1,4-Dioxane Biodegradation Potential in Aerobic and Anaerobic Microcosms Inoculated with Agricultural Soil and Contaminated Sediments

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ABSTRACT: 1,4-Dioxane, a probable human carcinogen, was commonly used as a stabilizer in 1,1,1-trichloroethane formulations and is now frequently detected at sites where the chlorinated solvents are present. A major challenge in addressing 1,4-dioxane contamination concerns chemical characteristics that result in migration and persistence. This study aims to examine the biodegradation potential of 1,4-dioxane in aerobic and anaerobic microcosms by developing enrichment cultures. Microcosms using agricultural soils, river sediments (Michigan) and contaminated site sediments (California and Maine) were established under a range of redox conditions (aerobic, nitrate amended, iron amended, sulfate amended and methanogenic). The experimental setup included triplicates of sample microcosms and abiotic controls amended with approximately 5 – 12 mg/L 1,4-dioxane. Selected samples were subjected to Compound Specific Isotope Analysis (CSIA) analysis to determine the ratio of stable isotopes $^{13}C/^{12}C$ and $^{2}H/^{1}H$ of 1,4-dioxane. 1,4-Dioxane concentrations over time were determined using a GC/MS combined with solid phase micro extraction technique. Biodegradation of 1,4-dioxane was observed in all of the aerobic microcosms within 40 to 50 days. However, the time required for the degradation of this chemical under anaerobic conditions was substantially longer (often >300 days). Significant differences between live microcosms and abiotic controls were most commonly noted for treatments that involved no electron acceptor amendment or those initially amended with nitrate (given the long incubation period, the nitrate amended samples likely transitioned into being methanogenic). Compound specific isotope analysis involving $^{13}C/^{12}C$ measurements confirmed the 1,4-dioxane degradation in one set of microcosms. This work is important because limited previous research has documented 1,4-dioxane biodegradation under anaerobic conditions.

INTRODUCTION

1,4-Dioxane, a commonly used stabilizer in 1,1,1-trichloroethane (TCA) formulations, is frequently detected at sites contaminated with TCA and other chlorinated solvents (Adamson et al., 2015; Mohr et al., 2010). It is commonly detected in federal sites due to its usage as a stabilizer in solvents, paint strippers and greases. Due to its toxicity, 1,4-dioxane has been classified as a probable human carcinogen by the U.S. EPA (U.S. EPA, 2017). There is a critical need to develop management strategies for this contaminant due to its widespread occurrence.

1,4-Dioxane shows high persistence and migration potential in the environment due to its chemical characteristics. Its low vapor pressure, low octanol-water partition coefficient and high solubility ensures complete miscibility in water and high migration potential (U.S. EPA, 2017). Thus, it is a challenge to treat the contamination in surface water and groundwater. A low organic carbon partition coefficient and Henry's Law constant, make traditional remediation methods such as air stripping or activated carbon largely ineffective. Treatment options using hydrogen peroxide, ozone, UV light or sonication are possible (Adams et al., 1994; Coleman et al., 2007; Son et al., 2009; Stefan and Bolton, 1998). However, the ex-situ methods can be costly at high concentrations.

Although natural attenuation can be an option, it may be too slow and thus reduces the feasibility of this option (Adamson et al., 2015).

Given the limitations associated with traditional remediation methods, interest has turned to the use of microorganisms to degrade 1,4-dioxane. Towards this goal, this study is aimed at developing enrichment cultures and determining the degradation potential in aerobic and anaerobic microcosms.

