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Anaerobic biotransformations of pollutant chemicals in aquifers

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SUMMARY

Anaerobic microbial communities sampled from either a methanogenic or sulfate-reducing aquifer site have been tested for their ability to degrade a variety of groundwater pollutants, including halogenated aromatic compounds, simple alkyl phenols and tetrachloroethylene. The haloaromatic chemicals were biodegraded in methanogenic incubations but not under sulfate-reducing conditions. The primary degradative event was typically the reductive removal of the aryl halides. Complete dehalogenation of the aromatic moiety was required before substrate mineralization was observed. The lack of dehalogenation activity in sulfate-reducing incubations was due, at least in part, to the high levels of sulfate rather than a lack of metabolic potential. In contrast, the degradation of cresol isomers occurred in both types of incubations but proved faster under sulfate-reducing conditions. The requisite microorganisms were enriched and the degradation pathway for *p*-cresol under the latter conditions involved the anaerobic oxidation of the aryl methyl group. Tetrachloroethylene was also degraded by reductive dehalogenation but under both incubation conditions. The initial conversion of this substrate to trichloroethylene was generally faster under methanogenic conditions. However, the transformation pathway slowed when dichloroethylene was produced and only trace concentrations of vinyl chloride were detected. These results illustrate that pollutant compounds can be biodegraded under anoxic conditions and a knowledge of the predominant ecological conditions is essential for accurate predictions of the transport and fate of such materials in aquifers.

INTRODUCTION

In recent years, it has become evident that aquifers can serve as sinks for a variety of organic chemical contaminants. As with surface habitats, the environmental and health risks associated with the pollution of groundwater reserves needs to be evaluated in light of the fate of the chemicals as well as their rate of transformation. An appreciation of subsurface microbiology is also relatively recent and the biodegradation pathways employed by the indigenous subsurface microflora is an area of intense scientific inquiry. The major factor prompting this interest in natural attenuation mechanisms is the realization that physicochemical processes like hydrolysis or volatilization may, at best, result in

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the partial alteration of aquifer pollutants. Microbial metabolism is the only process that can completely destroy the offending substances in situ and convert them to innocuous endproducts. There is also a growing commercial interest in the restoration of contaminated aquifers by stimulating the biodegradation abilities of the aquifer microbiota.

While information continues to emerge on the aerobic biodegradation pathways for aquifer pollutants, little research has concerned the fate of such materials when oxygen does not serve as the terminal electron acceptor. Yet, anaerobic conditions are known to develop in aquifers whenever the rate of microbial respiration of organic matter exceeds the rate of oxygen input via recharge, reaeration from soil gases or mixture of anoxic waters with surrounding oxygenated waters [5]. One reason for this lack of interest reflects historical attitudes toward anaerobic microbiology in general. Oxygen depletion was often found to limit the biodegradation of organic matter and the techniques for culturing anaerobes were tedious and costly. In addition, anaerobic bacteria were considered much less nutritionally versatile and slower-growing than aerobic counterparts. During the last two decades, advances in microbiological theory, such as the concept of interspecies hydrogen transfer [9], and improvements in laboratory culture techniques have stimulated a variety of studies to reevaluate these historical attitudes. Current research indicates that anaerobes are much more nutritionally diverse than previously imagined and capable of catalyzing novel biotransformations that are of environmental and commercial interest.

When labile organic matter enters oxygenated groundwater, the indigenous microflora use the contaminants as electron donors to support heterotrophic microbial respiration. Since oxygen is used by these organisms as a co-substrate and as an electron acceptor it is rapidly depleted. Microbial consumption of oxygen brings into action other groups of microorganisms with different biodegradation capabilities. Under conditions of anoxia, these organisms can use organic chemicals or several inorganic anions such as nitrate, sulfate or carbonate as alternate electron acceptors. Available organic matter thus represents electron donors to support anaerobic microbial metabolism under fermentative, denitrifying, sulfate-reducing or methanogenic conditions. While these anaerobic activities are not mutually exclusive, one can often observe a spatial separation of dominant metabolic processes depending on the availability of electron acceptors, the presence of suitable microorganisms and the energetic benefit of each process to the catalyzing microbial communities. Typically, nitrate reduction occurs first, followed by sulfate-reduction and then methanogenesis. As organic matter is transported in the flow of groundwater, a series of redox zones can be established which range from highly oxidized to highly reduced sites. Not surprisingly, different types of biodegradation activities can be observed within these redox zones. It is thus important to explore biodegradation potential linked to the consumption of different electron acceptors. To be most useful, anaerobic metabolic activities should be interpreted relative to the dominant ecological processes influencing carbon and electron flow. Ultimately, it may prove possible to stimulate desirable metabolic sequences in aquifers through the intentional introduction of suitable electron donor and acceptor combinations.

Indeed, the in situ anaerobic biodegradation of aquifer pollutants offers several potential advantages over aerobic biorestoration efforts. First, despite the fact that in situ biorestorations provide a cost-effective method of cleaning up contaminated aquifers, much of the expense associated with such procedures is tied to the cost of providing a suitable oxygen source; either with air or by providing ozone or hydrogen peroxide as alternate oxygen sources. This cost would be unnecessary with anaerobic treatment strategies. Secondly, some compounds may biodegrade at faster rates when incubated under anaerobic conditions. For example, Bouwer et al. [8] found that several halogenated aliphatic substrates were amenable to microbial metabolism under reducing conditions, but the same compounds persisted in aerobic incubations. Lastly, anaerobic biotransformations sometimes result in metabolic products that are less toxic and more amenable to subsequent aerobic metabolism.

