Tolerance of Anaerobic Bacteria to Chlorinated Solvents

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The aim of this research was to evaluate the effects of four chlorinated aliphatic hydrocarbons (CAHs), perchloroethene (PCE), carbon tetrachloride (CT), chloroform (CF) and 1,2-dichloroethane (1,2-DCA), on the growth of eight anaerobic bacteria: four fermentative species (Escherichia coli, Klebsiella sp., Clostridium sp. and Paenibacillus sp.) and four respiring species (Pseudomonas aeruginosa, Geobacter sulfurreducens, Shewanella oneidensis and Desulfovibrio vulgaris). Effective concentrations of solvents which inhibited growth rates by 50% (EC50) were determined. The octanol-water partition coefficient or log P_{o/w} of a CAH proved a generally satisfactory measure of its toxicity. Most species tolerated approximately 3-fold and 10-fold higher concentrations of the two relatively more polar CAHs CF and 1,2-DCA, respectively, than the two relatively less polar compounds PCE and CT. EC50 values correlated well with growth rates observed in solvent-free cultures, with fast-growing organisms displaying higher tolerance levels. Overall, fermentative bacteria were more tolerant to CAHs than respiring species, with iron- and sulfate-reducing bacteria in particular appearing highly sensitive to CAHs. These data extend the current understanding of the impact of CAHs on a range of anaerobic bacteria, which will benefit the field of bioremediation.

Key words: solvent tolerance, chlorinated solvents, anaerobic bacteria, toxicity

Chlorinated aliphatic hydrocarbons (CAHs) contaminate many soil and groundwater sites worldwide. Due to their significant hydrophobicity, resistance to degradation and toxicity, they create long-term sources of pollution and are the target of remediation efforts (8). Bioremediation, a technology relying on bacteria to degrade contaminants, is a proven treatment option for CAH remediation (2, 52). Highly chlorinated CAHs such as perchloroethene (PCE) and carbon tetrachloride (CT) are not susceptible to degradation by aerobic bacteria, but can be detoxified through the action of many types of anaerobic bacteria (21, 38). These include organohalide respiring bacteria (ORB), iron-, sulfate- and nitrate-reducing bacteria, and fermenters (13, 15, 36, 43, 48). These bacteria can either degrade CAHs enzymatically and derive energy in this way or mediate CAH removal cometabolically.

One important limitation of microbially mediated CAH degradation is the occurrence of toxic effects on bacteria. Being lipid-soluble solvents, CAHs partition in bacterial membranes, causing substantial damage to cells when present above certain concentrations (29). The magnitude of this toxicity depends on a solvent’s physicochemical attributes combined with the ability of a particular bacterial species to mount responses to solvent stress. Tolerance mechanisms employed to withstand solvent stress include solvent degradation, modification of membrane and cell wall composition, and active solvent extrusion by efflux pumps (20, 46, 47). Tolerance varies between species, and CAH bioremediation strategies that employ bacteria selected for their superior tolerance to the target solvents are more likely to achieve successful rates and extents of remediation.

However, most knowledge concerning the solvent tolerance of bacteria has been accumulated through studies of aerobic bacteria. For example, it has been shown that the toxicity of a particular solvent is correlated with its hydrophobicity, given by its log P_{o/w} value (28). Log P_{o/w}, the logarithm of a solvent’s partitioning in a standard octanol/water two-phase system, represents the ratio of solvent concentrations in each of the two liquids:

\[
\text{Log } P_{o/w} = \log \left( \frac{[\text{solvent}]_{\text{octanol}}}{[\text{solvent}]_{\text{water}}} \right)
\]

High log P_{o/w} solvents are more hydrophobic (or lipophilic) and those with low log P_{o/w} are more hydrophilic. Solvents with log P_{o/w} of between 1 and 4 are thought to be especially toxic to bacteria, as they are water-miscible enough to be bioavailable and lipid-soluble enough to disrupt bacterial membranes and cause cell death (49). Numerous aerobic bacteria able to grow in the presence of free-phase solvents have now been isolated, a large proportion of which belong to the genera *Pseudomonas*, *Bacillus* and *Rhdodococcus* (27, 29).

