Biodegradation **12:** 159–167, 2001.

Anaerobic degradation and dehalogenation of chlorosalicylates and salicylate under four reducing conditions

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Key words: chlorinated aromatic compounds, anaerobic degradation, alternate electron acceptors, dehalogenation

Abstract

The anaerobic biodegradability and transformation of the mono-and dichlorinated salicylates (2-hydroxybenzoates) was examined under denitrifying, Fe (III) reducing, sulfate reducing and methanogenic conditions. 3,6-Dichlorosalicylate and 6-chlorosalicylate are anaerobic microbial metabolites of dicamba, a widely used herbicide. Anaerobic microcosms were established with dicamba treated soil from Wyoming, and golf course drainage stream sediments from New Jersey, which were each spiked with salicylate, 3,6-dichlorosalicylate or one of the four monochlorosalicylate isomers. Salicylate was degraded under denitrifying, sulfidogenic and methanogenic conditions. In methanogenic enrichments 5-chlorosalicylate and 3-chlorosalicylate were reductively dehalogenated to salicylate which was then utilized. Dehalogenation of monochlorinated salicylates to salicylate was also observed in denitrifying chlorosalicylate degrading cultures. The study revealed that the position of the chlorine substituent as well as the predominant electron accepting process affect the rate and extent of chlorosalicylate degradation in anoxic environments.

Introduction

Dicamba (3,6-dichloro-2-methoxybenzoate) is a widely used post-emergence broadleaf herbicide. The anaerobic biodegradation of the herbicide dicamba proceeds through O-demethylation to 3,6-dichlorosalicylate, which in turn is reductively dehalogenated to 6-chlorosalicylate and salicylate under methanogenic conditions (Taraban et al. 1993; Milligan & Häggblom 1999). Complete degradation of dicamba to CH_4 and CO_2 has been demonstrated (Milligan & Häggblom 1999), but 6-chlorosalicylate and 3,6-dichlorosalicylate often accumulate in either methanogenic or sulfidogenic laboratory microcosms. The recalcitrance of these metabolites appears to be rate limiting for anaerobic mineralization.

Anaerobic microbial respiration in aquatic sediments and aquifers can take place via a variety of electron acceptors (Ghiorse et al. 1988; Gibson 1990; Nealson & Saffarini 1994; Zumft 1997) and microbial degradation of organic compounds, in particular the anaerobic degradation of halogenated aromatic compounds, can be influenced by the type of electron acceptors present (Myers & Nealson 1988; Lovley & Phillips 1986; Gibson 1990; Kuhn et al. 1990; & Colberg 1991). Often, stratification of electron accepting conditions develops in aquifers and sediments (Ghiorse et al. 1988; Nealson & Saffarini 1994). In general, the zonal distribution proceeds from aerobic to anaerobic with depth, in the following sequence O_2, NO_3^-, Mn(IV), Fe(III), SO_4^{2-}, HCO_3^-. The primary electron accepting process in anoxic soils and sediments has been shown to influence the type and extent of transformations that occur (Gibson & Suflita 1986; Kuhn et al. 1990; Colberg 1991; Häggblom et al. 1993; Kazumi et al. 1995). Determining how anaerobic biodegradation of toxic organic compounds is affected by the predominant electron accepting process is pertinent, as contaminated sites vary from location to location and are often heterogeneous with respect to microbial respiration.

Anaerobic biodegradation of chlorinated monoaromatic compounds is well documented (for reviews see Häggblom 1992; Mohn & Tiedje 1992; Häggblom & Milligan 2000). Reductive dehalogenation is a principal mechanism for transformation of these compounds and often is prerequisite to subsequent ring cleavage and mineralization (Häggblom 1992; Häggblom & Milligan 2000). Dehalogenation of halophenols and halobenzoic acid compounds has been observed under methanogenic, sulfidogenic and iron reducing conditions (Häggblom et al. 1993; Kazumi et al. 1995). Previous work (Taraban et al. 1993; Milligan & Häggblom 1999) indicates that chlorinated salicylates may accumulate in the environment following anaerobic biotransformation of dicamba. To date, the anaerobic degradation of halogenated salicylates has not been examined in detail. This study examines the effect of the position of the chlorine substituent on the potential for dehalogenation and degradation of chlorosalicylates under different reducing conditions.

Materials and Methods

Enrichment cultures. Methanogenic, denitrifying, sulfate reducing and iron reducing enrichment cultures were established under strictly anaerobic conditions using a 10% inoculum from a golf course drainage stream sediment in New Jersey and a dicamba treated soil from Wyoming as described previously (Milligan & Häggblom 1999). The $NaHCO_3$ (30 mM) buffered medium was amended with either KNO_3 (20 mM), Na_2SO_4 (20 mM), amorphous ferric oxyhydroxide (200 mM) or left with carbonate as the primary electron acceptor before the sediment inoculum was added. Triplicate cultures were spiked with either 3,6-dichlorosalicylate, 3-chlorosalicylate, 4-chlorosalicylate, 5-chlorosalicylate, 6-chlorosalicylate, or salicylate (100 μM), which were provided as a sole carbon source in order to enrich for bacteria which utilize these compounds under each condition. Background controls for carbonate, nitrate, sulfate, and Fe (III) reduction consisted of active sediment slurries with no added carbon source. Sterile controls were established by autoclaving cultures for 1 hour on three consecutive days before spiking with substrate. Samples were taken for chemical analysis following strict anaerobic techniques. At each sampling point the cultures were rigorously shaken and sampled with sterile syringes flushed with either argon or 70%N2 / 30%CO_2. After initial loss of substrate, active cultures were refed and repeatedly transferred into fresh media over a period of one year. Cultures were incubated in the dark at 20 °C.

