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Reductive dechlorination of 2,4-dichlorophenol and related microbial processes under limiting and non-limiting sulfate concentration in anaerobic mid-Chesapeake Bay sediments

Kimberly A. Warner [a,*], Cynthia C. Gilmour [b], Douglas G. Capone [a,1]

[a] *University of Maryland Center for Environmental Science, Chesapeake Biological Laboratory, P.O. Box 38, Solomons, MD 20688, USA*
[b] *Academy of Natural Sciences Estuarine Research Center, 10545 Mackall Road, St. Leonard, MD 20685, USA*

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Abstract

The effects of sulfate, added electron donors, and metabolic inhibitors on reductive dechlorination of 2,4-dichlorophenol (DCP) in anaerobic sediment from a relatively unpolluted site in the mesohaline region of Chesapeake Bay were investigated. The onset of *ortho* reductive dechlorination of DCP was often rapid in sulfate-depleted sediments and the metabolite, 4-chlorophenol, persisted for >44–60 days at nearly stoichiometric levels, relative to the DCP lost, with no further degradation or dechlorination observed. Dechlorination rates increased by a factor of 6–10 after re-feeding DCP, suggesting enrichment of dechlorinators. Sulfate inhibited reductive dechlorination greatly in fresh sediments, but only partially in aged, methanogenic or DCP-acclimated sediments. Exogenous H_2 stimulated rates of reductive dechlorination in the absence, but not presence of sulfate. Molybdate severely inhibited reductive dechlorination and also significantly inhibited methanogenesis in sulfate-depleted sediments. Direct inhibition of methanogenesis with bromoethane sulfonic acid did not negatively affect dechlorination rates, suggesting that molybdate may have inhibited production of electron donors for methanogenesis and reductive dechlorination. These results demonstrate a potential for rapid dechlorination of DCP in relatively uncontaminated estuarine sediments and reveal confounding effects of a putative 'specific' metabolic inhibitor, molybdate.

Keywords: Reductive dehalogenation; 2,4-Dichlorophenol; Sulfate reduction; Methanogenesis; Estuarine sediment; Molybdate

1. Introduction

Estuarine sediments are often the repository of many classes of chlorinated aryl compounds of anthropogenic origin. Factors that affect the persistence of such compounds need to be carefully evaluated if we are to understand their fate in these environments. Sulfate reduction (SR) is normally the dominant terminal electron accepting process in estuarine anaerobic carbon flow and prevails as long as the supply of labile substrates and rate of sediment metabolism do not exceed the supply of sulfate [1]. Under anaerobic conditions, reductive dechlorination is generally the first step in the microbial degradation of aryl halides. The bulk of early observations of dehalogenation came from methanogenic habitats (for review, see [2,3]), with fewer under sulfate reducing (or other electron accepting) conditions in marine sediments [4–17]. Whereas sulfate often inhibited dehalogenation, more recent studies have demonstrated prompt dehalogenation in marine or estuarine sediments, concomitant with SR, especially when previous exposure to polluted conditions [11] or halogenated compounds of anthropogenic or biogenic origin was indicated [5,12].

We investigated the capacity for reductive dechlorination of the model compound 2,4-dichlorophenol (DCP) in relatively unpolluted surficial sediments from a well-studied mesohaline site in the Chesapeake Bay. SR was the dominant terminal electron accepting process at this site, but sulfate concentration often limited SR in relatively shallow sediment horizons in late summer [18]. Here we examine the effect of sulfate, exogenous electron donors (mainly H_2), and molybdate and bromoethane sulfonic acid (BES), 'specific' metabolic inhibitors of sulfate

* Corresponding author. Present address: The University of Alabama, Department of Biological Science, P.O. Box 870206, Tuscaloosa, AL 35487-0206, USA. Tel.: +1 (205) 348-5191; Fax: +1 (205) 348-1403.
E-mail address: kwarner@biology.as.ua.edu (K.A. Warner).