The same level of information is not available concerning anaerobic bacteria. Certain species such as *Clostridium acetobutylicum* and *Zymomonas mobilis* employed in the production of solvents and biofuels have been studied in detail (10, 45); however, very few data exist regarding the solvent tolerance of other anaerobic species important in CAH bioremediation settings. Because CAH-contaminated environments such as soil and aquifers are often complex and inhabited by a variety of species, it is useful to not only understand how solvents influence the species targeted as the catalyst of remediation but also co-existing species which may modulate bioremediation outcomes through mutualism or competition. In this sense, a broad view of the
solvent tolerance of different metabolic types of anaerobes is beneficial. While reports of solvent levels tolerated by ORB are available (1, 5, 55), this is not the case for other metabolic types. Dulldhardt et al. (18, 19) recently determined solvent tolerance levels for the iron reducer *Geobacter sulfurreducens*, the sulfate reducer *Desulfococcus multivorans* and the nitrate reducer *Thauera aromatica* with a number of hydrocarbons relevant to oil contamination; however, no CAHs were tested.

The present article focuses on the sensitivity of anaerobic bacteria to chlorinated solvents. The aim was to generate EC50 values for a diverse range of bacteria potentially relevant to CAH bioremediation. The impact of perchloroethene (PCE), carbon tetrachloride (CT), chloroform (CF) and 1,2-dichloroethane (1,2-DCA) on bacterial growth rates was assessed. These four CAHs were chosen as they are classified as priority pollutants (8) and are amenable to degradation by anaerobic bacteria (16, 24, 39, 40).

A broad range of bacteria was chosen based on their environmental origin, relevance to bioremediation, and presence in the existing solvent tolerance literature for ease of data comparison. Of the four fermentative bacteria selected, *Escherichia coli* was chosen due to its widespread use as a model organism for bacteria in general and the large body of literature concerning various aspects of its tolerance to xenobiotic compounds. *Klebsiella* sp. was chosen as a fast-growing fermenter and was isolated from a wastewater treatment plant, which potentially exposed it to a range of compounds and influenced its ability to tolerate solvents. *Clostridium* sp. and *Paenibacillus* sp. were isolated from a CAH-contaminated site and were of particular interest since their long-term exposure to CAHs hypothetically affected their response to solvent stress. Of the four respiratory lineages examined, the nitrate reducer *Pseudomonas aeruginosa* was chosen because of the considerable amount of knowledge regarding the resistance of *Pseudomonas* spp. to organic solvents. The iron reducers *Geobacter sulfurreducens* and *Shewanella oneidensis* were chosen as representatives of ubiquitous respiring anaerobes with bioremediation potential, and finally the sulfate reducer *Desulfovibrio vulgaris* was selected as a representative of sulfate-reducing bacteria, also widespread in anaerobic environments and with potential applications as agents of bioremediation.

### Materials and Methods

**Bacteria and culture conditions**

All bacteria included in this study were cultivated under conditions known to favor optimal growth in order to minimize the impact of growth-limiting factors other than CAHs. *Escherichia coli* strain K12 substrain W3110 and *Pseudomonas aeruginosa* strain PA01 were obtained from the University of New South Wales culture collection. Both species were cultivated under N2 in Luria Bertani broth (LB), containing per L: 10.0 g tryptone, 5.0 g yeast extract and 10.0 g NaCl. For *P. aeruginosa*, 2.0 g L⁻¹ NaNO₃ was added as this organism requires NO₃⁻ as an electron acceptor in the absence of oxygen.

*Klebsiella* sp. strain SM1, *Clostridium* sp. strain BIP1 and *Paenibacillus* sp. strain BIP2 were isolated in our laboratory and deposited in the Belgian Co-ordinated Collection of Microorganism (BCCM) under the respective strain numbers LMG 26928, LMG 26929 and LMG 26926. Isolation and identification procedures are described below. These three bacteria were grown under N₂ in half-concentrated Brain–Heart Infusion broth (Oxoid, Basingstoke, UK) containing, per L: 8.75 g beef heart infusion solids, 5.0 g protein peptone, 1.0 g glucose, 2.5 g NaCl and 1.25 g NaHPO₄ and 0.4 g L⁻¹ cysteine-HCl as a reducing agent.

