitroaromatic Compounds. *Groundwater Monit. Rem.* **2023**, *43* (3), 108–120. https://doi.org/10.1111/gwmr.12596.

(2) Barreto, P.; Lemes, M.; Jimenez, J.; Mack, E. E.; Henderson, J.; Freedman, D. L. Evaluation of Strategies to Remediate Mixed Wastes at an Industrial Site in Brazil. *Groundwater Monit. Rem.* **2023**, *43* (3), 93–107. https://doi.org/10.1111/gwmr.12607.

(3) Buser, H.-R.; Bosshardt, H.-P. Studies on the Possible Formation of Polychloroazobenzenes in Quintozene Treated Soil. *Pestic Sci* **1975**, *6*, 35–41.

(4) Roldán, M. D.; Pérez-Reinado, E.; Castillo, F.; Moreno-Vivián, C. Reduction of Polynitroaromatic Compounds: The Bacterial Nitroreductases. *FEMS Microbiol. Rev.* **2008**, pp 474–500. https://doi.org/10.1111/j.1574-6976.2008.00107.x.

(5) Bunce, N. J.; Merrick, R. L.; Corke, C. T. Reductive Transformations of Nitrate with 3,4-Dichloroaniline and Related Compounds by *Escherichia Coli*. *J Agric Food Chem* **1983**, *31* (5), 299–326.

(6) Spain, J. C. Biodegradation of Nitroaromatic Compounds. *Annu Rev Microbiol* **1995**, *49*, 523–555.

(7) Esteve-Nunez, A.; Lucchesi, G.; Philipp, B.; Schink, B.; Ramos, J. L. Respiration of 2,4,6-Trinitrotoluene by *Pseudomonas* sp. strain JLR11. *J Bacteriol* **2000**, *182* (5), 1352–1355.

(8) Huang, J.; Ning, G.; Li, F.; Sheng, G. D. Biotransformation of 2,4-Dinitrotoluene by Obligate Marine *Shewanella marisflavi* EP1 under Anaerobic Conditions. *Bioresour Technol* **2015**, *180*, 200–206. https://doi.org/10.1016/j.biortech.2014.12.108.

(9) Madeira, C. L.; Menezes, O.; Park, D.; Jog, K. V.; Hatt, J. K.; Gavazza, S.; Krzmarzick, M. J.; Sierra-Alvarez, R.; Spain, J. C.; Konstantinidis, K. T.; Field, J. A. Bacteria Make a Living Breathing the Nitroheterocyclic Insensitive Munitions Compound 3-Nitro-1,2,4-Triazol-5-One (NTO). *Environ Sci Technol* **2021**, *55* (9), 5806–5814. https://doi.org/10.1021/acs.est.0c07161.

(10) Struijs, J.; Rogers, J. E. Reductive Dehalogenation of Dichloroanilines by Anaerobic Microorganisms in Fresh and Dichlorophenol-Acclimated Pond Sediment. *Appl Environ Microbiol* **1989**, *55* (10), 2527–2531.

(11) Kuhn, E. P.; Suflita, J. M. Sequential Reductive Dehalogenation of Chloroanilines by Microorganisms from a Methanogenic Aquifer. *Environ Sci Technol* **1989**, *23* (7), 848–852. https://doi.org/10.1021/es00065a014.

(12) Kuhn, E. P.; Townsend, G. T.; Suflita, J. M. Effect of Sulfate and Organic Carbon Supplements on Reductive Dehalogenation of Chloroanilines in Anaerobic Aquifer Slurries. *Appl Environ Microbiol* **1990**, *56* (9), 2630–2637. https://doi.org/10.1128/aem.56.9.2630-2637.1990.

(13) Travkin, V.; Baskunov, B. P.; Golovlev, E. L.; Boersma, M. G.; Boeren, S.; Vervoort, J.; Berkel, W. J. H.; Rietjens, I. M. C. M.; Golovleva, L. A. Reductive Deamination as a New Step in the Anaerobic Microbial Degradation of Halogenated Anilines. *FEMS Microbiol Lett* **2002**, *209* (2), 307–312. https://doi.org/10.1111/j.1574-6968.2002.tb11149.x..