[1] Present address: Wrigley Institute for Environmental Studies and Department of Biological Science, University of Southern California, Los Angeles, CA 90089-0371, USA.

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reducing bacteria (SRB) and methanogens, respectively, on DCP dechlorination.

2. Materials and methods

2.1. Site description and sediment collection

Sediments were sampled on several occasions from a mesohaline mid-Chesapeake Bay site (MB) on the western slope of the central channel (14 m depth; 38°34.1′N × 76°26.6′W). This region of the bay experiences hypoxic/anoxic bottom water conditions in summer, which largely excludes the presence of infauna. These highly reduced, sulfidic sediments likely limit any possible nitrate or metal reduction to brief periods in spring [19,20]. Surface sediments from this site have an average porosity of 0.9 and organic matter content of 10% [18]. Sediments were collected as cores, the top 0–2 or 4 cm extruded, and stored in glass jars (250 or 500 ml) filled to capacity. Surficial sediment sulfate levels were typically 7–15 mM at the time of collection. Sediments were held at room temperature after collection until used in experiments.

2.2. Experimental preparations

Slurries were prepared inside an anaerobic chamber (Coy Laboratory Products) with an atmosphere of N_2 (85%), CO_2 (10%), and H_2 (5%). Sediments were gently sieved (1-mm mesh) and mixed with diluents (1:2 or 1:3, v/v) that had been deoxygenated by bubbling with N_2 for 45–60 min. Diluent for the first preliminary and third experiment was an artificial salt water (ASW) solution (30 ppt; pH 8) which contained, before dilution, in grams per liter deionized water: NaCl, 24.72; KCl, 0.67; $CaCl_2{\cdot}2H_2O$, 1.36; $MgSO_4{\cdot}7H_2O$, 6.29; $MgCl_2{\cdot}6H_2O$, 4.46; $NaHCO_3$, 0.18. For sulfate-free assays, magnesium chloride replaced magnesium sulfate in the ASW. The ASW was diluted with deionized water to ambient bottom water salinity (13–21 ppt) prior to use. Salinity was checked with a hand-held refractometer. Diluent for the second experiment was bottom water from site MB. For sulfate-free assays, barium chloride was added to bottom water at equivalent equimolar amounts to achieve sulfate precipitation with low residual barium chloride, followed by filtration. Slurries were dispensed into serum bottles, sealed with Teflon-faced butyl stoppers, removed from the anaerobic chamber and flushed with N_2. All additions to bottles were made with sterile degassed syringes. Small volumes ($<0.1\%$) of an anoxic concentrated stock of DCP (10 mg ml^{-1}) prepared in acetone were added to achieve the stated concentrations. This resulted in acetone concentrations of 14–21 mM, sufficient to reduce or produce twice as much sulfate or methane, respectively. All other solutions were added from concentrated, filtered (0.2 μm) anoxic stocks prepared with deionized water to achieve the stated concentrations. Hydrogen (ca. 10%) was added to sealed vials with a wetted ground glass syringe and needle and measured immediately. Hydrogen additions were repeated when H_2 concentrations fell below the detection limit (3 Pa). Killed controls were autoclaved (121°C, 45 min) on three successive days. Treatments consisted of two replicates, except for the preliminary experiment, where methane concentrations were monitored in triplicate. All experimental treatments were incubated in the dark without shaking, at room temperature ($\sim$24°C).

Gas samples (0.1 ml) were withdrawn from sealed vials with N_2-flushed, gastight syringes, after shaking vials to equilibrate dissolved and gaseous phases. The volume of overpressure in vials was measured prior to gas analyses by inserting an N_2-flushed wetted glass syringe, measuring the deflected volume (precision of this measurement was 0.1 ml) and then re-injecting this volume. For methane, the headspace and slurry volumes were noted at each sampling event in order to calculate total moles produced per unit volume of slurry. The moles of gas in the headspace (converted from partial pressure) plus the moles dissolved in the porewater, calculated from Bunsen coefficients corrected for salinity and temperature [21], were divided by the total sediment volume, in order to calculate slurry volume-normalized cumulative methane production.