Carbon, nitrogen, and methane stoichiometry. Stoichiometry experiments were undertaken in order to establish whether the degradation of 3-chlorosalicylate, 5-chlorosalicylate and salicylate was coupled to either methanogenesis or dissimilatory denitrification. Methane stoichiometry experiments were performed in triplicate in 60 ml sealed bottles containing 50 ml of inoculum and 10 ml 70%N_2 / 30%CO_2 head space. For each substrate tested, triplicates were dispensed from one 250 ml homogenous culture into 50 ml aliquots in a Coy anaerobic chamber (Coy Laboratory Products Inc.) and sealed. Due to varying toxicity of chlorosalicylates and salicylates in cultures under different reducing conditions, substrates were added in varying concentrations depending on substrate tolerance. Salicylate utilizing cultures were fed 250 μM of substrate four times over the 28 day experiment. Chlorosalicylate using cultures were fed 225 μM three times over 35 days. Two additional cultures for each substrate and inoculum were established with no external carbon source in order to measure endogenous methanogenesis. The volume of gas produced was measured with a water lubricated glass syringe. End point methane concentration was measured by gas chromatography one day after complete substrate utilization had been confirmed by HPLC.

Denitrifying chlorosalicylate and salicylate utilizing enrichments were collected by centrifugation at 10000 × G for 20 minutes, then resuspended into nitrate free medium under a 97%N_2 / 3%H_2 atmosphere in a Coy chamber. This process was repeated to remove all residual nitrate. Cultures were then resuspended in 10 mM nitrate-amended medium. Nitrate stoichiometry experiments were performed in triplicate in 30 ml sealed bottles containing 25 ml of inoculum and 5 ml argon head space. Cultures were fed 150 μM of 3- or 5-chlorosalicylate or 250 μM salicylate three to four times over the experimental period (16 days for salicylate and 60 days for chlorosalicylates). Two additional cultures were established with 10 mM nitrate and no external carbon source in order to measure endogenous denitrification. After complete substrate utilization endpoint measurements were taken for nitrate and nitrite.

Chemicals. All aromatic compounds were certified >98% pure. 3,6-Dichlorosalicylate was a gift from Sandoz Agro. Inc. (Des Plaines, IL). Salicylate, 3-, 4-, and 5-chlorosalicylate, o-anisate, m-anisate, 4-, and 5-chloro-o-anisate, vanillate, protocatechuate, benzoate, 3-chlorobenzoate, 3-hydroxybenzoate, 2-bromobenzoate, 2-fluorobenzoate, phenol, 2-chlorophenol, 3-chlorophenol, and 3-chlorocatechol and were purchased from Aldrich Chemical Co. (Milwaukee, WI). 6-Chlorosalicylate was synthesized by Berry & Associates, Inc. (Ann Arbor, MI). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Sigma (St. Louis, MO).

Analysis. Liquid samples for chemical analysis were filtered through 0.45 μm pore size filters (Millipore, Bedford, Mass.). Aromatic compounds were analyzed using a Shimadzu (LC-10AS) HPLC, (SCL-10A) System Controller, (SPD-10A) UV-VIS Detector, and (SIL-10A) Autoinjector (Shimadzu Scientific Instruments, Columbia, MD.), equipped with a Spherisorb ODS(2) column, 250 by 4.6 mm (Phenomonex, Torrance, CA.). UV detection was at 280 nm. Chromatograms were recorded with a Spectra-Physics Chrome Jet integrator (Spectra-Physics, San Jose, CA.) and quantified using external standards. 3,6-Dichlorosalicylate, 6-chlorosalicylate, 5-chlorosalicylate, 4-chlorosalicylate, 3-chlorosalicylate and salicylate were separated using a solvent gradient consisting of isocratic concentrations of 10% methanol and 2% acetic acid held constant over a 24 minute gradient of distilled water (decreasing from 64.8 to 51.8%) and acetonitrile (increasing from 23.2 to 36.2%) at a flow rate of 1 ml/minute. Another method was developed which shortened the retention times for 3-chlorosalicylate, 5-chlorosalicylate and salicylate analysis. This method utilized isocratic concentrations of methanol (10%) and acetic acid (2%) while running a gradient of distilled water (decreasing from 51.8 to 41.4 %) and acetonitrile (increasing from 36.2 to 46.6%) over 12 minutes at a flow rate of 1 ml/minute.