*Geobacter sulfurreducens* strain 12177 and *Desulfovibrio vulgaris* subsp. vulgaris strain 2119 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *G. sulfurreducens* was cultivated under N₀–CO₂ (80/20 v/v) in the DSMZ-recommended defined medium DSM 826, with 10 mmol L⁻¹ acetate as an electron donor and 50 mmol L⁻¹ fumarate as an electron acceptor (http://www.dsmz.de, accessed 30/07/2013). *Desulfovibrio vulgaris* was cultivated under N₂–CO₂ in DSMZ medium 334, replacing the acetate with 10 mmol L⁻¹ lactate as an electron donor and adding 5 mmol L⁻¹ sulfate as an electron acceptor. *Shewanella oneidensis* strain MR-1 was purchased from the American Type Culture Collection (ATCC strain 700550) and cultivated under N₂–CO₂ in a defined medium described in Löfler et al. (37) with 20 mmol L⁻¹ lactate as an electron donor and 20 mmol L⁻¹ fumarate as an electron acceptor.

**Solvent tolerance tests**

Solvent tolerance tests were carried out in 25 mL Hungate tubes containing 10 mL medium, capped with Teflon-lined rubber stoppers and sealed with aluminum crimp caps. Incubation temperatures were as follows: *E. coli* 30°C, *P. aeruginosa* 37°C, *S. oneidensis* 30°C, *Klebsiella* sp. 26°C, *Paenibacillus* sp. 30°C, *Clostridium* sp. 24°C, *G. sulfurreducens* 30°C and *D. vulgaris* 30°C. Each species was tested with PCE, CT, CF and 1,2-DCA, each at five different concentrations in triplicate. One set of control triplicates did not include any solvent and served as the benchmark for growth rate comparisons. Each species was tested with all four CAHs at the same time in order to minimize any variations between cultures used on different days.

Solvents were added to autoclaved tubes directly as the free phase or as dilute ethanolic stocks (1 to 100 µL). When ethanolic stocks were used, one set of triplicates was set up including the maximum ethanol concentration (analytical grade ethanol; Ajax Finechem, Sydney, Australia) used in order to control the effect of ethanol on growth. No differences were observed between no-solvent and
ethanol-only controls. Tubes were left at room temperature overnight to allow for solvent equilibration between the aqueous phase and headspace before inoculation. Tests were initiated by inoculating exponentially growing parent cultures (5% transfers). Growth was monitored by measuring optical density at 600 nm against medium blanks. Monitoring ceased as soon as the stationary phase was reached, which took between 5 h for fast-growing organisms such as Klebsiella sp. and 70 h for slow growers such as D. vulgaris.

**Mathematical treatment of data**

Actual solvent concentrations in the aqueous phase were calculated using Henry’s constants for each temperature, aqueous and gas phase volumes and volumes of solvent added. For PCE, solvent concentrations above the maximum aqueous solubility of 0.9 mmol L\(^{-1}\) are nominal and do not reflect actual dissolved concentrations but rather indicate increasing amounts of free-phase PCE.

Specific growth rates \( \mu \) (h\(^{-1}\)) were determined by plotting the natural logarithm of OD\(_{600}\) at time “t” \((X_t)\) over that at time “0” \((X_0)\) versus time:

\[
\mu = \frac{\ln \left( \frac{X_t}{X_0} \right)}{t}
\]

Time “0” was taken as the beginning of exponential growth. No differences in lag or exponential phase lengths were observed between no-solvent controls and solvent-containing cultures: bacteria consistently started and ceased growing at the same time and growth only varied in rate. Average specific growth rates were calculated for each set of triplicates and are expressed as percentages of the no-solvent controls’ average growth rate \( \mu_0 \) set at 100%. These percentages were plotted against solvent concentrations and the effective concentration causing a 50% decrease in growth rate \( (EC_{50}) \) was estimated by linear interpolation and in a few cases by the effective concentration causing a 50% decrease in growth rate \( (EC_{50}) \) was estimated by extrapolation at 4.95 mmol L\(^{-1}\). Except for S. oneidensis, D. vulgaris and G. sulfurreducens, all bacteria had EC\(_{50}\) values for PCE above this solvent’s maximum solubility of 0.9 mmol L\(^{-1}\) which means they were still able to grow at or above 50% of \( \mu_0 \) when PCE was present as a separate phase. Further increases in nominal PCE concentrations led to eventual complete inhibition of growth, indicating that free-phase PCE itself exerted toxic effects. S. oneidensis, D. vulgaris and G. sulfurreducens were affected by PCE below the point of free-phase appearance.