(14) Zhang, S.; Wondrousch, D.; Cooper, M.; Zinder, S. H.; Schüürmann, G.; Adrian, L. Anaerobic Dehalogenation of Chloroanilines by *Dehalococcoides* m*ccartyi* strain CBDB1 and *Dehalobacter* strain 14DCB1 via Different Pathways as Related to Molecular Electronic Structure. *Environ Sci Technol* **2017**, *51* (7), 3714–3724. https://doi.org/10.1021/acs.est.6b05730.

(15) Susarla, S.; Yonezawa, Y.; Masunaga, S. Reductive Dehalogenation of Chloroanilines in Anaerobic Estuarine Sediment. *Environ. Technol.* **1997**, *18* (1), 75–83. https://doi.org/10.1080/09593331808616514.

(16) Susarla, S.; Yonezawa, Y.; Masunaga, S. Reductive Transformations of Halogenated Aromatics in Anaerobic Estuarine Sediment: Kinetics, Products and Pathways. *Water Res* **1998**, *32* (3), 369–348.

(17) Duc, H. D.; Oanh, N. T. Anaerobic Degradation of Chloroanilines by *Geobacter* sp. KT5. *Curr Microbiol* **2019**, *76* (2), 248–257. https://doi.org/10.1007/s00284-018-1617-7.

(18) Oanh, N. T.; Duc, H. D. Anaerobic Degradation of Propanil in Soil and Sediment Using Mixed Bacterial Culture. *Curr Microbiol* **2021**, *78* (4), 1499–1508. https://doi.org/10.1007/s00284-021-02419-7.

(19) Lu, H.; Zhang, T.; Zhou, Y.; Zhou, J.; Wang, J.; Wang, X. Enhanced Dechlorination and Biodegradation of 2-Chloroaniline by a 2-Aminoanthraquinone-Graphene Oxide Composite

under Anaerobic Conditions. *Sci Rep* **2019**, *9* (1), 12376. https://doi.org/10.1038/s41598-019-48904-9.

(20) Kraus, S. Aerobic and Anaerobic Biotransformation of Chloroanilines, Chlorobenzenes, and Dichlonitrobenzenes at a Complex Industrial Site in Brazil and Analysis of Associated Microbial Communities. Master Thesis, University of Toronto, Toronto, 2018. https://hdl.handle.net/1807/91484 (accessed 2024-06-11).

(21) Edwards, E. A.; Grbic-Galic, D. Anaerobic Degradation of Toluene and O-Xylene by a Methanogenic Consortium. *Appl Environ Microbiol* **1994**, 313–322.

(22) Toth, C. R. A.; Luo, F.; Bawa, N.; Webb, J.; Guo, S.; Dworatzek, S.; Edwards, E. A. Anaerobic Benzene Biodegradation Linked to the Growth of Highly Specific Bacterial Clades. *Environ Sci Technol* **2021**, *55* (12), 7970–7980. https://doi.org/10.1021/acs.est.1c00508.

(23) Engelbrektson, A.; Kunin, V.; Wrighton, K. C.; Zvenigorodsky, N.; Chen, F.; Ochman, H.; Hugenholtz, P. Experimental Factors Affecting PCR-Based Estimates of Microbial Species Richness and Evenness. *ISME Journal* **2010**, *4* (5), 642–647. https://doi.org/10.1038/ismej.2009.153.

(24) Ferris, M. J.; Muyzer, G.; Ward, D. M. Denaturing Gradient Gel Electrophoresis Profiles of 16S rRNA-Defined Populations Inhabiting a Hot Spring Microbial Mat Community. *Appl Environ Microbiol* **1996**, *62* (2), 340–346.

(25) Bolyen, E.; Rideout, J. R.; Dillon, M. R. Reproducible, Interactive, Scalable and Extensible Microbiome Data Science Using QIIME 2. *Nat. Biotechnol*, **2019**, pp 852–857. https://doi.org/10.1038/s41587-019-0190-3.

(26) Callahan, B. J.; McMurdie, P. J.; Rosen, M. J.; Han, A. W.; Johnson, A. J. A.; Holmes, S. P. DADA2: High-Resolution Sample Inference from Illumina Amplicon Data. *Nat Methods* **2016**, *13* (7), 581–583. https://doi.org/10.1038/nmeth.3869.