Fogel et al. [14] observed the mineralization of methoxychlor when it was subjected to a combined anaerobic-aerobic soil incubation regime, but either process alone resulted in only slight degradation of the parent substrate. It is important to note that solid information on the predominant anaerobic and aerobic biodegradation pathways is required for accurate fate predictions and biorestoration strategy development. Substrate disappearance is not synonymous with mineralization. The partial degradation of organic substrates in either oxic or anoxic aquifers can result in the production of breakdown products which generate their own environmental and health consequences. Such contaminants may be of more toxicological concern than the parent substrates.

Over the past several years, we have studied the fate of various substrates in anoxic aquifer slurries or with microorganisms known to inhabit the terrestrial subsurface. The present contribution summarizes results from previous investigations on the anaerobic biodegradation of several halogenated and alkylated benzenoid compounds. In addition, more recent and as yet unpublished results on the transformation of tetrachloroethylene, a known groundwater pollutant, are included for comparison. Collectively, these findings help establish the fate of such compounds in reducing environments and illustrate the need to understand the governing microbial ecology in aquifers as it is influenced by the availability of various electron acceptors before the results of biodegradation tests can be interpreted successfully.

THE MICROBIAL ECOLOGY OF AN ANOXIC AQUIFER

An anoxic aquifer that receives leachate from the municipal landfill in Norman, OK was chosen as a study site. The landfill is located in central Oklahoma on the north bank of the South Canadian River in Cleveland County, OK. The landfill was operated as an open dump for 38 years. During this time, there were no restrictions on the type of materials disposed and open burning was practiced. In the 1960s, it was considered desirable to dig about 3 m deep trenches into the aquifer to deposit refuse. The trenches containing groundwater were filled with refuse and covered with permeable sand obtained from the immediate area. This practice continued until 1972 when state regulations required changes in operation to prevent further groundwater contamination. The modified disposal method was to place the refuse in layers at least 2 feet above the water table and cover at least weekly, but daily cover was commonly practiced [29].

The approximately 12-hectare landfill has now been closed and a clay capping was started in 1985. The water table at the site is about 1–2 m below the soil surface. The soil consists of quaternary recent alluvium of silt, sand, clay and dune sand. The soil is moderately to highly permeable. The alluvial sand layer extends 10–14 m in depth and is underlain by a 100 m layer of dense clay and chert gravel known locally as the 'red bed'. Groundwater flow was previously determined to be about 7° west of south at 0.6 m/year toward the river [29]. In a study of compounds leaching from the landfill, Robertson et al. [29] found over 40 organic chemicals of industrial or commercial importance in the groundwater including *p*-cresol, phthalates and a variety of straight and branched-chain volatile fatty acids.

Initial attempts at understanding the microbial ecology of the aquifer involved an assay for the presence of methanogenic bacteria at a site adjacent to one of the refuse mounds that was relatively accessible. A mineral salts enrichment medium was inoculated with aseptically obtained aquifer solids [4] and placed under an atmosphere which contained mainly hydrogen. With time, the hydrogen was consumed but no methane production was detected. After several more substrate additions, the aquifer solids blackened and a microscopic examination of the culture fluid showed the presence of numerous vibriod bacteria, suggesting the possible involvement of hydrogen-utilizing sulfate-reducing bacteria. Since there was no sulfate in the enrichment medium, the only source of this anion was the aquifer material itself. Subsequent analysis of the groundwater at this site showed that it had high

levels of sulfate year-round while water sampled from other areas did not (Table 1).

A microbiological and chemical characterization of several sampling locations has been recently completed [4]. We concluded that at least two spatially distinct sites exist within the aquifer. Sulfate reduction appeared to be the terminal metabolic process governing carbon dissimilation at the site originally sampled. However, carbon and electron flow were largely coupled with methanogenesis at another site located only about 100 m from the first. The evidence supporting this conclusion is summarized in Table 1.

The two sites differed in terms of observations

Table 1

Microbiological and chemical characterization of two sites within a shallow anoxic aquifer (adapted from Ref. 4)

Characteristic	Sampling site	
	methanogenic	sulfate-reducing
Field observations		
aquifer sediment color	sandy	dark grey/black
temperature (varies seasonally)	4–22°C	1–21°C
pH (varies seasonally)	6.0–7.9	3.9–7.2
dissolved O_2 (ppm)	un[a]	un–2
Groundwater analysis		
dissolved organic matter (ppm)	285–1100	14–160
chloride (ppm)	2000	250
NO_3^- (ppm)	un	un
SO_4^{2-} (ppm)	un–230 (mostly absent)	57–2000 (always present)
Microbial activities		
methane production in situ	yes	no
rate of methane production from endogenous substrates[b]	3–17	0.06–1.0
rate of methane production from endogenous substrates with SO_4^{2-} amendment[b]	1–3	0.06–1.0
% CH_4 recovery from:[c]		
acetate	92	3
acetate + SO_4^{2-}	4	1
formate	100	1
formate + SO_4^{2-}	9	1
hydrogen	51	41
hydrogen + SO_4^{2-}	4	1
methanol	100	70
methanol + SO_4^{2-}	74	75
trimethylamine	96	89
trimethylamine + SO_4^{2-}	87	89
Microbial enumerations		
numerically dominant bacterial population utilizing:		
acetate	methanogens	sulfate-reducers
formate	methanogens	sulfate-reducers
hydrogen	methanogens	sulfate-reducers

[a] Undetectable.

[b] Range (ppm · day^{-1} · g^{-1}) measured with aquifer material sampled on 7/83 and 2/84, respectively.