All eight anaerobic bacteria tested were negatively affected by the presence of CAHs in the growth medium, and solvent concentrations found to decrease their growth rates by half are reported in Fig. 1. All eight species were able to grow at 50% of no-solvent rates \( (\mu_0) \) with 0.3 mmol L\(^{-1}\) PCE or more (Fig. 1A). Klebsiella sp. was the species most tolerant to PCE, and could still grow at 54% of \( \mu_0 \) at the maximum nominal concentration tested of 4.58 mmol L\(^{-1}\); hence, its EC\(_{50}\) value for PCE was estimated by extrapolation at 4.95 mmol L\(^{-1}\). For S. oneidensis, D. vulgaris and G. sulfurreducens, all bacteria had EC\(_{50}\) values for PCE above this solvent’s maximum solubility of 0.9 mmol L\(^{-1}\) which means they were still able to grow at or above 50% of \( \mu_0 \) when PCE was present as a separate phase. Further increases in nominal PCE concentrations led to eventual complete inhibition of growth, indicating that free-phase PCE itself exerted toxic effects. S. oneidensis, D. vulgaris and G. sulfurreducens were affected by PCE below the point of free-phase appearance.

With CT (Fig. 1B), S. oneidensis, D. vulgaris and G. sulfurreducens were unable to grow even at the lowest levels tested of 80, 80 and 30 \( \mu \)M, respectively. The other five species displayed EC\(_{50}\) values between 0.5 and 2.4 mmol L\(^{-1}\), corresponding to between 10 and 50% of CT’s aqueous solubility of 5 mmol L\(^{-1}\). Paenibacillus sp., isolated from a CAH-contaminated site, was the organism most tolerant to CT with an EC\(_{50}\) value of 2.4 mmol L\(^{-1}\).

In general, the anaerobic bacteria under study could tolerate more CF and 1,2-DCA than PCE and CT (Fig. 1C and 1D). With the exception of G. sulfurreducens and D. vulgaris, all microorganisms displayed EC\(_{50}\) values for CF above 3.5 mmol L\(^{-1}\). G. sulfurreducens showed an EC\(_{50}\) for CF of 0.2 mmol L\(^{-1}\), while D. vulgaris was the organism most sensitive to CF and was completely inhibited even at the lowest test concentration of 0.2 mmol L\(^{-1}\). All eight lineages could tolerate more 1,2-DCA in the medium than any other solvent, EC\(_{50}\) values being consistently above 6.5 mmol L\(^{-1}\). G. sulfurreducens and D. vulgaris could still grow at 70% and 91% of \( \mu_0 \) at the respective highest 1,2-DCA levels tested of 3.72 and 4.65 mmol L\(^{-1}\), while an EC\(_{50}\) of 6.5 mmol L\(^{-1}\) could be calculated by extrapolation for G. sulfurreducens, it could not be calculated for D. vulgaris.
given the lack of a decreasing trend of growth rate versus 1,2-DCA. Because *D. vulgaris* was at least as tolerant to 1,2-DCA as *G. sulfurreducens*, a minimum EC\textsubscript{50} of 6.5 mmol L\textsuperscript{-1} is reported for this species. Overall, the most solvent-tolerant anaerobic bacteria were *Klebsiella* sp. and *E. coli*, consistently appearing in the top three highest EC\textsubscript{50} values for any of the four solvents tested. The least tolerant organisms were *D. vulgaris* and *G. sulfurreducens*.

The ability of a species to tolerate CAHs, as given by EC\textsubscript{50}, correlated well with its growth rate in solvent-free cultures, \( \mu_0 \) (Fig. 2). Coefficients of determination for linear regressions fitted to the data were above 0.7 for all solvents except CT (Fig. 2B), validating growth rate as an important parameter influencing bacterial solvent tolerance. In the case of CT, the ability of a species to grow rapidly under stress-free conditions did not influence its capacity to tolerate the chlorinated compound, as it did with the other three CAHs. Nevertheless, when scoring a species’ solvent tolerance by normalizing and averaging its EC\textsubscript{50} values across all four CAHs, a very good correlation appears between the maximum growth rate and tolerance (\( R^2 = 0.89 \), Fig. 2E). Slow-growing organisms such as *G. sulfurreducens* and *D. vulgaris* were the most sensitive overall, while fast-growing species such as *Klebsiella* sp. and *E. coli* demonstrated marked resistance to CAH toxicity.