(27) Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F. O. The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools. *Nucleic Acids Res* **2013**, *41*, D590-6. https://doi.org/10.1093/nar/gks1219.

(28) Ritalahti, K. M.; Amos, B. K.; Sung, Y.; Wu, Q.; Koenigsberg, S. S.; Löffler, F. E. Quantitative PCR Targeting 16S rRNA and Reductive Dehalogenase Genes Simultaneously Monitors Multiple *Dehalococcoides* strains. *Appl Environ Microbiol* **2006**, *72* (4), 2765–2774. https://doi.org/10.1128/AEM.72.4.2765-2774.2006.

(29) Lane, D. J. 16S/23S rRNA Sequencing. In *Nucleic acid techniques in bacterial systematics*; Stackebrandt, E., Goodfellow, M., Eds.; John Wiley and Sons: Chichester, United Kingdom, 1991; pp 115–175.

(30) Yu, Y.; Lee, C.; Kim, J.; Hwang, S. Group-Specific Primer and Probe Sets to Detect Methanogenic Communities Using Quantitative Real-Time Polymerase Chain Reaction. *Biotechnol Bioeng* **2005**, *89* (6), 670–679. https://doi.org/10.1002/bit.20347.

(31) Cheng, J.; Suidan, M. T.; Venosa, A. D. Abiotic Reduction of 2,4-Dinitrotoluene in the Presence of Sulfide Minerals under Anoxic Conditions. *Wat. Sci. Tech.* **1996**, *34* (10), 25–33. https://doi.org/10.1016/S0273-1223(96)00693-2.

(32) Elsner, M.; Schwarzenbach, R. P.; Haderlein, S. B. Reactivity of Fe(II)-Bearing Minerals toward Reductive Transformation of Organic Contaminants. *Environ Sci Technol* **2004**, *38* (3), 799–807. https://doi.org/10.1021/es0345569.

(33) Klupinski, T. P. Abiotic Degradation of Pentachloronitrobenzene by Fe (II): Reactions on Goethite and Iron Oxide Nanoparticles. *Environ. Sci. Technol* **2004**, *38*, 4353–4360. https://doi.org/10.1021/es035434j.

(34) Niedźwiecka, J. B.; Drew, S. R.; Schlautman, M. A.; Millerick, K. A.; Grubbs, E.; Tharayil, N.; Finneran, K. T. Iron and Electron Shuttle Mediated (Bio)Degradation of 2,4-Dinitroanisole (DNAN). *Environ Sci Technol* **2017**, *51* (18), 10729–10735. https://doi.org/10.1021/acs.est.7b02433.

(35) Khatiwada, R.; Root, R. A.; Abrell, L.; Sierra-Alvarez, R.; Field, J. A.; Chorover, J. Abiotic Reduction of Insensitive Munition Compounds by Sulfate Green Rust. *Environ. Chem.* **2018**, *15* (5), 259–266. https://doi.org/10.1071/EN17221.

(36) Cárdenas-Hernández, P. A.; Anderson, K. A.; Murillo-Gelvez, J.; Di Toro, D. M.; Allen, H. E.; Carbonaro, R. F.; Chiu, P. C. Reduction of 3-Nitro-1,2,4-Triazol-5-One (NTO) by the Hematite-Aqueous Fe(II) Redox Couple. *Environ Sci Technol* **2020**, *54* (19), 12191–12201. https://doi.org/10.1021/acs.est.0c03872.

(37) Niedźwiecka, J. B.; McGee, K.; Finneran, K. T. Combined Biotic-Abiotic 2,4-Dinitroanisole Degradation in the Presence of Hexahydro-1,3,5-Trinitro-1,3,5-Triazine. *Environ Sci Technol* **2020**, *54* (17), 10638–10645. https://doi.org/10.1021/acs.est.0c02363.