[c] All substrates at 1 mM and recoveries based on known theoretical stoichiometries.

made in the field, the chemical analysis of groundwater and a variety of microbiological parameters. Particularly revealing are the data on methane formation from both endogenous and exogenous electron donors (Table 1). Sulfate-reducers and methanogens were isolated from both sites, but only a slow rate of methane production was detected in laboratory experiments with aquifer material sampled from the sulfate-reducing site. This rate was unaffected by further sulfate amendments and was several orders of magnitude slower than the rate observed in samples from the methanogenic site. Sulfate is an inhibitor of methanogenesis in environments where sulfate-reducers and methanogens coexist [1,10,23,26,33,43]. This effect is especially obvious with the so-called 'competitive' electron donors (acetate, formate and hydrogen) but not so with substrates that sulfate-reducers apparently do not use to an ecologically significant extent (methanol and trimethylamine).

A mapping of the methane levels and sulfate concentrations in groundwater samples corroborated the conclusions reached from the results shown in Table 1 and revealed that the sulfate-reducing site was localized in a relatively small area [4]. While the reasons for this condition are not clear, the presence of two ecologically distinct sites in close physical proximity in a single anoxic aquifer provided an ideal site to study pollutant biodegradation under anoxic conditions.

HALOGENATED AROMATIC COMPOUNDS

The presence of the two ecologically well-characterized sites within the same aquifer provided an ideal opportunity to examine the anaerobic biotransformation of xenobiotic substrates under both methanogenic and sulfate-reducing conditions. Since haloaromatic compounds are known contaminants of the terrestrial subsurface [21,38], a variety of these substrates were incubated in serum bottles containing aquifer sediment and groundwater obtained from the various sites (Table 2). The results of these experiments are summarized below and are adapted from Gibson and Suflita [15].

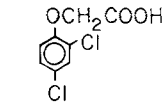
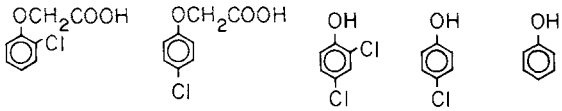
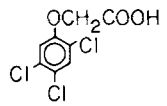
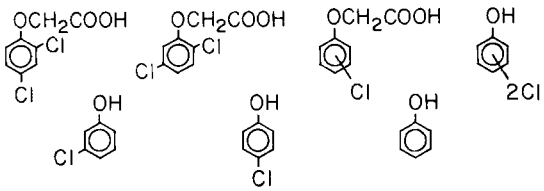
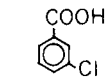
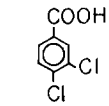
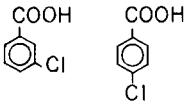
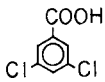
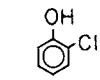
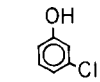
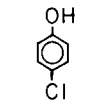
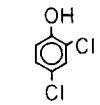
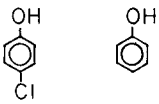
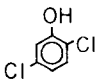
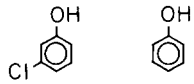
When a variety of individual halogenated benzoates, phenols or phenoxyacetic acid substrates (Table 2) was incubated in aquifer slurries, only methanogenic incubations showed any removal of the parent compounds relative to controls sterilized by autoclaving. Primary degradation of 300–500 μM of most substrates was generally initiated within 4–8 weeks. However, up to 4 months were required before complete mineralization of some substrates was detected. No evidence for the anaerobic decomposition of either 3,4-dichlorophenol, 2,4,5-trichlorophenol or 4-chlorobenzoate was obtained following a 4-week incubation, but the latter compound was eventually removed following extended incubation (up to 2 years).

During this time, little or no evidence for biodegradation of the halogenated substrates was detected in sulfate-reducing aquifer incubations. That is, disappearance of the parent substrates was either not observed or the removal rate in experimental incubations was not greater than the rate measured in sterile controls. With one exception, all of the compounds listed in Table 2 were detected in the sulfate-reducing aquifer slurries even after a year of incubation. Only 4-chlorophenol was removed in experimental bottles relative to controls following the lengthy incubation, but no metabolites were demonstrated. Unfortunately, it cannot be stated conclusively that the serum bottles truly represented sulfate-reducing conditions throughout the entire incubation period.

In methanogenic incubations, the fate of the halogenated substrates that were transformed could be determined from the predominant intermediates produced during the experiment. Generally, HPLC co-chromatography with authentic compounds was used to help identify the metabolites, which are shown in Table 2. Based on the identity and the order of appearance of the metabolites, the primary biodegradative reaction for the tested substrates involved the reductive removal of an aryl halide and its replacement by a hydrogen atom (Table 2). Aromatic intermediates were formed that contained at least one less halogen than the parent substrate. Usually, the dehalogenation process continued until all halogens were removed from the chloroben-

Table 2

Fate of several haloaromatic compounds in samples from a shallow anoxic aquifer in Norman, OK (adapted from Ref. 15)

	Substrates Examined	Intermediates Detected
Phenoxyacetic acids		
		
Benzoates		
		
		
Phenols		Mineralized but no intermediate detected
		
		
		
		

zoates and chlorophenols. The resulting completely dehalogenated benzoate and phenol moieties were then completely mineralized to methane and presumably carbon dioxide.