2.3. Analytical

Slurry subsamples were withdrawn periodically from sealed vials with N_2-flushed sterile plastic syringes and needles while shaking to homogenize vial contents. Samples (1 ml) for DCP and potential dechlorinated metabolites, 4-chlorophenol (4CP), 2-chlorophenol and phenol analyses were mixed with analytical-grade acetonitrile at a 1:1 or 2:1 volume ratio, vortexed, and centrifuged. Supernatants were filtered (0.2-μm nylon syringe filters) and stored at 5°C, usually less than 24 h prior to analyses. Phenol and chlorophenols were analyzed by reverse phase high-performance liquid chromatography with UV detection at 280 nm after a modified method of Bryant et al. [22]. Analytes were separated on a 250×4.6-mm C-18 column using a mobile phase of acetonitrile, methanol, water and acetic acid (25:20:54:1) at a flow rate of 0.95 ml min^{-1}. Analytes were identified based on retention times of authentic standards (Sigma) and quantified by linear regression of external standards of phenolic compounds freshly prepared and diluted in 50% aqueous acetonitrile. Phenol and chlorinated phenols were never detected in any method blanks or in sediments unamended with DCP.

Sulfate samples were placed in N_2-flushed tubes, centrifuged (15 min, 2000×g) and the supernatant quickly transferred to vials with 10% (by weight) zinc acetate and stored at 5°C until analyses. Sulfate samples were filtered (0.45 μm), diluted with deionized water and ana-

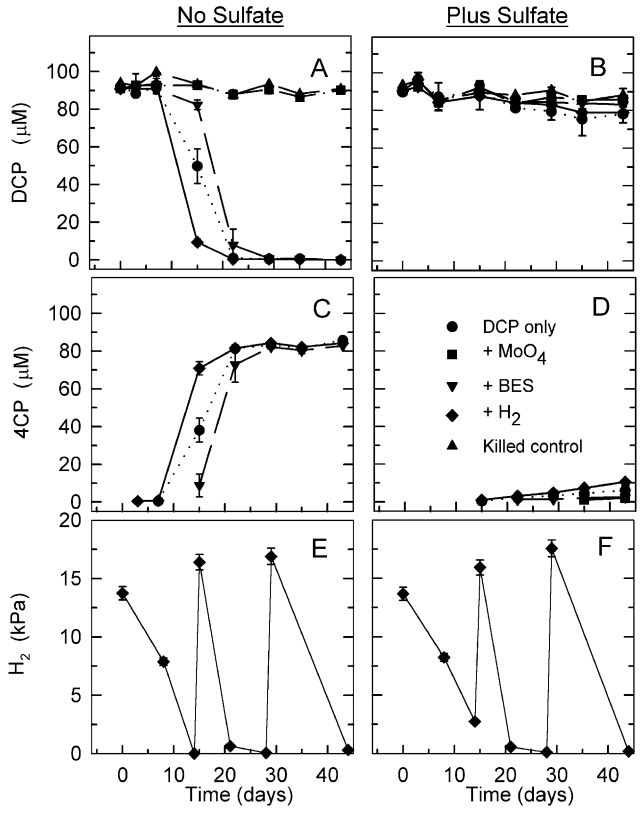

Fig. 1. DCP dechlorination in (A, C) sulfate-depleted and (B, D) sulfate-replete treatments of Experiment 2 and corresponding H_2 concentration (E, F) in selected treatments. Data points are the average of duplicates ± 1 S.D.

lyzed by ion exchange chromatography with conductivity detection as previously described [18].