(38) Menezes, O.; Yu, Y.; Root, R. A.; Gavazza, S.; Chorover, J.; Sierra-Alvarez, R.; Field, J. A. Iron(II) Monosulfide (FeS) Minerals Reductively Transform the Insensitive Munitions

Compounds 2,4-Dinitroanisole (DNAN) and 3-Nitro-1,2,4-Triazol-5-One (NTO). *Chemosphere* **2021**, *285*, 131409. https://doi.org/10.1016/j.chemosphere.2021.131409.

(39) Yu, H. Y.; Wang, Y. kui; Chen, P. cheng; Li, F. bai; Chen, M. jia; Hu, M.; Ouyang, X. Effect of Nitrate Addition on Reductive Transformation of Pentachlorophenol in Paddy Soil in Relation to Iron(III) Reduction. *J Environ Manage* **2014**, *132*, 42–48. https://doi.org/10.1016/j.jenvman.2013.10.020.

(40) Kruse, T.; Maillard, J.; Goodwin, L.; Woyke, T.; Teshima, H.; Bruce, D.; Detter, C.; Tapia, R.; Han, C.; Huntemann, M.; Wei, C. L.; Han, J.; Chen, A.; Kyrpides, N.; Szeto, E.; Markowitz, V.; Ivanova, N.; Pagani, I.; Pati, A.; Pitluck, S.; Nolan, M.; Holliger, C.; Smidt, H. Complete Genome Sequence of *Dehalobacter restrictus* PER-K23T. *Stand Genomic Sci* **2013**, *8* (3), 375-388. https://doi.org/10.4056/sigs.3787426.

(41) Molenda, O.; Puentes Jácome, L. A.; Cao, X.; Nesbø, C. L.; Tang, S.; Morson, N.; Patron, J.; Lomheim, L.; Wishart, D. S.; Edwards, E. A. Insights into Origins and Function of the Unexplored Majority of the Reductive Dehalogenase Gene Family as a Result of Genome Assembly and Ortholog Group Classification. *Environ Sci Process Impacts* **2020**, *22* (3), 663–678. https://doi.org/10.1039/c9em00605b.

(42) Bulka, O.; Webb, J.; Dworatzek, S.; Mahadevan, R.; Edwards, E. A. A Multifunctional *Dehalobacter*? Tandem Chloroform and Dichloromethane Degradation in a Mixed Microbial Culture. *Environ Sci Technol* **2023**, *57* (48), 19912–19920. https://doi.org/10.1021/acs.est.3c06686.

(43) Tang, S.; Gong, Y.; Edwards, E. A. Semi-Automatic in Silico Gap Closure Enabled de Novo Assembly of Two *Dehalobacter* Genomes from Metagenomic Data. *PLoS One* **2012**, *7* (12), e52038. https://doi.org/10.1371/journal.pone.0052038.

(44) Tang, S.; Wang, P. H.; Higgins, S. A.; Löffler, F. E.; Edwards, E. A. Sister *Dehalobacter* Genomes Reveal Specialization in Organohalide Respiration and Recent Strain Differentiation Likely Driven by Chlorinated Substrates. *Front Microbiol* **2016**, *7*, 100. https://doi.org/10.3389/fmicb.2016.00100.

(45) Nelson, J. L.; Fung, J. M.; Cadillo-Quiroz, H.; Cheng, X.; Zinder, S. H. A Role for *Dehalobacter* spp. in the Reductive Dehalogenation of Dichlorobenzenes and Monochlorobenzene. *Environ Sci Technol* **2011**, *45* (16), 6806–6813. https://doi.org/10.1021/es200480k.

(46) Wang, S.; Araujo, S. P.; Lomheim, L.; Mack, E. E.; Edwards, E. A.; Passport, E. *Dehalobacter converts 2,3-dichloroaniline to aniline via 2-chloroaniline in a methanogenic enrichment culture*; 2025. Manuscript in preparation (preprint on *BioRxiv*).

(47) Cole, J. R.; Cascarelli, A. L.; Mohn, W. W.; Tiedjel, J. M. Isolation and Characterization of a Novel Bacterium Growing via Reductive Dehalogenation of 2-Chlorophenol. *Appl Environ Microbiol* **1994**, *60* (10), 3536–3542.