MATERIALS AND METHODS

Chemicals and Materials. 1,4-dioxane was purchased from Thermo Fischer Scientific Chemicals Inc. (MA, USA) and Sigma-Aldrich (MO, USA). Ethylenediaminetetraacetic acid (EDTA) iron (III) sodium salt, sodium sulfate, sodium nitrate and humic acid were purchased from Sigma-Aldrich (MO, USA). All stock solutions and dilutions were prepared using DI water. The agricultural samples were collected from three locations in East Lansing (soils E,F and G) and two other locations (agricultural soils T1 and T2) at Michigan State University's Main Cropping System Experiment at Kellogg Biological Station Long-Term Ecological Research (KBS LTER). Contaminated site samples were obtained from two sampling locations at a site in California (contaminated site 1 and 2) and one from Maine (contaminated site 3). The sites 1 and 2 were contaminated with trichloroethylene (TCE), 1,1-dichloroethene (1,1-DCE) and 1,4-dioxane. The contaminated site 3 had traces of 1,4-dioxane contamination. The river sediment samples (Sediment H) were collected from Red Cedar River in Okemos, Michigan. All the samples were stored in the dark at 6 $^{\circ}$C until use.

Experimental Setup. The aerobic microcosms were established using six different inocula, including agricultural soils F, G, T1 and T2 (Michigan) along with contaminated sediments from site 1 and 2 (California). The microcosms were set up in triplicates of live controls (no 1,4-dioxane added), samples and abiotic controls with 5g of inocula in 30ml amber serum vials. The abiotic controls were autoclaved thrice with 24 hrs intervals before being setup. Basal salts medium containing K_2HPO_4 (32.4 g/L), KH_2PO_4 (10 g/L), NH_4Cl (20 g/L) and a trace metal solution with disodium salt (1.23 g/L), $MgSO_4.7H_2O$ (2 g/L), $FeSO_4.7H_2O$ (0.12 g/L), $MnSO_4.H_2O$ (0.03 g/L), $ZnSO_4.7H_2O$ (0.03 g/L) and $CoCl_2.6H_2O$ (0.01 g/L) was added to the microcosms (Parales et al., 1994). 1,4-Dioxane was added to the microcosms after adding 25 ml of the Basal salts media. The microcosms were established to have an initial concentration of 12 mg/L of 1,4-dioxane. Nucleic acids were extracted using MO BIO UltraClean Microbial DNA Isolation kit (following the manufacturer's directions) from the samples and live controls following the depletion of each amendment of 1,4-dioxane.

The anaerobic microcosms were established in 30 ml amber serum bottles with 5 g of each agricultural soil and 5g of contaminated sediments. Each of the three agricultural soils (E, F and G) were setup under two redox conditions (methanogenic and nitrate reducing). Triplicates of samples and the abiotic controls were established to result in 36 microcosms in total. The river sediment H was set up under methanogenic condition. Each of the contaminated sediments (site 1 and 3) were set up under four redox conditions (nitrate amended, iron amended, sulfate amended and no amendment). The abiotic controls were autoclaved thrice at an interval of 24 hrs before introducing them to anaerobic conditions. A 9 ml solution was added to the agricultural soils for each treatment, as follows: a) sodium nitrate (10 mM) b) ethylenediaminetetraacetic acid (EDTA) iron(III) sodium salt (10 mM) with humic acid (0.25 g/l), b) sodium sulfate (10 mM), and d) water for methanogenic condition. All solutions were purged under a stream of nitrogen gas (oxygen free) for 60 minutes before adding each to the microcosms. The same stock solutions (25ml) were used for the contaminated sediments under each

treatment. Sodium lactate (5 mM) was added as a carbon source for microcosms established with contaminated sediments. Two weeks after the initial set up, 1 ml of 1,4-dioxane was added to each microcosm for a final concentration of 5 mg/L.

The samples, closed with septa, were incubated in the anaerobic chamber at 20 ˚C. The anaerobic chamber was maintained with gaseous mix of approximately 5% H_2, 90% N_2 and 5% CO_2. The vials were sealed using BiMetal vial crimp with PTFE/silicone septas to maintain the cultures at anaerobic conditions

All microcosms were transferred on a shaker at 200 rpm and maintained at 20 ˚C. The aerobic microcosms were opened periodically to replenish oxygen. The nitrate amended microcosms were tested for methane after 200 days of incubation using a GC (Hewlett Packard 5890).