When considering individual CAHs, a good correlation could be found between a compound’s hydrophobicity, as denoted by its log \( P_{ow} \), and its ability to inhibit bacterial growth (Fig. 3). For all eight bacterial lineages tested, 1,2-DCA (log \( P_{ow} = 1.48 \)) was consistently less toxic than CF (log \( P_{ow} = 1.97 \)), CT (log \( P_{ow} = 2.64 \)) and PCE (log \( P_{ow} = 2.88 \)). For all but two species, CF was less toxic than CT and PCE, the exceptions to this rule being the two respiring species *G. sulfurreducens* and *D. vulgaris*. The delineation between CT and PCE was less clear. Except for *Paenibacillus* sp. and despite a marginally lower log \( P_{ow} \), CT was consistently more toxic than PCE, as it took less CT to diminish growth rates by half than PCE. Of particular note is the severe impact of CT on the three respiring strains *S. oneidensis*, *G. sulfurreducens* and *D. vulgaris*, which showed no growth at all at or below 80 \( \mu M \).

**Discussion**

The rapid and complete bioremediation of CAHs in soil and groundwater hinges on the capacity of the bacteria involved to tolerate high concentrations of these solvents. Depending on the solvents present and on the cleanup protocol to be implemented, one or several anaerobic genera can be selected as detoxifying agents. Very few reports concerning the solvent tolerance of anaerobic bacteria are available, which represents a gap in the bioremediation knowledge base. In this report, the CAH tolerance of fermenting, iron-, sulfate-, nitrate- and fumarate-reducing bacterial species was studied.

Studies on the sensitivity of aerobic bacteria to solvents have led to an empirical rule concerning solvent toxicity, stating that a bacterium can grow with a free phase of any solvent with a log \( P_{ow} \) above that of the index value, equivalent to the log \( P_{ow} \) of the most toxic solvent tolerated (6, 7, 28). Solvents with log \( P_{ow} \) below the index value are too toxic to allow growth when present as a free phase.

Results of the current study support the validity of this rule for anaerobic bacteria. The CAH with the highest log \( P_{ow} \) was PCE, and five out of eight species tolerated the presence of a small volume of free-phase PCE, indicated by EC\textsubscript{50} values above the solubility of PCE (0.9 mmol L\textsuperscript{-1}). No species could tolerate the presence of free phases of any of the other three CAHs with lower log \( P_{ow} \). While EC\textsubscript{50} values for the more polar CF and 1,2-DCA were higher than for the less polar
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CT and PCE, these EC_{50} values actually represent smaller percentages of the solvents’ maximum aqueous solubility. The average EC_{50} for CT was 1 mmol L^{-1}, equivalent to 20% of CT solubility, while average EC_{50}s for CF and 1,2-DCA were 3.5 and 12.3 mmol L^{-1}, equating to 5 and 14% of maximum solubility respectively. This indicates that, overall, the anaerobic bacteria tested would be less able to grow with free phases of more polar than less polar compounds. This has implications for the bioremediation of solvents present as DNAPL, suggesting that more polar solvents such as 1,2-DCA are less likely to be tolerated as free phases than as dissolved plumes. With less polar compounds such as PCE, the likelihood of bacteria establishing themselves near DNAPL is higher, leading to faster dissolution times as degradation proceeds (12).

The increased toxicity of PCE to bacteria beyond its aqueous solubility threshold observed here reflects phenomena noted by other authors (9, 56). One potential explanation involves direct contact between cells and free-phase solvent increasing as the number and size of solvent droplets increased (56), thereby amplifying solvent partitioning in

Fig. 2. Relationship between no-solvent growth rate (\(\mu_0\)) and EC_{50}. Panel E comprises a plot of species’ averaged normalized EC_{50}s versus no-solvent growth rates. Normalization was carried out by setting the EC_{50} of Klebsiella sp. at 100% for each solvent and expressing EC_{50}s for all other species as percentages of klebsiella’s EC_{50}. All four values for each species were then averaged.

Fig. 3. Relationship between EC_{50} and CAH log P_{o/w} (dimensionless) for each species. Log P_{o/w} values were obtained from (35): 1,2-DCA: 1.48; CF: 1.97; CT: 2.64; PCE: 2.88. Legend: ◆ E. coli; □ P. aeruginosa; ▲ Klebsiella sp.; × Clostridium sp.; ○ Paenibacillus sp.; ● G. sulfurreducens; ◇ D. vulgaris; Δ S. oneidensis.
membranes and causing cell death.