Nitrate and nitrite were measured using a DX-100 ion chromatograph (Dionex, Sunnyvale, CA.), equipped with a IonPac Asg-Sc (Dionex) column. The mobile phase consisted of a carbonate buffer (1 mM Na2CO3, 0.75 mM NaHCO3) and a sulfuric acid column regenerant (25 mM). All compound concentrations were quantified with external standards using a Spectra-Physics Chrome Jet integrator (Spectra-Physics, San Jose, CA.). Methane gas chromatography was conducted on a Varian 3300 GC, equipped with a Porapak-Q 100/120 column (Supelco) and a TCD detector (Varian Instruments, Sugar Land Texas).

Samples for mass spectrometry were anaerobically drawn from active cultures with N_2 flushed syringes. Samples (0.5 ml) were salted and acidified with saturated NaCl solution (approximately 100 μl) and 1N HCl (1 to 3 drops, final pH 2). Samples were then extracted into pentane (0.5 ml) twice, which was combined and evaporated under nitrogen. Dried samples were derivatized using 100 μl bis(trimethylsilyl)-trifluoroacetamide (BSTFA), analyzed by GC-MS and compared to silylated standards. Gas chromatography-mass spectrometry (GC-MS) was done on a Hewlett Packard 5890 GC equipped with a DB-5MS capillary column and a HP 5971 Mass Selective Detector.

Results

Anaerobic degradation of chlorinated salicylates under different reducing conditions. The utilization of chlorinated salicylates by anaerobic microcosms established under methanogenic, denitrifying, sulfidogenic, or iron reducing conditions is summarized in Table 1. Methanogenic enrichments from golf course drainage stream sediment dehalogenated 3,6-dichlorosalicylate to 6-chlorosalicylate, which accumulated stoichiometrically within 90 days (Figure 1). Methanogenic enrichments from the golf course sediment utilized 3-chlorosalicylate within 40 days and 5-chlorosalicylate within 160 days. During 3- and 5-chlorosalicylate degradation, salicylate appeared as a transient intermediate, indicating that the substrates were dehalogenated as an initial step in metabolism. 6-Chlorosalicylate was also dehalogenated under methanogenic conditions to salicylate in trace amounts (between 10 and 18%) in the golf course sediment microcosms. 6-Chlorosalicylate was transformed to an unidentified metabolite in methanogenic cultures from the Wyoming site. HPLC analysis using different mobile phases during chromatography determined that the compound was neither 3-chlorosalicylate, 3-chlorophenol, 2-chlorophenol, 2-chlorobenzoate, nor 3-chlorocatechol. No metabolites were identified by GC-MS of silylated culture extracts. Salicylate was utilized by enrichments from both sites. The methanogenic cultures were refed either 3-, or 5-chlorosalicylate or salicylate and maintained for over a one-year period (data not shown).

Table 1. Loss of chlorosalicylates and salicylate in anaerobic enrichments from two sites

	Methanogenic		Denitrifying		Sulfate reducing		Iron reducing	
Substrate	RGS	WAS	RGS	WAS	RGS	WAS	RGS	WAS
3,6-Dichlorosalicylate	++^{a}	–	–	–	±^{a}	nd	–	–
3-Chlorosalicylate	+++^{a}	–	+++	+++	–	nd	–	–
4-Chlorosalicylate	–	–	–	+	–	nd	–	–
5-Chlorosalicylate	+^{a}	–	+++	+++	–	nd	–	–
6-Chlorosalicylate	±^{a}	+^{b}	–	–	–	nd	–	–
Salicylate	+++	+++	+++	+++	+++^{c}	nd	+^{d}	+

RGS: Rutgers Golf Course; WAS: Wyoming agricultural soil.

+++ initial loss of 100 μM within 40 days; ++ initial loss of 100 μM within 90 days; + initial loss of 100 μM within 160 days; ± partial loss of compound; – no loss of compound in 160 days; nd no data. All substrates were stable in sterile controls with less than 15% loss in 160 days.

[a] Dehalogenation of substrate.

[b] Unknown metabolite accumulated.

[c] Fed 50 μM substrate.

[d] Methane present in head space.

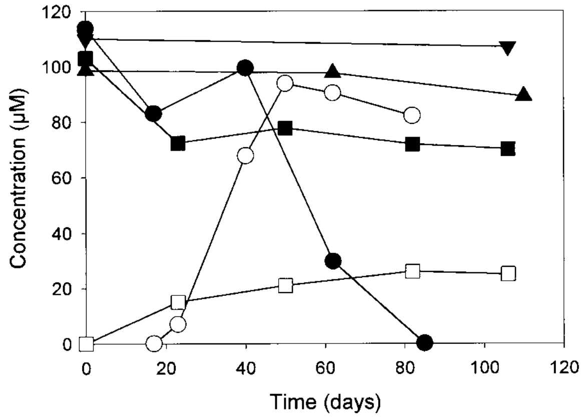

Figure 1. Dechlorination of 3,6-dichlorosalicylate to 6-chlorosalicylate in methanogenic and sulfidogenic golf course sediment (RGS) microcosms. Data points represent the mean from duplicate cultures. Symbols: methanogenic 3,6-dichlorosalicylate (●), 6-chlorosalicylate (○), 3,6-dichlorosalicylate sterile control (▲); sulfidogenic 3,6-dichlorosalicylate (■), 6-chlorosalicylate (□), 3,6-dichlorosalicylate sterile control (▼).