Methane and exogenous H_2 were separated on a 1-m column (Poropak R) with N_2 carrier gas and measured by gas chromatography (GC) with thermal conductivity detection (Shimadzu GC-9A). Low-level methane samples were analyzed by GC (Shimadzu GC-9A) with a flame ionization detection with a 1-m Poropak R column and N_2 carrier gas (50 ml min^{-1}). Analytes were identified based on retention times of external standards.

2.4. Statistical analyses

Significant differences among treatment means were tested by one-way analysis of variance, followed by Scheffé's *F*-test using Statview software (SAS Institute, Cary, NC, USA). Differences between two means were tested by Student's *t*-test.

3. Results

A preliminary study (Experiment 1) revealed rapid dechlorination of DCP in spring-collected MB sediments aged 3 months to deplete sulfate. Over 90% of the DCP added (0.6 μmol g^{-1}; 99 μM) was lost within 1 month (results not shown). The only detected metabolite, 4CP, appeared after 1 day and increased over time, demonstrating that *ortho*-dechlorination was primarily responsible for the DCP loss. Molybdate (20 mM) completely inhibited dechlorination and significantly inhibited methanogenesis by 25%, compared to uninhibited treatments (results not shown). The effect of sulfate on dechlorination was mixed. Sulfate (28 mM) added to the aged sediment inhibited neither DCP transformation nor methane formation. In contrast, no loss of DCP was observed within the same 1-month time frame with freshly collected sulfate-rich sediments. The rapid dechlorination observed in this preliminary experiment and confounding effects of sulfate led to further investigations at this site.

To determine whether natural in situ depletion of sulfate would provide the same result, an experiment (Experiment 2) with sediments freshly collected in late summer and recently depleted of sulfate was conducted. Similar to our preliminary results DCP dosed at a higher concentration (1.3 μmol g^{-1}; 96 μM) was rapidly dechlorinated within 21 days in the sulfate-free control treatment, following a 7-day lag (Fig. 1A). The initial addition of H_2 (∼14 kPa) (Fig. 1E) led to earlier detection of 4CP (Fig. 1C) and stimulated reductive dechlorination rates. 4CP production was nearly stoichiometric (≥90%) to the amount of DCP lost, and persisted through the remaining assay period. Checks on residual incubations after 1 year showed continued persistence of 4CP (results not shown).

Sulfate added back to these fresh sulfate-depleted sediments severely inhibited reductive dechlorination of DCP in all treatments (Fig. 1B). Excess H_2 did not relieve the inhibitory effect of sulfate on reductive dechlorination rates. H_2 additions (Fig. 1F) slightly, but significantly (Student's *t*-test; $P < 0.05$), increased the percentage of DCP recovered as 4CP, relative to the control (11 vs. 6%, respectively), although the total loss of DCP was similar between the two treatments (Fig. 1B). Consistent with our preliminary findings, molybdate (20 mM) completely inhibited reductive dechlorination in the absence of sulfate (Fig. 1A). BES (12 mM) delayed the onset of reductive dechlorination, but subsequent rates were more rapid, relative to the sulfate-depleted control. The effect of the metabolic inhibitors in sulfate-replete sediments was difficult to assess, due to the overall inhibition of reductive dechlorination by sulfate (Fig. 1B,D). While small losses of DCP were observed in killed controls in this (Fig. 1A,B) and other experiments, no dechlorination (as evidenced by 4CP production) was detected.

Beyond the first week of the experiment no appreciable loss of sulfate occurred in the treatments with sulfate, with the exception of the treatment with added H_2, where ∼6 mM was lost (data not shown). This demonstrates that SRB were present in the sulfate-replete sediment, but apparently limited by labile carbon or electron donor availability.