The pesticide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was also reductively dehalogenated as the primary degradative event in methanogenic aquifer slurries. This reaction resulted in the formation of two isomeric dichlorophenoxyacetic acid (-D) intermediates: 2,4-D and 2,5-D indicating a *meta* and *para* dehalogenation, respectively (Table 2). However, in most experiments, mass balance calculations indicated that the latter isomer was produced in excess of the former. After the formation of the dichlorophenoxyacetates (or when 2,4-D was used as a parent substrate), intermediates were observed which indicated at least two possible degradative routes. That is, both monochlorophenoxyacetate and dichlorophenols were detected, indicating another dehalogenation reaction or a cleavage of the two-carbon side chain of the substrate, respectively. None of the intermediates accumulated to a large extent and at this point it is unclear which of the possible pathways is predominant. However, phenol was formed and it appeared that both routes eventually converged on this compound, and it was subsequently mineralized.

Cleavage of the two-carbon side chain of 2,4,5-T generally did not occur until at least one halogen had been removed. If this apparent hydrolysis had occurred rapidly relative to aryl dehalogenation, 2,4,5-trichlorophenol would have been formed. This was not the usual finding and only trace concentrations ($<0.5\%$) of this compound were found in methanogenic aquifer slurries. As noted earlier, this trichlorophenol isomer remained persistent when tested as a parent substrate in methanogenic aquifer incubations. In this case, the major degradative route in aquifer slurries was one that led to the complete mineralization of the test substrate. The same two reaction mechanisms are also potentially operative for dichlorophenoxyacetate substrates, but the apparent hydrolysis of these substrates and reductive dehalogenation reactions seemed to proceed simultaneously. Thus, when 2,4-D served as the parent substrate, it was converted to the corresponding dichlorophenol or to a monochlorophenoxyacetate as the primary degradative event and only subsequently mineralized following the complete removal of chlorines from the aromatic moiety (Table 2). Neither the disappearance of the substrates listed in Table 2 nor the formation of intermediates were detected in autoclaved control incubations as evidenced by HPLC analysis.

To investigate whether the lack of dehalogenation activity in sulfate-reducing incubations was due to a fundamental lack of metabolic potential or other possible reasons, serum bottles containing aquifer solids and filter-sterilized groundwater from the various sites were constructed and amended with 2,4,5-T as a model substrate. The bottles were treated as indicated in Fig. 1 and 2,4,5-T depletion was monitored with time. The results are summarized in Fig. 1.

Typically, when groundwater and aquifer solids from the methanogenic site were incubated with 2,4,5-T, no parent substrate was detected at the end of the incubation period. In contrast, only slight amounts of the substrate disappeared from aquifer slurries with inoculum and groundwater from the sulfate-reducing site and only trace quantities of 2,5-D and 2,4-D were detected in these cultures. When mixtures of aquifer solids from the methanogenic site were combined with groundwater from

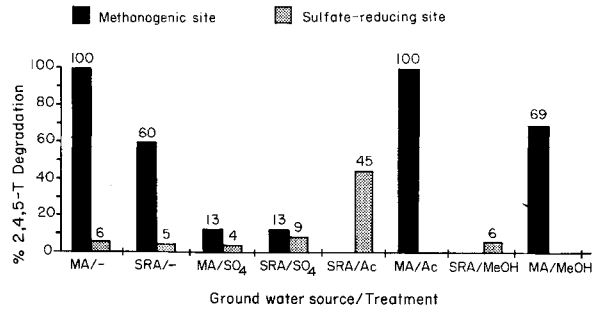


Fig. 1. Degradation of 400 μM 2,4,5-T after 2 months in aquifer slurries containing various combinations of filter-sterilized groundwater and aquifer solids from either the methanogenic (MA) or sulfate-reducing (SRA) site. The incubations were also treated with various amendments: – = no amendment; SO_4 = sulfate (29 mM); Ac = acetate (29 mM); MeOH = methanol (5 mM) (adapted from Ref. 15).

the sulfate-reducing site, 2,4,5-T removal was inhibited (60%); the opposite incubation mixture exhibited only a 5% removal of the parent substrate (Fig. 1). This result indicated that a soluble inhibitor of dehalogenation existed in the groundwater at the sulfate-reducing site or that the requisite microorganisms were simply absent from that site. Since sulfate concentrations differed drastically between the two sites at most times of the year (Table 1), the influence of this anion on dehalogenation of the model substrate was examined. Amendment of the same four combinations of aquifer solids and groundwater with sulfate uniformly inhibited 2,4,5-T dehalogenation, regardless of the inoculum source. After 2 months, 87–96% of the parent substrate persisted and only trace quantities (< 15 μM) of dichlorophenoxyacetate intermediates were detected (Fig. 1).

To further implicate sulfate as an inhibitor of aryl dehalogenation reactions, experiments were conducted in aquifer slurries amended with acetate or methanol as exogenous electron donors. As shown in Table 1, of the two electron donors, only methanol was efficiently converted to methane regardless of the sulfate status of the incubation mixture. As noted previously, this is because sulfate-reducing bacteria apparently do not metabolize high concentrations of methanol to an ecologically significant extent. Methanol was rapidly converted to methane in aquifer slurries sampled from the sulfate-reducing site but little degradation of 2,4,5-T was noted and no dehalogenated intermediates were found (Fig. 1). Some sulfate-reducing bacteria will use acetate as an electron donor to support dissimilatory sulfate reduction and in the process convert sulfate to hydrogen sulfide. When enough acetate was added to (i) stimulate biological sulfate-reduction, (ii) consume the endogenous levels of sulfate, and (iii) shift the flow of carbon and energy in the aquifer incubation mixtures toward methanogenesis, 2,4,5-T dehalogenation reached 45% (Fig. 1). These results strongly suggest that the lack of dehalogenation in sulfate-reducing aquifer slurries was not due to a fundamental lack of appropriate microorganisms at the site and that methane formation is not necessarily a good indicator of aryl dehalogenation potential. While dehalogenating bacteria were present at both aquifer sites, their potential activity was at least partially inhibited by the high concentrations of inorganic sulfate at the sulfate-reducing site.