Denitrifying enrichments from both sites utilized salicylate, 3-chlorosalicylate, and 5-chlorosalicylate within 40 days. Cultures were refed and maintained over a one-year period (data not shown). In addition, initial loss of 4-chlorosalicylate was observed in enrichments from the Wyoming site within 160 days, but activity could not be maintained upon refeeding. There was no transformation of 4-chlorosalicylate in enrichments from the golf course sediment. Sulfate reducing enrichments partially dehalogenated 3,6-dichlorosalicylate (approximately 25%) within 106 days (Figure 1). Salicylate was utilized under sulfate reducing conditions and cultures were maintained by refeeding. None of the monochlorinated isomers were degraded in the presence of 20 mM sulfate. Sulfate loss (4.75 ± 1.15 mM) was observed over a sixty day period and no methane was produced, indicating that sulfate reduction was the primary electron acceptor. Iron(III) reducing enrichments from both sites utilized salicylate within 160 days. The color of the iron floc turned from rust to black, though changes in Fe(III)/Fe(II) were not measured. Methane was present in the head space of golf course sediment microcosms at 160 days, suggesting that iron (III) had largely been depleted and that iron (III) reduction was no longer the terminal electron accepting process. Further enrichment of these cultures was not pursued due to low activity. Sterile controls for all substrates under all conditions showed no appreciable loss of substrate (<15 %) demonstrating that loss was biological.

Stoichiometry for salicylate and chlorosalicylate utilization and methanogenesis. Methane production from the degradation of chlorosalicylates was measured in order to determine whether mineralization of salicylate and chlorosalicylates was linked to methanogenesis. Methane was produced above background in salicylate degrading cultures from the Wyoming site and salicylate and chlorosalicylate degrading cultures from the golf course drainage sediment (Table 2). The measured amount of methane produced was compared to the amount of methane expected, which was calculated from the balanced equations for salicylate and chlorosalicylate oxidation coupled to methanogenesis (Table 2. Equations 1 and 2). Protein was not measured in the system due high background levels and low yields during methanogenesis. Salicylate degrading cultures from the Wyoming site and golf course sediment produced 110% and 121% of expected CH_4, respectively. Methanogenic chlorosalicylate cultures from the golf course site utilized 3-chlorosalicylate with 114% of expected CH_4 production and 5-chlorosalicylate with 112% of expected CH_4 production. All four cultures tested showed a clear relationship between substrate utilization and methanogenesis.

Nitrate dependent degradation of chlorosalicylate. Stable denitrifying enrichment cultures from the golf course site utilizing 3-chlorosalicylate or 5-chlorosalicylate (approximately 1:10 dilution of the original sediment slurry equivalent to 1 to 2 % sediment content) were used to examine the requirement for nitrate during substrate degradation. Cells were collected by centrifugation and resuspended into nitrate free medium under a 97%N2 / 3%H2 atmosphere. The cultures were spiked with approximately 100 μM of the respective substrates, either 3-chlrorosalicylate or 5-chlorosalicylate, with or without 10 mM nitrate. The time course for 3-chlorosalicylate and 5-chlorosalicylate utilization is shown in Figure 2. 3-Chlorosalicylate and 5-chlorosalicylate were utilized within 50 days in the presence of nitrate. Without nitrate, both 3-chlorosalicylate and 5-chlorosalicylate were stable over the experimental period of 125 days with less than 23% loss of 3-chlorosalicylate and less than 15% loss of 5-chlorosalicylate. When unamended cultures received a 10mM nitrate spike on day four, utilization of 3-chlorosalicylate and 5-chlorosalicylate proceeded indicating that nitrate was required as an electron acceptor for degradation.

Stoichiometry for chlorosalicylate degradation and denitrification. The stoichiometry of substrate utilization and nitrate reduction was determined to examine whether degradation of chlorosalicylates and salicylate was coupled to denitrification. The stoichiometry (Table 3) was obtained by measuring the difference between substrate fed cultures and control cultures for endogenous denitrification. The values for electrons produced are calculated from the theoretical half reactions for the oxidation of salicylate and chlorosalicylate to CO_2. The values for electrons consumed are calculated from the values of nitrate reduced to nitrite and N2. The ratio of electrons consumed to electrons produced is a percentage of electrons generated during substrate oxidation which are accounted for by the reduction of electron acceptors measured in the system.

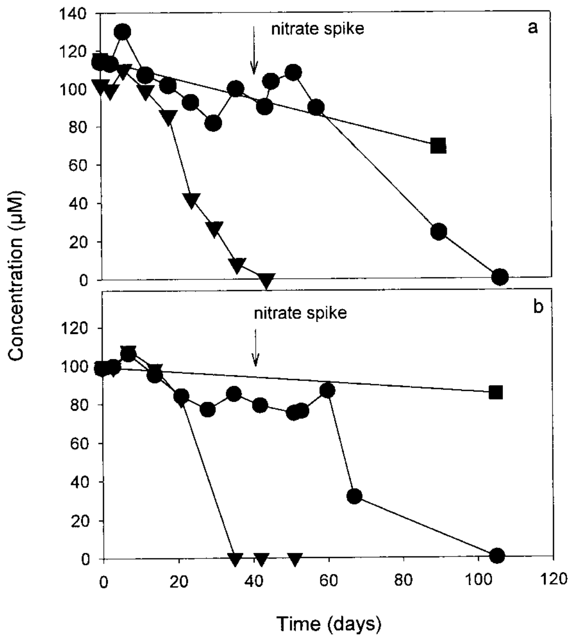

Figure 2. Nitrate dependency for (a) 3-chlorosalicylate and (b) 5-chlorosalicylate degradation in golf course sediment (RGS) microcosms. Data points represent the mean from duplicate cultures containing chlorosalicylate without nitrate (■), chlorosalicylate with 10 mM nitrate (▼), and chlorosalicylate with 10 mM nitrate spiked on day 40 (●). Standard deviation ±12.5 μM.