Methane production was active in sulfate-depleted treat-

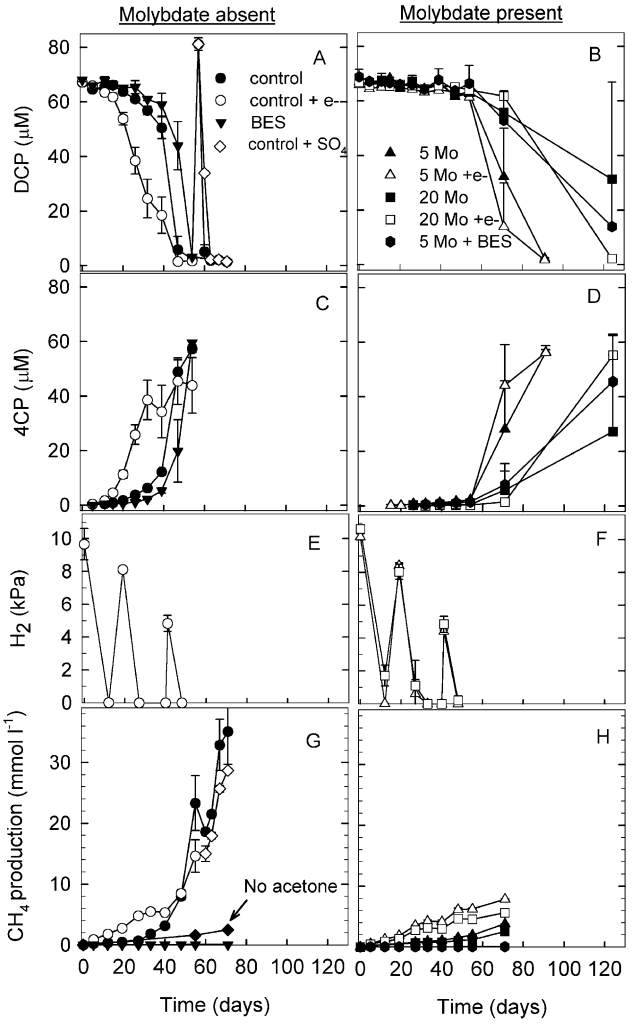

Fig. 2. DCP dechlorination (A, B) and corresponding 4CP production (C, D), H_2 concentration (E, F) and cumulative methane production (G, H) over time in the presence and absence of molybdate in low sulfate sediments in Experiment 3. H_2 and acetate (1 mM) ('+e' treatments) were added on day 0; H_2, only thereafter. DCP (with acetone carrier) was re-fed to the control treatment with DCP only on day 57 with and without the addition of 11 mM sulfate. Error bars represent 1 S.D. of duplicates. Mo: molybdate; BES: bromoethane sulfonic acid.

ments without inhibitors and stimulated by H_2 in both the presence and absence of sulfate (Table 1). Unlike results obtained in the preliminary study, sulfate added to sediments recently depleted of sulfate effectively suppressed methanogenesis. Molybdate stimulated methane production rates in the presence of sulfate, relative to the control (Student's t-test; $P < 0.001$), but greatly inhibited methanogenesis (94% of the control value) in the absence of sulfate. Yet, there was no significant difference between methane production rates in the absence or presence of sulfate in treatments with molybdate.

In a third experiment we tested the effect of molybdate at two different concentrations (5 and 20 mM), with and without added electron donors (H_2 and acetate), on dechlorination of DCP (0.6 μmol g^{-1}; 65 μM) in sulfate-limited (25 μM) MB sediments. The treatments with H_2 and acetate provided typical products of fermentation, production of which may have been blocked by molybdate. Similar to the first experiment, sediments were pre-incubated for 3 months to deplete sulfate before starting the experiment.

DCP dechlorination rates in uninhibited treatments were much slower in the third experiment than in previous sulfate-limited experiments (Fig. 2A). Dechlorination began after an apparent lag period of 25 days and another 25 days were required to completely dechlorinate DCP in the DCP-only treatment. Small amounts of 4CP were detected by day 5 indicating that a low rate of dechlorination was occurring during the 25-day apparent lag period (Fig. 2C). The additions of electron donors, H_2 and acetate, (hereafter referred to as 'e-donors') decreased the lag period and increased the initial rate of reductive dechlorination in the uninhibited control. BES slightly inhibited dechlorination when dosed alone (Fig. 2A), but had a synergistic negative effect on reductive dechlorination when dosed in combination with 5 mM molybdate (Fig. 2B).