ALKYLATED AROMATIC COMPOUNDS

Essentially the same comparative approach was used to study the anaerobic biodegradation of alkylated aromatic compounds in aquifer samples under both methanogenic and sulfate-reducing conditions [35]. Substrates belonging to this chemical class are groundwater contaminants emanating from a variety of municipal and industrial waste disposal practices [11,17,20,30]. When cresol isomers were incubated in anoxic aquifer slurries, the parent substrates were depleted after varying lengths of time. Under both sulfate-reducing and methanogenic conditions, *o*-cresol proved to be the most persistent of the isomeric series, exhibiting a lag period of over 90 days before any substrate disappearance was detected. In contrast, *m*-cresol showed an intermediate lag time (46–90 days), while *p*-cresol was the least persistent of the tested substrates. Under methanogenic conditions, *p*-cresol exhibited a lag of 46 days and a rate of decomposition of 18 nmol/h/g dry wt. of sediment. The same substrate exhibited a much shorter lag (< 10 days) and faster biodegradation rate (330 nmol/h/g dry wt. of sediment) when incubated in sulfate-reducing aquifer slurries.

Since *p*-cresol was the most easily degraded of the three isomers, subsequent experimentation focused on characterizing the biodegradation of this substrate. Fig. 2 shows that an inhibitor of sulfate reduction, molybdate, reduced the rate of *p*-cresol degradation by 75% in sulfate-reducing aquifer slurries. This indicates that *p*-cresol metabolism was coupled to sulfate reduction rather than to methanogenesis.

In consistent fashion, an inhibitor of methanogenesis (bromoethanesulfonic acid) had very little impact on the rate of *p*-cresol decomposition. Sulfate-reducing aquifer slurries which received an ad-

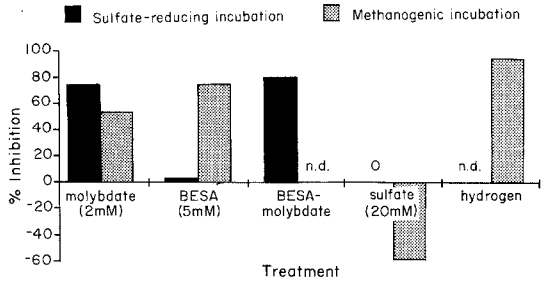


Fig. 2. The effect of various treatments on the relative rate of *p*-cresol metabolism in both sulfate-reducing and methanogenic aquifer slurries. A 2 atm headspace pressure of 80% H_2/20% CO_2 was used for the hydrogen treatment. BESA = bromoethanesulfonic acid; n.d. = not determined (adapted from Ref. 35).

ditional sulfate amendment did not show a significant change in the rate of *p*-cresol metabolism under these conditions.

In contrast, a different pattern of *p*-cresol inhibition was observed with methanogenic aquifer slurries. In these samples, bromoethanesulfonic acid and hydrogen were more inhibitory to *p*-cresol metabolism than molybdate (Fig. 2), indicating that the metabolism was a coupled fermentation process and that methanogens were involved as important members of the degradative bacterial consortium. Interestingly, sulfate amendments to methanogenic incubations actually stimulated biodegradation. Presumably, the degradation of *p*-cresol was more thermodynamically favorable when it was coupled with sulfate reduction.

In experiments designed to examine the biodegradation of *p*-cresol under sulfate-reducing conditions, several small, transient, and more polar peaks were noted during HPLC analysis of the aquifer slurries. These peaks were not present at the start of the incubation, nor were they present in sterile control cultures. The peaks were chromatographically and spectrophotometrically compared to standards of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate, respectively, and found to be identical. Simultaneous adaptation studies revealed that these two compounds were utilized without a lag by aquifer slurries acclimated to the anaerobic decomposition of *p*-cresol, suggesting that they may indeed be on the biodegradation pathway [35]. It thus appeared that the methyl group of *p*-cresol was oxidized by the aquifer microflora under sulfate-reducing conditions. However, there was no simultaneous adaptation to the anticipated initial product of such an oxidation, *p*-hydroxybenzylalcohol.

Repeated attempts were made without success to isolate a pure culture of a sulfate-reducer capable of using *p*-cresol as sole source of carbon and energy. While this task has recently been accomplished in another laboratory [2], we were only able to enrich a stable mixture consisting of at least four morphologically distinct bacteria that could completely degrade this substrate under sulfate-reducing conditions. The enrichment completely mineralized *p*-[*ring*-U-^{14}C]cresol and exhibited a Michaelis constant of 126 μM [34]. The initial steps in the pathway of *p*-cresol metabolism by the enrichment were investigated both by HPLC co-chromatography with standard materials and by GC/MS analysis of the culture fluids (Fig. 3). In addition to the previously suggested intermediates, *p*-hydroxybenzylalcohol and benzoic acid were also detected in spent culture fluids, but not in identically treated autoclaved control samples. These findings confirmed the earlier suggestion that the methyl group of *p*-cresol was initially oxidized under sulfate-reducing conditions.

Biodegradation of *p*-cresol by the enrichment was completely inhibited when sulfate was omitted from the incubation mixture or when oxygen, thiosulfate, or a methanogen were used as potential substitute terminal electron acceptors. However, sulfite and nitrate could replace sulfate as terminal electron acceptors for the enrichment. The latter result is particularly interesting since some sulfate-reducing bacteria also denitrify.