Despite CT having a log $P_{\text{ow}}$ similar to that of PCE, it was more toxic than PCE for most species examined. The higher aqueous solubility limit of CT possibly accounts for this divergence. While PCE forms a free phase above 0.9 mmol L$^{-1}$, CT’s aqueous solubility is 5 mmol L$^{-1}$, meaning greater dissolved levels of CT can cause stress to bacterial cells before a separate phase is formed. Between 0.9 and 5 mmol L$^{-1}$, where many EC$_{50}$s are found, CT presents a much greater threat to cell integrity compared with PCE, by still existing in the dissolved form while being almost as hydrophobic as PCE.

In terms of solvent tolerance, the eight anaerobes investigated here can be classified into three groups. Firstly, the two fermenters *E. coli* and *Klebsiella* sp. displayed the highest resistance to CAH stress. They belong to the family Enterobacteriaceae of the class Gammaproteobacteria, are facultatively anaerobic, and were also the fastest growing bacteria tested. In the second category are the other two fermenting species *Clostridium* sp. and *Paenibacillus* sp., both belonging to the phylum *Firmicutes*, together with the two other Gammaproteobacteria *P. aeruginosa* and *S. oneidensis*. These four species displayed intermediate growth rates and EC$_{50}$ values. In the third category are the two Deltaproteobacteria *G. sulfurreducens* and *D. vulgaris*, the most CAH-sensitive species in this investigation and also the slowest growing and only strictly anaerobic respiring species.

When comparing EC$_{50}$ values obtained in this study with those given in the literature for aerobic bacteria, tolerance levels appear similar between aerobes and the fast-growing anaerobes of the first two groups outlined above. In a study by Heipieper et al. (26), EC$_{50}$s of 3.6 and 3 mmol L$^{-1}$ were reported for aerobic *Pseudomonas putida* exposed to toluene and styrene, two solvents with log $P_{\text{ow}}$ of 2.48 and 3.0, respectively. Hage et al. reported EC$_{50}$s of 8.0, 13.0, 1.5, 3.0, 0.5 and 1.9 mmol L$^{-1}$ for *P. putida* growing in the presence of various organic compounds with log $P_{\text{ow}}$ in the range 1.72–3.46 (25). Another anaerobe, *Acinetobacter calcoaceticus*, showed EC$_{50}$ of between 6.0 and 0.11 mmol L$^{-1}$ when exposed to alkanols of log $P_{\text{ow}}$ of between 1.87 and 3.97, with EC$_{50}$s decreasing with increasing log $P_{\text{ow}}$ (30). In this study, most bacteria tested showed EC$_{50}$ >0.5–23 mmol L$^{-1}$ with solvents of log $P_{\text{ow}}$ of 1.48–2.88. Hence, we conclude that anaerobic bacteria can be at least as tolerant as aerobic bacteria, depending on metabolism and growth rate.

The apparent relationship between growth rate and CAH tolerance could be explained by a high turnover of cell components in fast-growing species compensating for the damage inflicted through contact with CAHs. Further, a number of solvent tolerance mechanisms elucidated in Gram-negative bacteria, such as changes in membrane fatty acid composition and production of stress proteins, require *de novo* synthesis, strongly linked to cell growth (30, 32, 46). Gram-negative bacteria have also been shown to possess energy-dependent solvent efflux pumps (31, 54), pointing to energy status as an important factor in solvent resistance. In Gram-positive bacteria, changes in membrane composition and the appearance of stress proteins have also been demonstrated (3, 51). The sensitivity of slow-growing organisms in this study is likely linked to lower energy generation rates, leading to a delay in resistance mechanism activation and thus greater vulnerability to solvent attack. Duldhardt et al. (19) recently demonstrated that the anaerobic respirers *G. sulfurreducens*, *Desulfooccus multivorans* and *Thauera aromatica* respond to solvent toxicity through increases in the saturated fatty acid component of their cell membranes, a mechanism dependent on cell growth.