Denitrifying microcosms from both the golf course sediment and the Wyoming soil degraded salicylate, and nitrate/substrate stoichiometry accounted for 90% and 137% of electron acceptors utilized by the cultures, respectively (Table 3). These data are supportive of the assumption that salicylate utilization is coupled to denitrification. Similarly, electrons consumed by denitrification accounted for 101% and 112% of electrons generated through utilization of 5-chlorosalicylate in golf course enrichment cultures and Wyoming soil cultures. 3-Chlorosalicylate was also degraded by Wyoming soil cultures with an electron balance of 117%. These values show a definitive relationship between the oxidation of substrates and reduction of nitrate.

Reductive dehalogenation during degradation of chlorosalicylates under denitrifying conditions. During degradation of 100 μM chlorosalicylate in denitrifying cultures from both sites, formation of 5 to 19 μM salicylate was observed by HPLC, indicating that reductive dehalogenation of 3-chlorosalicylate

Table 2. Stoichiometry of methane production during salicylate and chlorosalicylate utilization

Substrate	μmol used	CH_4 expected μmol	CH_4 produced μmol	Produced/expected
Salicylate[a]	22.8	74.1	85.0	110
Salicylate[b]	28.7	93.5	118	121
3-CS[b]	20.6	66.8	76.7	114
5-CS[b]	19.5	63.5	71.6	112

[a] Wyoming Agricultural soil enrichments.
[b] Rutgers Golf Course enrichments.
Balanced equations:
Salicylate: $C_7H_6O_3 + 4.25\ H_2O \rightarrow 3.375\ CH_4 + 3.625\ CO_2$
Chlorosalicylate: $C_7H_5O_3Cl + 4.5\ H_2O \rightarrow 3.25\ CH_4 + 4.75\ CO_2 + Cl^- + H^+$

Table 3. Balance of electrons in during utilization of chlorosalicylate and salicylate by denitrifying enrichment cultures

Substrate	μ mol used	NO_3^- used μmol	NO_2^- produced μmol	e^- produced μmol	e^- used μmol	e^- used/ produced
5-Chlorosalicylate[a]	8.6	56.6	19.8	223	224	101
Salicylate[a]	22.5	128	37.7	631	566	90
3-Chlorosalicylate[b]	23.8	158	22.5	620	727	117
5-Chlorosalicylate[b]	8.8	65.9	24.2	229	256	112
Salicylate[b]	22.5	214	103	631	863	137

[a] Rutgers Golf Course enrichments.
[b] Wyoming Agricultural soil enrichments.
Half reactions:

$$C_7H_6O_3 + 11\ H_2O \rightarrow 7\ CO_2 + 28\ H^+ + 28\ e^-$$

$$C_7H_5O_3Cl + 11\ H_2O \rightarrow 7\ CO_2 + Cl^- + 27\ H^+ + 26\ e^-$$

$$NO_3^- + 2\ H^+ + 2\ e^- \rightarrow NO_2^- + H_2O$$

$$NO_3^- + 6\ H^+ + 5\ e^- \rightarrow 0.5\ N_2 + 3\ H_2O$$

Due to the low volume of gas produced N_2 was not measured. Reduction of nitrate to N_2 was calculated from NO_3^- consumption less accumulated NO_2^-.

and 5-chlorosalicylate was occurring under denitrifying conditions. Identification of salicylate as a reductive dehalogenation product from 3-chlorosalicylate and 5- chlorosalicylate was confirmed by GC-MS in denitrifying chlorosalicylate degrading cultures from the Wyoming site (Figure 3) and denitrifying 3-chlorosalicylate degrading cultures from the golf course site (data not shown). During this experiment salicylate (1 to 4 μM) also accumulated in HPLC chromatograms of controls that did not contain nitrate, although loss of chlorosalicylate was negligible and may be attributed to residual nitrate in the medium (0.1–0.3mM).