The biodegradation of *p*-cresol has been noted in a variety of studies involving aerobic [18,19], de-

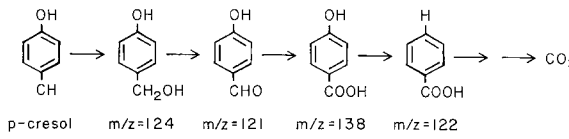


Fig. 3. Proposed pathway for the anaerobic biodegradation of *p*-cresol under sulfate-reducing conditions and an indication of the mass spectral parent ions detected for the intermediates.

nitrifying [6], sulfate-reducing [2,35] and methanogenic [16,31,35] incubation conditions. The observation that *p*-cresol was initially oxidized under sulfate-reducing conditions was at first surprising since this is the same reaction sequence previously demonstrated for the aerobic microbial metabolism of many methyl-substituted aromatic compounds. However, Hopper [19] showed the incorporation of [^{18}O]water rather than molecular oxygen into the methyl group of *p*-cresol by a purified methylhydroxylase from an aerobically grown pseudomonad. Similarly, Bossert and Young [6] described a denitrifying bacterium that oxidized the methyl group of *p*-cresol to form *p*-hydroxybenzoic acid. The latter compound was also detected as a *p*-cresol metabolite in methanogenic incubations by Senior and Balba [31]. Therefore, it seems quite likely that a similar mechanism may also account for the biodegradation of *p*-cresol by the sulfate-reducing aquifer enrichment, and future experiments will be designed to test that possibility.

It is interesting to note that unlike the halogenated aromatic substrates, the anaerobic biodegradation of simple alkylated aromatic compounds was stimulated by the presence of sulfate as a terminal electron acceptor in aquifer slurries. The lag times were generally shorter and the rates of degradation faster when cresol isomers were incubated under sulfate-reducing rather than methanogenic conditions.

HALOGENATED ALIPHATIC COMPOUNDS

Halogenated aliphatic compounds are widely used as cleaning and degreasing agents and can be produced as a result of the chlorination of water. Sources of these contaminants are varied, but a recent survey indicated that 10% of the industrial effluents monitored contained tetrachloroethylene and 20% contained trichloroethylene [36]. These two solvents are among the most pervasive pollutants of U.S. groundwaters [40]. Environmental regulatory agencies have raised serious concerns over human health risks associated with the pollution of potable water supplies with short-chain halogenated solvents.

Consequently, the metabolic fate of tetra- and trichloroethylene in aquifers is a matter of intense investigation. Current evidence suggests that several aerobic microorganisms or microbial communities synthesize oxygenase enzyme systems that cometabolize trichloroethylene when the organisms are grown on other substrates [13,25,42]. However, a number of studies attest to the recalcitrance of tetrachloroethylene when it is incubated under aerobic conditions [8]. In contrast, microbiological investigations employing anaerobic incubation conditions indicate that tetrachloroethylene can be transformed by reductive dehalogenation reactions [7,8,27,39]. Since most studies have employed either methanogenic or denitrifying incubation conditions, we compared the biodegradation of tetrachloroethylene in aquifer slurries under methanogenic and sulfate-reducing conditions. The aquifer slurries were constructed in serum bottles as previously described [15]. Methane and sulfate were monitored by gas chromatography and HPLC, respectively, as previously reported [4]. Autoclaved and substrate-unamended aquifer slurries served as controls.

Our initial investigations with this substrate were unsatisfactory in that we did not have suitable closures for the serum bottle incubation systems. That is, the volatile compound partitioned to some types of commercially available septa and escaped detection, or anaerobic conditions could not be maintained over extended incubation periods with others. To solve these problems, we closed serum bottles with autoclaved 1-cm-thick butyl rubber septa which were lined on their inner surface with sterile aluminum foil. This operation was performed in a well-working anaerobic glovebox (Coy Laboratory Products, Inc., Ann Arbor, MI). Following closure, the serum bottles were wrapped with foil to prevent photodecomposition of the solvent and incubated upside down at room temperature. These closures minimized the loss of tetrachloroethylene and still maintained long-term anaerobic conditions as determined with the redox indicator resazurin. However, the bottles could not be subsampled since the integrity of the foil lining would then be compromised. Therefore, at each sampling period triplicate bottles were sacrificed

and analyzed for substrate removal and intermediate formation by purge and trap gas chromatography [41].

Briefly, solvent traps consisting of a tenax resin and silica gel (Tekmar, Cincinnati, OH) were used for the headspace analysis of the serum bottles. The trap was removed from a Liquid Sample Concentrator (LSC-2, Tekmar) and placed in a burette holder. A piece of teflon tubing (0.4 m × 0.15 cm i.d.) was connected to the trap at one end using standard gas-tight connections. The tubing had an 18-gauge needle fastened to the opposite end which was used to pierce the serum bottle closures. This needle was positioned in the headspace of the serum bottles. Halocarbon-free nitrogen was then bubbled through the serum bottle liquid phase via a second gassing needle. The opposite end of the trap was in turn connected to a flow meter that was used to monitor the rate of solvent purge. A solution of fluorobenzene was added to the serum bottles as an internal purge standard. The bottles were purged at 30 ml/min for 5 min while agitating them on a rotary shaker at 250 rpm. Traps were then reconnected to the LSC-2 instrument and the halocarbons were thermally desorbed to a Varian 3300 gas chromatograph. The gas chromatograph was equipped with a 60/80 Carbopack 1% SP-1000 packed column (2 m × 0.32 cm), and nitrogen served as the carrier gas at a flow of 30 ml/min. Detection was by flame ionization. Injector and detector temperatures were 200°C and 250°C, respectively. A thermal program was used in the analysis which consisted of an initial hold time of 5 min at 45°C, followed by an 8°C/min increase until the final column temperature of 210°C was achieved and then held for 5 min. Unamended and autoclaved controls were similarly analyzed at all time points and both internal and external standards were employed in the analytical procedure.