The compound CT was particularly toxic to *G. sulfurreducens*, *D. vulgaris* and *S. oneidensis*. When comparing EC$_{50}$ values obtained for *G. sulfurreducens* and *D. multivorans* in a study by Duldhardt et al. (18) to those obtained here for *G. sulfurreducens* and *D. vulgaris*, close similarities are found for chlorinated and non-chlorinated solvents of similar log $P_{\text{ow}}$. For instance, EC$_{50}$s for 1,2-DCA of 6.5 mmol L$^{-1}$ obtained here approximate the EC$_{50}$ of 7.6 to 8.9 mmol L$^{-1}$ obtained by Duldhardt et al. for phenol, which has the same log $P_{\text{ow}}$ as 1,2-DCA (log $P_{\text{ow}} = 1.48$). Also, EC$_{50}$s of 0.3–0.4 mmol L$^{-1}$ were determined here for PCE (log $P_{\text{ow}} = 2.88$) compared with EC$_{50}$s of 0.1–0.2 mmol L$^{-1}$ for solvents of log $P_{\text{ow}}$ near 3.0, such as 1-octanol and ethylbenzene, found by Duldhardt et al. The same correspondence, however, did not apply to CT and CF and non-chlorinated compounds with similar log $P_{\text{ow}}$ values, suggesting that CT and CF have additional distinct mechanisms of toxicity. A specific toxicity mechanism has previously been reported for CT, with the formation of radicals from CT interacting with reduced bacterial proteins and co-factors (14, 42). This is termed “reactive toxicity” and solvents which cause this type of toxicity do not fit models based on properties such as log $P_{\text{ow}}$ (9, 44). It is possible that electron chain components located in the cell membrane of respiring bacteria are more vulnerable to this toxic action than cytoplasm-based energy generation through fermentation. The toxicity of CT and CF to organohalide-respiring bacteria such as *Dehalococcoides* is well-documented (17, 41), suggesting the general susceptibility of anaerobic respiring bacteria to these compounds.

The nitrate respirer *P. aeruginosa*, despite possessing an electron chain, was not more susceptible to CT or CF than other solvents. Its high growth rate coupled with the well-known ability of *Pseudomonas* species to tolerate organic solvents in general (46) likely account for this exception. Moreover, the sensitivity of electron transport chain proteins to solvents might differ between nitrate respiration and other respiration pathways and is worthy of further research efforts.

Of note are the two fermenting bacteria *Clostridium* sp. strain BIP1 and *Paenibacillus* sp. strain BIP2, both isolated from a CAH-contaminated aquifer as part of this study. Several members of the Clostridia have been found to be associated with organochlorine-respiring bacteria (ORB) in dechlorinating cultures, where they are thought to participate in substrate fermentation and hydrogen supply to the ORB (22, 23, 50). Bowman et al. (11) tested the effects of 1,2-DCA and PCE on hydrogen production by 18 strains of *Clostridium* isolated from CAH-contaminated groundwater, and inhibitory concentrations fit well with EC$_{50}$ values obtained here for *Clostridium* sp. strain BIP1. Interestingly, the 16S rRNA gene of strain BIP1 most closely matched *Clostridium* sp. strain BL-26 isolated by Bowman et al., and it was also isolated from acidic (pH 3.5 to 5.8) CAH-contaminated
groundwater, suggesting that similar strains of *Clostridium* sp. may exist in acidic chlorinated solvent-polluted groundwater sites around the world. The *Paenibacillus* sp. strain examined here was isolated from the same contaminated site and was the most CT-tolerant bacterium amongst all those tested. It is possible that long-term *in situ* exposure to DNAPL comprising CT led to the increased tolerance of this organism. The potential of these groundwater-dwelling bacteria to act as hydrogen providers, together with their relatively high CAH tolerance compared with respiring species, suggests that encouraging the co-existence of fermenting and respiring species may have advantages over the stimulation of respiring species only in bioremediation strategies.

In summary, the data presented indicate that anaerobic bacteria are sensitive to CAHs, and their level of toxicity can generally be predicted by their log $P_{ow}$. However, log $P_{ow}$ is not always a reliable indicator and other parameters such as aqueous solubility and potential for reactive toxicity need to be evaluated when assessing the impact of CAH contamination in an aquifer or planning a bioremediation strategy. Fast-growing bacteria, especially fermenters, were found to be more tolerant overall than slow-growing anaerobic respiring bacteria, likely due to their capacity to rapidly activate energy-dependent resistance mechanisms. Efforts are underway in our laboratory to further elucidate possible links between growth rate and solvent tolerance. Bioremediation protocols would benefit from including fermenting organisms in their CAH detoxification plan, as not only can they often degrade contaminants through cometabolism but also new strategies.

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