After extended incubation of these sulfate-limited sediments, some treatments with molybdate eventually dechlorinated DCP (Fig. 2B,D). Molybdate still had a pronounced inhibitory effect on reductive dechlorination, delaying the onset and subsequent rates of reductive dechlorination, and these effects were dose dependent. In the treatment with 20 mM molybdate, noticeable degradation commenced after 70 days in one replicate only. The additions of e-donors did not relieve molybdate inhibition of dechlorination. Nonetheless treatments with molybdate plus e-donors displayed slightly faster rates of dechlorination compared to treatments with molybdate only. These enhanced rates occurred more than 10 days after the last of the H_2 had been consumed (Fig. 2F), suggesting H_2 may have sustained molybdate-sensitive populations during the time when its inhibitory effects were most effective.

DCP was re-fed to the treatment with DCP only on day 57 of the third experiment with and without the addition of 11 mM sulfate. The second dose of DCP was rapidly dechlorinated in the treatment without sulfate (Fig. 2A), suggesting that this activity could be enriched. Although sulfate, which was reduced in this treatment (results not shown), did not block dechlorination of the second dose of DCP, the rate was slowed by nearly half that of the control.

In treatments without inhibitors, methane production increased exponentially after a 25–35-day lag period (Fig. 2G), coinciding with the timing of increased rates of reductive dechlorination (Fig. 2A). By day 55, methane production reached 15–23 mmol l^{-1} in these treatments, whereas production in background incubations (no acetone) and those with molybdate (no e-donors) were 1–2 mmol l^{-1}. Acetate and H_2 additions stimulated methane production in the presence of molybdate (Fig. 2H). Ace-

Table 1
Methane production rates (μmol (l sediment)$^{-1}$ day^{-1}) (±1 S.D.) in treatments from the second experiment with fresh, sulfate-depleted sediments incubated 44 days with and without sulfate

Treatment[a]	Methane production rate[b]	
	−sulfate	+sulfate
No additions	28 (2.8)	0.1 (0.01)
DCP+acetone	31 (1.8)	0.5 (0.06)
+MoO_4^{2-} (20 mM)	2.0 (0.3)	1.6 (0.17)
+BES (12 mM)	–	–
+H_2 (47 kPa)	130 (8.0)	37 (9.3)

Rates were calculated from the slope of linear increase in methane concentration over time. Dashes: no detectable rate.
[a]All treatments (except 'no additions') received 96 μM DCP and 21 mM acetone (as carrier).
[b]Values represent the averages of duplicates.

tone (14 mM), which was present, but apparently not utilized in previous experiments, was supplying electron donors for increased methanogenesis, and possibly dechlorination, in this experiment. Here, molybdate apparently inhibited acetone-based methanogenesis.

4. Discussion

The presence of sulfate exerted various degrees of inhibition of dehalogenation in these experiments, ranging from nearly total inhibition in fresh, sulfate-replete sediments to partial or practically no inhibition in aged methanogenic or DCP-acclimated sediments. The mechanism of sulfate inhibition is thought to result from SR outcompeting dehalogenation for electron donors, either within an organism or community [23–26]. However, our results may not be completely consistent with this view. Whereas excess H_2 has been shown to relieve the inhibition of dechlorination by sulfate in other systems [24,27], in our experiments excess H_2 only stimulated SR. The addition of molybdate should have also relieved sulfate inhibition of dechlorination, by freeing up electron donors used in SR for dechlorination, as has been observed in other systems [25,28]. Although we have previously observed the accumulation of both H_2 and acetate upon the addition of molybdate to sulfate-replete sediments from this site [29] and noted stimulation of methanogenesis here (Table 1) the increased availability of e-donors was not channeled to dechlorinators in our system. These results initially suggested that SRB might be involved in dechlorination, yet the effect of molybdate on microbial processes not involving SR (Table 1, Fig. 2H) raises some issues worth considering.