When necessary, mass spectral evidence was obtained by extracting the serum bottle contents suspected of containing daughter products with 5 ml of pentane. The pentane extracts were then analyzed by GC/MS with a Hewlett-Packard 5890 gas chromatograph interfaced with an HP 5970 mass selective detector. The GC was equipped with an HP-1 capillary column (12 m × 0.2 mm × 0.33 μm) and helium served as the carrier gas at 3.0 ml/min. The GC oven was temperature-programmed from 35°C to 100°C at 8°C/min. The injection and transfer line temperatures were 150°C and 280°C, respectively. The electron impact detector was operated at 70 eV. Alternately, mass spectra were collected from selected samples by purge and trap GC/MS. After trapping the volatiles as described earlier, the traps were stored on dry ice (−70°C) until analysis. Clean traps were also stored with the purged ones as a control for extraneous volatile contaminants. No contaminants were found on the control traps when they were analyzed by GC/MS. The volatiles on stored traps were desorbed to a Finnigan 4000 GC/MS system using an LSC-2 concentrator and analyzed as previously described [41].

Table 3 summarizes the findings of tetrachloroethylene biodegradation in both methanogenic and sulfate-reducing aquifer slurries. Trace quantities of tetrachloroethylene disappeared in aquifer slurries within several weeks of incubation and a variety of transformation products were produced.

Under both methanogenic and sulfate-reducing conditions, tetrachloroethylene underwent sequential reductive dehalogenation reactions to form tri- and dichloroethylene. The identity of the latter compounds was confirmed by mass spectrometry. In contrast to previous reports, the initial transformation of tetrachloroethylene occurred without a significant lag period [41]. In methanogenic incubations, the rate of parent compound transformation was somewhat faster and trace quantities of vinyl chloride were also detected by gas chromatography at the end of the 10-week incubation period. None of transformation products was detected in autoclaved or substrate-unamended controls. The autoclaved controls remained reduced throughout the incubation period as evidenced by the resazurin indicator. The same rate of endogenous methane formation was detected in both experimental and substrate-unamended controls made with aquifer materials sampled from the methanogenic site. Similarly, no increase in methane formation was detected in comparable bottles made with aquifer ma-

Table 3

The biotransformation of tetrachloroethylene in anoxic aquifer slurries and the appearance of transformation products

Compound[a]	Concentration ± 1 S.D. (nM) at:					
	sulfate-reducing site			methanogenic site		
Incubation time (week):	0.5	5	10	0.5	5	10
PCE	241 ± 5	271 ± 5	152 ± 15	217 ± 13	165 ± 67	101 ± 72
TCE	3 ± 2	16 ± 1	33 ± 6	31 ± 6	87 ± 35	73 ± 20
DCE	n.d.	n.d.	3 ± 1	n.d.	n.d.	3 ± 2
VC	n.d.	n.d.	n.d.	n.d.	n.d.	trace
Control	282	259	185	223	294	185

[a] PCE = tetrachloroethylene; TCE = trichloroethylene; DCE = dichloroethylene; VC = vinyl chloride; Control = autoclaved control; n.d. = not detected.

terials sampled from the sulfate-reducing site, and the initial sulfate concentration was about 2 mM. However, the parent substrate was not mineralized to any significant extent in either type of incubation, since mass balance calculations at the end of the incubation period showed near-quantitative recovery of the initial amount of substrate as residual tetrachloroethylene or one of the halogenated daughter products.

Similar results were also obtained in other experiments which used material from the methanogenic aquifer where the concentration of tetrachloroethylene was varied between 12 and 1500 nM. At the high concentration, all of the parent substrate was converted to 1,2-dichloroethylene in 7 weeks while the same transformation product and traces of vinyl chloride were detected at the end of the incubation period when the low substrate concentration was employed. While we were unable to separate the *cis* and *trans* 1,2-dichloroethylene isomers using the gas chromatographic procedure outlined above, we separated 1,1-dichloroethylene. However, the latter intermediate was never detected in either sulfate-reducing or methanogenic aquifer slurries amended with tetrachloroethylene. The absence of 1,1-dichloroethylene as a transformation product of tetrachloroethylene differs from other studies [27,39] but agrees with the results of Wilson et al. [41] and Kloepfer [22].

Our results suggest that tetrachloroethylene was biodegraded under anaerobic conditions, but to our knowledge no microorganism capable of degrading this solvent has yet been isolated from any environment. We therefore tested a dehalogenating bacterial consortium originally enriched for its ability to completely mineralize 3-chlorobenzoic acid to CH_4 and CO_2 [37]. The consortium was tested with 150–170 nM tetrachloroethylene in both the presence and absence of its normal substrate, 3-chlorobenzoate. The same serum bottle incubation procedure and headspace analysis used for the aquifer slurries was employed in these experiments. All treatments were performed in duplicate. However, in addition to unamended and autoclaved controls, aerobic controls were included by oxidizing the microbiological media with sterile O_2 prior to substrate amendment and closure of the serum bottles with foil-lined stoppers. The headspace composition of the latter control bottles was room air and the resazurin indicator in the media was intense pink throughout the incubation period. Traces of trichloroethylene were formed by the consortium within 2 weeks. However, at the end of a 5-month incubation, only 10% of the substrate was trans-