Discussion

Results from our study indicate that the anaerobic utilization of chlorosalicylates depended on the electron acceptors available and on the bacterial populations at the two sites tested. Furthermore, the position of the chlorine substituent greatly affected biodegradability. 3,6-Dichlorosalicylate, 6-chlorosalicylate and salicylate are anaerobic metabolites of dicamba (Taraban et al. 1993; Milligan & Häggblom 1999). Reductive dehalogenation of 3,6-dichlorosalicylate at the 3-position with accumulation of 6-chlorosalicylate, was consistent with previously observed preferential anaerobic dehalogenation of chlorobenzoic acids at the *meta* position and of chlorophenols at the *ortho* position (Suflita et al. 1982; Horowitz et al. 1983; Boyd & Shelton 1984; Kohring et al. 1989; Genthner et al. 1989; Zhang & Wiegel 1990; Madsen & Aamand

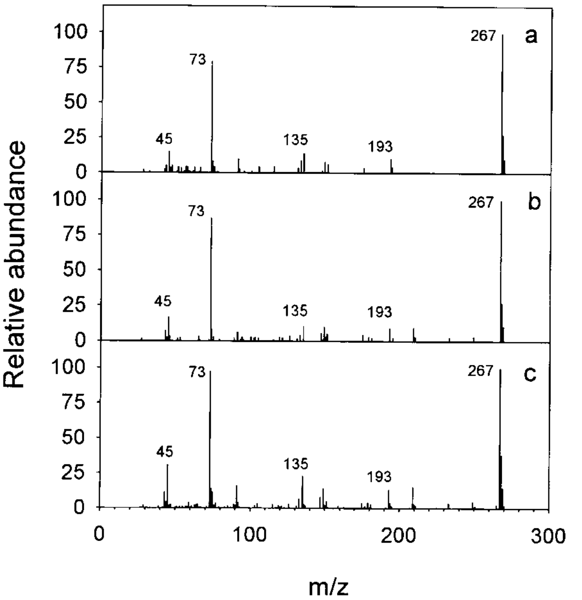

Figure 3. Mass spectra of silylated dehalogenation products from Wyoming soil microcosms. Panels show (a) silylated 3-chlorosalicylate metabolite, (b) silylated 5-chlorosalicylate metabolite, and (c) silylated salicylate standard.

1992). While dehalogenation of 3,6-dichlorosalicylate occurred under methanogenic and sulfate reducing conditions in microcosms from the golf course site, only trace amounts of activity were observed under methanogenic conditions with the microcosms from the Wyoming site. This discrepancy may have been due to many factors such as differences in available electron donors for dehalogenation, or lack of indigenous dehalogenating microbes. Our data indicate that 3,6-dichlorosalicylate produced by O-demethylation of dicamba may accumulate in anaerobic environments.

In microcosms established with monochlorinated salicylates, transformation of 6-chlorosalicylate occurred only under methanogenic conditions with the golf course sediment, and initial activity was not sustained. The recalcitrance of 6-chlorosalicylate is consistent with previous observations on anaerobic dehalogenation of ortho-chlorobenzoates and meta-chlorophenols (Suflita et al. 1982; Horowitz et al. 1983; Boyd & Shelton 1984; Kohring et al. 1989; Genthner et al. 1989; Zhang & Wiegel 1990; Madsen & Aamand 1992), and in dicamba degrading methanogenic cultures (Milligan & Häggblom 1999). Reductive dechlorination of 6-chlorosalicylate, has been demonstrated prior to methanogenic mineralization of dicamba, but this appears to be a rate limiting step (Milligan & Häggblom 1999).

Reductive dechlorination of 3- and 5-chlorosalicylate, but not 4-chlorosaliclyate under methanogenic conditions is also consistent with the positional preferences for dechlorination of monoaromatic compounds observed in previous studies. While initial dechlorination *meta* to the hydroxyl group of pentachlorophenol and 2,4,5-trichlorophenol in methanogenic cultures has been observed, (Mikesell & Boyd 1986; Bryant, 1992; Madsen & Aamand 1992) *ortho* attack is the dominant pathway reported for chlorophenols (Häggblom 1992). Similarly, when *para* dechlorination was observed in a methanogenic 4-chlorobenzoate culture it was only after a 12 month lag period and comparatively slow to 3- and 2-chlorobenzoate dehalogenation (Genthner et al. 1989). The degradation of 3- and 5-chlorosalicylate in the methanogenic enrichment cultures from the golf course sediment was stoichiometrically linked to methane production, demonstrating that the compounds were mineralized.

The chlorinated salicylates were not transformed under sulfate reducing conditions, with the exception of partial dechlorination of 3,6-dichlorosalicylate to 6-chlorosalicylate observed in the golf course sediment cultures. This lack of activity was not totally unexpected, as several studies have indicated that sulfur oxyanions inhibit reductive dechlorination (see Häggblom & Milligan 2000). However, the reductive dehalogenation of substituted aromatic compounds has been shown to be thermodynamically feasible under both methanogenic and sulfidogenic conditions (Dolfing & Harrison 1992; Dolfing & Harrison 1993) and *ortho*-dehalogenation of 2,4-dichloropenol in the presence of 25 mM sulfate has been reported in freshwater sediment cultures (Khoring et al. 1989). In addition, 2,4-dichlorophenoxy acetic acid was reductively dechlorinated in estuarine and marine sediments in the presence of sulfate (Boyle et al. 1999). Sulfate dependent reductive dehalogenation and degradation of chlorophenol has been demonstrated with a stable 4-chlorophenol degrading consortium (Häggblom & Young 1990; Häggblom & Young 1995; Häggblom 1998). Conversely, inhibition of dehalogenation of monoaromatics by sulfate is well documented (Gibson & Suflita 1986; Kuhn et al. 1990; Madsen & Aamand 1991). Thus, the effects of sulfate on reductive dechlorination are mixed and compound and/or culture specific. Similarily, although the anaerobic degradation of halogenated phenols and benzoic acids under iron (III) reducing conditions has been demonstrated in other studies (Kazumi et al. 1995; Monserrate & Häggblom 1997), no utilization of chlorinated salicylates was observed in the presence of iron (III).