The inhibition of methanogenesis by 20 mM molybdate in sulfate-depleted sediments (Table 1) was unexpected as this concentration is often used in marine systems to ensure inhibition of all SRB without affecting other community members [30–32]. This suppression of methanogenesis by 20 mM molybdate in the first two experiments led to concern that this putative specific inhibitor of SRB could be exerting non-specific toxic effects on methanogens and possibly dechlorinators. Molybdate (20 mM) was shown to be toxic to some pure cultures of methanogens and to mixed populations in a freshwater lake [33]. On the other hand, other researchers have found no toxic effects on methanogens at concentrations up to 10 mM, either in pure or co-culture with SRB [34]. In our last experiment, even low concentrations (5 mM) of molybdate inhibited methanogenesis. If the inhibition of methanogenesis and dechlorination by 20 mM molybdate was not due to direct toxicity, we hypothesized that molybdate could be specifically inhibiting SRB which were supplying electron donors through fermentation for these reactions under sulfate limitation. The recent findings of Drzyzga and Gottschal [35] support this view, wherein dehalogenation of PCE by *Desulfitobacterium frappieri* (and subsequently, methanogenesis by another organism) was strictly dependent on syntrophic fermentation by a SRB species in continuous co-culture. Whereas some non-SRB fermentative bacteria have been shown to be sensitive to molybdate [36,37], others have found inhibition of methanogenesis by molybdate in systems where SRB are supplying electron donors through fermentation [33,38–40], but apparently not in marine or estuarine settings. The SRB at the MB site are frequently faced with sulfate limitation in late summer and it is not unreasonable to assume that they would switch to alternative forms of metabolism available to them, such as fermentation.

If the above suppositions are correct, then some of the variation in response of methanogenesis to molybdate noted here could be the result of changing population densities of methanogens and SRB. In the third experiment with aged, methanogenic sediment, 20 mM molybdate did not affect methanogenesis with endogenous electron donors, relative to background controls with no added acetone (Fig. 2G,H), suggesting that the native methanogenic population (i.e. those not enriched by added e-donors) was not affected by molybdate. This finding is in contrast to results obtained in Experiment 2, where 20 mM molybdate severely inhibited methanogenesis with endogenous electron donors. That this inhibited methane production rate in the absence of sulfate was equivalent to the 'stimulated' rate in the presence of sulfate (Table 1) suggests that the methanogenic population that directly competed with SRB for common substrates was small. The remaining methanogenic population was plausibly dependent on molybdate-sensitive fermentations. The presumed greater supply of fermentable substrates in those fresh, sulfate-depleted sediments and a more active and abundant SRB population able to ferment these substrates could, in part, account for these differences.

Although the exact mechanism of DCP dechlorination at this site cannot be gleaned from this study, we have identified an unpolluted estuarine location where DCP dechlorination can, at times, proceed rapidly and without

lengthy acclimation or lag periods (weeks to months) commonly observed to accompany DCP dechlorination under methanogenic conditions [6,7,41–43]. Whereas lag periods are thought to result largely from the time required to induce or de-repress dehalogenating enzymes or enrich an initially small population of dechlorinators in the presence of an aryl halide [2], these conditions apparently were not consistently required for dechlorinators at the MB site. However, other unpublished data from this site [29] suggest to us that some of the variability in lag periods and dechlorination rates shown here are influenced by seasonal variability in labile carbon availability and microbial community composition (which will be explored more fully elsewhere). Nonetheless, these observations and the results of molybdate' effect on non-target microbial processes point to an intriguing site for further study.

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