(48) Sanford, R. A.; Cole, J. R.; Tiedje, J. M. Characterization and Description of *Anaeromyxobacter dehalogenans* gen. nov., sp. nov., an Aryl-Halorespiring Facultative Anaerobic Myxobacterium. *Appl Environ Microbiol* **2002**, *68* (2), 893–900. https://doi.org/10.1128/AEM.68.2.893-900.2002.

(49) Thomas, S. H.; Wagner, R. D.; Arakaki, A. K.; Skolnick, J.; Kirby, J. R.; Shimkets, L. J.; Sanford, R. A.; Löffler, F. E. The Mosaic Genome of *Anaeromyxobacter dehalogenans* strain 2CP-C Suggests an Aerobic Common Ancestor to the Delta-Proteobacteria. *PLoS One* **2008**, *3* (5), e2103. https://doi.org/10.1371/journal.pone.0002103.

(50) Fincker, M.; Spormann, A. M. Biochemistry of Catabolic Reductive Dehalogenation. *Annu Rev Biochem* **2017**, *86*, 357–386. https://doi.org/10.1146/annurev-biochem.

(51) Qian, Y.; Xu, M.; Deng, T.; Hu, W.; He, Z.; Yang, X.; Wang, B.; Song, D.; Chen, L.; Huang, Y.; Sun, G. Synergistic Interactions of *Desulfovibrio* and *Petrimonas* for Sulfate-Reduction Coupling Polycyclic Aromatic Hydrocarbon Degradation. *J Hazard Mater* **2021**, *407*, 124385. https://doi.org/10.1016/j.jhazmat.2020.124385.

(52) Manchester, M. J.; Hug, L. A.; Zarek, M.; Zila, A.; Edwards, E. A. Discovery of a Trans-Dichloroethene-Respiring *Dehalogenimonas* Species in the 1,1,2,2-Tetrachloroethane-Dechlorinating WBC-2 Consortium. *Appl Environ Microbiol* **2012**, *78* (15), 5280–5287. https://doi.org/10.1128/AEM.00384-12.

(53) Kato, S.; Yumoto, I.; Kamagata, Y. Isolation of Acetogenic Bacteria That Induce Biocorrosion by Utilizing Metallic Iron as the Sole Electron Donor. *Appl Environ Microbiol* **2015**, *81* (1), 67–73. https://doi.org/10.1128/AEM.02767-14.

(54) Ise, K.; Suto, K.; Inoue, C. Microbial Diversity and Changes in the Distribution of Dehalogenase Genes during Dechlorination with Different Concentrations of Cis -DCE. *Environ Sci Technol* **2011**, *45* (12), 5339–5345. https://doi.org/10.1021/es104199y.

(55) Menezes, O.; Kocaman, K.; Wong, S.; Rios-Valenciana, E. E.; Baker, E. J.; Hatt, J. K.; Zhao, J.; Madeira, C. L.; Krzmarzick, M. J.; Spain, J. C.; Sierra-Alvarez, R.; Konstantinidis, K. T.; Field, J. A. Quinone Moieties Link the Microbial Respiration of Natural Organic Matter to the Chemical Reduction of Diverse Nitroaromatic Compounds. *Environ Sci Technol* **2022**, *56* (13), 9387–9397. https://doi.org/10.1021/acs.est.2c01329.

(56) Murillo-Gelvez, J.; Di Toro, D. M.; Allen, H. E.; Carbonaro, R. F.; Chiu, P. C. Reductive Transformation of 3-Nitro-1,2,4-Triazol-5-One (NTO) by Leonardite Humic Acid and Anthraquinone-2,6-Disulfonate (AQDS). *Environ Sci Technol* **2021**, *55* (19), 12973–12983. https://doi.org/10.1021/acs.est.1c03333.

(57) Ratasuk, N.; Nanny, M. A. Characterization and Quantification of Reversible Redox Sites in Humic Substances. *Environ Sci Technol* **2007**, *41* (22), 7844–7850. https://doi.org/10.1021/es071389u.

(58) Uchimiya, M.; Stone, A. T. Reversible Redox Chemistry of Quinones: Impact on Biogeochemical Cycles. *Chemosphere* **2009**, *77* (4), 451–458. https://doi.org/10.1016/j.chemosphere.2009.07.025.