Degradation of 3- and 5-chlorosalicylate occurred in denitrifying microcosms from both sites and nitrate stoichiometry linked substrate oxidation to denitrification. Nitrate dependency was further demonstrated in chlorosalicylate degrading cultures from golf course sediments. To our knowledge this is the first report of chlorosalicylate degradation linked to denitrification. Previous work has shown degradation of chlorobenzoates under denitrifying conditions (Genthner et al. 1989; Häggblom et al. 1993; Häggblom et al.1996), but not of chloroaromatics with both hydroxyl and carboxyl substituents. Utilization of 4-chlorosalicylate was unique to the Wyoming site in our study, demonstrating that inoculum is important, as well as chlorine position for the degradation of chlorinated salicylates. Degradation of 3- and 5-chlorosalicylate in the golf course sediment cultures was nitrate-dependent. Between 5 and 20 μM salicylate accumulated as a transient metabolite, which was then further degraded, indicating that reductive dechlorination occurred under denitrifying conditions in the presence of 10 mM nitrate. Reductive dechlorination of chloroaromatics (chlorophenols) has previously been demonstrated in denitrifying cultures only when the nitrate concentration was below 5 mM (Sanford & Tiedje 1997) and other studies have indicated that halophenols are stable under denitrifying conditions (Genthner et al. 1989; Häggblom et al. 1993). Further examination of these chlorosalicylate degrading cultures is necessary in order to determine whether a reductive dehalogenating organism is synergistically linked with a denitrifying salicylate degrading population or if one organism is responsible for dehalogenation and ring cleavage in this system.

Anaerobic dehalogenation and biodegradation of dicamba degradation metabolites and related chlorosalicylates occurred in our experiments to a varied extent in the presence of different terminal electron acceptors and soil/sediment inoculum. Reductive dechlorination readily took place under methanogenic conditions with golf course sediment, but not the Wyoming agricultural soil. We speculate that differences in activity were due to variances in water content and organic carbon at either site prior to the experiment. The Wyoming agricultural soil was well aerated with a low organic content of 1.15%. Conversely, the golf course sediment was saturated with water and had an organic content of 6.65%, indicating previous anaerobic conditions and abundant carbon sources available as electron donors for dehalogenation. These variables could have accounted for differences in the microbial population present between the sites as well. Low activity under sulfate reducing conditions may have been due to either site or compound specificity for degradation. Sulfate is a known inhibitor of dehalogenation in methanogenic systems (Gibson & Suflita 1986; Kuhn et al. 1990), which may explain the low activity. Although 3- and 5-chlorosalicylate were degraded in both sites under denitrifying conditions, the dicamba metabolites, 3,6-dichlorsalicylate and 6-chlorosalicylate, were not transformed, suggesting that dicamba and its metabolites can be recalcitrant in denitrifying environments.

This study revealed that the predominant electron accepting process as well as the indigenous microbial populations can effect the rate and extent of chlorinated salicylate degradation in anaerobic environments. Mineralization of 3- and 5-chlorosalicylate was demonstrated under methanogenic and denitrifying conditions. Dehalogenation of 3,6-dichlorosalicylate occurs under methanogenic conditions with the concomitant accumulation of 6-chlorosalicylate, which is recalcitrant. Transformation of 3,6-dichlorosalicylate under sulfate reducing conditions does occur, but the extent of dehalogenation remains unclear. The data also suggests that the anaerobic half-life of applied pesticides can vary with the heterogeneous nature of ground water environments and different electron accepting processes.

Acknowledgments

This work was funded in part by Grant R822497 from the U.S. Environmental Protection Agency. We thank L. Y. Young for sharing analytical instrumentation.

References

Boyd SA, & Shelton DR (1984) Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge. Appl. Environ. Microbiol. 47: 272–277

Boyle AW, Knight VK, Häggblom MM & Young LY (1999) Transformation of 2,4-dichlorophenoxyacetic acid in four different marine and estuarine sediments: effects of sulfate, hydrogen and acetate on dehalogenation and side chain cleavage. FEMS Microbiol. Ecol. 29: 105–113

Bryant FO (1992) Biodegradation of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyaceatic acid by dichlorophenoladapted microorganisms from freshwater, anaerobic sediments. Appl. Microbiol. Biotechnol. 38: 276–281

Colberg PJS (1991) Role of sulfate in microbial transformations of environmental contaminants: chlorinated aromatic compounds. Geomicrobiol. J. 8: 147–165

Dolfing J & Harrison BK (1992) Gibbs free energy of formation of halogenated aromatic compounds and their potential role as electron acceptors in anaerobic environments. Environ. Sci. Technol. 26: 2213–2218

Dolfing J & Harrison BK (1993) Redox and reduction potentials as parameters to predict the degradation pathway of chlorinated benzenes in anaerobic environments. FEMS Microbiol. Ecol. 13: 23–30