Table 4

Reductive dehalogenation of 160 nM tetrachloroethylene (PCE) to trichloroethylene (TCE) by defined cultures of anaerobic bacteria

Defined anaerobic culture [Ref.]	Transformation of PCE to TCE (%)	Incubation conditions	
		carbon source[a]	growth medium from Ref.:
Dehalogenating bacterial consortium [32]	42	3-Cl-Bz (820 μM)[b]	32
Methanosarcina barkeri strain MS[c]	0	methanol	32
Methanosarcina sp. [4]	0	acetate	32
Desulfovibrio desulfuricans strain DD[d]	0	lactate	4
Desulfotomaculum sp. [4]	0	methanol	4
		acetate	4
Acetobacterium woodii ATCC 29683	0	methanol	3
Co-culture of *Syntrophomonas wolfei* and *Methanosprillum hungateii*[d]	0	butyrate (20 mM)	24
Dehalogenating bacterium strain DCB-1 [32]	0	pyruvate (18 mM)	32
	4	pyruvate and 3-Cl-Bz (820 μM)	32

[a] 10 mM unless otherwise indicated.
[b] 3-Chlorobenzoic acid.
[c] Received from Dr. M. Bryant.
[d] Received from Dr. M. McInerney.

formed by the enrichment without 3-chlorobenzoate whereas 42% was metabolized in the presence of the haloaromatic substrate. Mass balance calculations revealed that only trichloroethylene was formed in the former incubation while tri- and a trace of 1,2-dichloroethylene were produced in the latter. No substrate transformation or daughter product formation was found in any of the controls.

The dehalogenating enrichment has been well characterized and consists of five metabolic groups of bacteria which include (i) methanogens, (ii) sulfidogens, (iii) butyrate oxidizers, (iv) benzoate oxidizers, and (v) the dechlorinating strain, DCB-1 [32]. We tested pure or defined cultures of anaerobic bacteria representing most of the groups above for the ability to transform tetrachloroethylene (Table 4). Growth media for the various organisms are referenced in Table 4. All organisms were grown at room temperature to a density of at least 10^6 bacteria/ml as determined by microscopic examination before an experiment was started. As with the dehalogenating consortium, autoclaved, unamended and aerobic cultures served as controls.

None of the cultures transformed trace quantities of tetrachloroethylene during a 2-month incu-

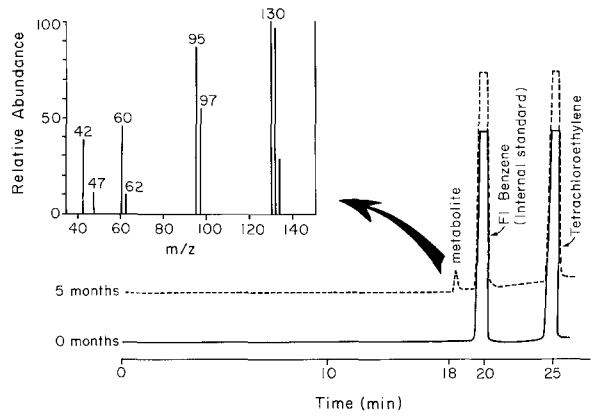


Fig. 4. Gas chromatograms showing a metabolite peak appearing with time when the dehalogenating strain DCB-1 was incubated with tetrachloroethylene. The inset shows the mass spectral profile of the metabolite and helps identify it as trichloroethylene.

bation period. However, after 5 months, the pure culture of DCB-1 showed about 4.0% conversion of the initial amount of halogenated solvent to a daughter product with the same retention time as trichloroethylene (Fig. 4). The mass spectrum of this metabolite showed a three-chlorine isotopic abundance pattern and was about a 96% match to the standard library spectrum for trichloroethylene. Autoclaved, substrate-unamended, and aerobic controls were uniformly negative for tetrachloroethylene depletion and product formation. Therefore, strain DCB-1 could transform a halogenated aliphatic compound in addition to haloaromatic substrates by reductive dehalogenation. It remains to be determined how closely related these two processes may be.

These findings support the field evidence of Reinhard et al. [28] in which they infer a rapid breakdown of both tetra- and trichloroethylene in a landfill leachate-polluted alluvial sand aquifer. To our knowledge, this is the first report of tetrachloroethylene biodegradation in aquifer sediments incubated under sulfate-reducing conditions and the first demonstration of a pure culture able to transform this solvent under anaerobic conditions.

CONCLUSIONS

Two sites within a single shallow aquifer were identified and characterized. The predominant flow of carbon and energy was through methanogenesis at one site and sulfate-reduction at the other. The biodegradation of haloaromatic compounds and tetrachloroethylene was faster under methanogenic conditions and involved the substitution of the halogen substituents by a proton. High levels of sulfate completely inhibited this process with the haloaromatic substrates. In contrast, the biodegradation of simple alkyl phenols occurred faster under sulfate-reducing conditions. The degradation pathway for *p*-cresol under sulfate-reducing conditions involved the initial hydroxylation of the aryl methyl group to form *p*-hydroxybenzylalcohol. This intermediate was subsequently completely mineralized. Different electron donor and acceptor combinations can drastically influence the transport and fate of contaminants in groundwaters.

NOTE ADDED IN REVIEW

Recently, Fathepure et al. [12] also found that strain DCB-1 reductively dehalogenated high concentrations of tetrachloroethylene (1 ppm). In addition, of three *Methanosarcina* strains tested, two cultures were able to similarly transform this substrate at low rates.

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