Genthner BRS, Price WAI & Pritchard PH (1989) Anaerobic degradation of chloroaromatic compounds in aquatic sediments under a variety of enrichment conditions. Appl. Environ. Microbiol. 55: 1466–1471

Ghiorse WC, Wilson JT & Kerr RS (1988) Microbial ecology of the terrestrial subsurface. Adv. Appl. Microbiol. 33: 107–172

Gibson GR (1990) Physiology and ecology of the sulfate-reducing bacteria. J. Appl. Bacteriol. 69: 769–797

Gibson SA & Suflita JM (1986) Extrapolation of biodegradation results to groundwater aquifers: reductive dehalogenation of aromatic compounds. Appl. Environ. Microbiol. 52: 681–688

Häggblom MM (1992) Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol. Rev. 103: 29–72

Häggblom MM (1998) Reductive dehalogenation by a sulfate-reducing consortium. FEMS Microbiol. Ecol. 26: 35–41

Häggblom MM & Milligan PW (2000) Anaerobic biodegradation of halogenated pesticides: influence of alternate electron acceptors. In: Bollag J-M, & Stotzky G (eds) Soil biochemistry, Vol. 10. (pp. 1–34) Marcel Dekker, New York

Häggblom MM & Young LY (1990) Chlorophenol degradation coupled to sulfate reduction. Appl. Environ. Microbiol. 56: 3255–3260

Häggblom MM & Young LY (1995) Anaerobic degradation of halogenated phenols by sulfate-reducing consortia. Appl. Environ. Microbiol. 61: 1546–1550

Häggblom MM, Rivera MD & Young LY (1996) Anaerobic degradation of halogenated benzoic acids coupled to denitrification observed in a variety of sediment and soil samples. FEMS Microbiol. Lett. 144: 213–219

Häggblom MM, Rivera MD & Young LY (1993) Influence of alternative electron acceptors on the anaerobic biodegradability of chlorophenols and benzoic acids. Appl. Environ. Microbiol. 59: 1162–1167

Horowitz A, Suflita JM & Tiedje JM (1983) Reductive dechlorination of halobenzoates by anaerobic lake sediment microorganisms. Appl. Environ. Microbiol. 45: 1459–1465

Kazumi, J, Häggblom MM & Young LY (1995) Diversity of anaerobic microbial processes in chlorobenzoate degradation: nitrate, iron, sulfate and carbonate as electron acceptors. Appl. Microbiol. Biotechnol. 43: 929–936

Kohring G, Zhang X & Wiegel J (1989) Anaerobic dechlorination of 2,4-dichlorophenol in freshwater sediments in the presence of sulfate. Appl. Environ. Microbiol. 55: 2735–2737

Kuhn EP, Townsend TG & Sufita JM (1990) Effect of sulfate and organic carbon supplements on reductive dehalogenation of chloroanilines in anaerobic aquifer slurries. Appl. Environ. Microbiol. 56: 2630–2637

Lovley DR & Phillips EJP (1986) Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl. Environ. Microbiol. 51: 683–689

Madsen T & Aamand J (1992) Anaerobic transformation and toxicity of trichlorophenols in a stable enrichment. Appl. Environ. Microbiol. 58: 557–561

Madsen T & Aamand J (1991) Effects of sulfuroxy anions on degradation of pentachlorophenol by a methanogenic enrichment culture. Appl. Environ. Microbiol. 57: 2453–2458

Mikesell MD & Boyd SA (1986) Complete reductive dechlorination and mineralization of pentachlorophenol by anaerobic microorganisms. Appl. Environ. Microbiol. 52: 861–865

Milligan PW & Häggblom MM (1999) Biodegradation and biotransformation of dicamba under different reducing conditions. Environ. Sci. Technol. 33: 1224–1229

Monserrate E, Häggblom MM (1997) Dehalogenation and biodegradation of brominated phenols and benzoic acids under iron-reducing, sulfidogenic, and methanogenic conditions. Appl. Environ. Microbiol. 63: 3911–3915

Mohn WW & Tiedje JM (1992) Microbial reductive dehalogenation. Microbiol. Rev. 56: 482–507

Myers CR & Nealson KH (1988) Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science 240: 1319–1321

Nealson KH & Saffarini D (1994) Iron and manganese in anaerobic respiration: environmental significance, physiology, and regulation. Annu. Rev. Microbiol. 48: 311–343

Sanford RA & Tiedje JM (1997) Chlorophenol dechlorination and subsequent degradation in denitrifying microcosms fed low concentrations of nitrate. Biodegradation 7: 425–234

Suflita JM, Horowitz A, Shelton DR & Tiedje JM (1982) Dehalogenation: a novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science 218: 1115–1117

Taraban RH, Berry DF, Berry DA & Walker HLJ (1993) Degradation of dicamba by an anaerobic consortium enriched from wetland soil. Appl. Environ. Microbiol. 59: 2332–2334

Zhang X & Wiegel J (1990) Sequential anaerobic degradation of 2,4-dichlorophenol in fresh water sediments. Appl. Environ. Microbiol. 56: 1119–1127

Zumft WG (1997) Cell biology and molecular basis of denitrification. Microbiol. Molec. Biol. Rev. 61: 533–604