GC/MS Analysis. GC/MS with Agilent 5975 GC/single quadrupole MS (Agilent Technologies, CA, USA) equipped with a CTC Combi Pal autosampler was used for analysis of 1,4-dioxane concentrations. A sterile 1 ml syringe with 22 Ga 1.5 in. (3.81 cm) needle was used to collect 0.7 ml sample from each microcosm in 40 ml amber glass vials for GC. The samples were filtered using a 0.22 µm nylon filter before injecting into the 40 ml vial. A method was developed to analyze 1,4-dioxane using solid phase micro extraction (SPME) technique. The SPME fiber was inserted in the headspace of the vial and exposed to the analyte for 1 minute before being injected into the GC for thermal desorption. The fiber coating can adsorb the analytes in the vapor phase. Splitless injection was executed and the vials were maintained at 40 ˚C. In this experiment, SPME fiber assembly with 50/30µm Divinylbenzene/ Carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS) and 24 Ga needle was utilized. The initial oven temperature was 35 ˚C and was programmed to increase at a rate of 20 ˚C/min to 120 ˚C. Once it reached 120 ˚C, it increased at a rate of 40 ˚C/min to 250 ˚C, which was maintained for 3 min. VF5MS column was used with Helium as the carrier gas in constant flow mode at a flow rate of 1 ml/min. The conditioning of the SPME fiber was at 270 ˚C for 60 min at the beginning of each sequence.

Compound Specific Isotope Analysis (CSIA). The CSIA was performed at the University of Waterloo Environmental Isotope Laboratory (UWEIL), Ontario, Canada. The ratios of $^{13}C/^{12}C$ and $^{2}H/^{1}H$ were measured using a recently developed method. For this, the dilute 1,4-dioxane samples were concentrated on to a sorbent and subjected to thermal desorption in a GC coupled with an isotope ratio mass spectrometer (Bennett et al., 2018).

RESULTS

Aerobic Microcosms

1,4-Dioxane concentrations were determined in the samples and aerobic controls over a period of five months. During this time, aerobic biodegradation of the chemical was observed (red numbers in Table 1). Following 1,4-dioxane depletion, the samples were re-spiked with 1,4-dioxane. The estimated initial concentration (day 0) of the compound was 12 mg/L in samples and controls. All further time points measured using the GC/MS indicates significant difference between samples and controls ($p<0.05$) after each addition of 1,4-dioxane (Table 1). All of the agricultural soils and one contaminated soil indicate more than 50% reduction in 1,4-dioxane concentration after each addition. These experiments are on-going and future research will include another time point for DNA extraction. Following this, the DNA extracts will be submitted for shotgun

sequencing (HiSeq Illumina) to quantify the functional genes associated with 1,4-dioxane degradation in the samples and live controls.

Table 1: 1,4-Dioxane concentrations in the samples and abiotic controls over time.

Inocula	Time (days)	Sample concentration (mg/L)	Control concentration (mg/L)	t.test
Agricultural soil KBS T1	Day 0	12	12	
	Day 50	5.73±0.1	11.28±0.22	0.000
	Day 90	14.68±0.17	12.28±0.07	
	Day 135	6.15±1.68	13.21±1.59	0.006
	Day 181	14.83±1.52	11.13±0.08	
Agricultural soil KBS T2	Day 0	12	12	
	Day 50	5.03±0.47	11.46±0.4	0.000
	Day 90	15.45±0.18	12.64±0.25	
	Day 135	6.95±1.05	12.94±0.26	0.002
	Day 181	16.25±1.5	11.54±0.18	
Agricultural soil F	Day 0	12	12	
	Day 34	6.16±1.05	12.35±0.78	0.001
	Day 60	13.57±0.31	12.19±0.22	
	Day 107	5.28±0.78	12.52±0.15	0.000
	Day 159	12.99±0.18	11.61±0.22	
Agricultural soil G	Day 0	12	12	
	Day 38	5.07±0.48	11.42±0.3	0.000
	Day 60	14.28±0.35	12.55±0.22	
	Day 112	6.25±1.01	12.69±0.15	0.000
	Day 158	13.74±0.36	12.94±0.08	
Contaminated Site 1	Day 0	12	12	
	Day 38	7.24±1.25	12.63±0.72	0.003
	Day 62	14.44±0.62	13.14±0.05	
	Day 107	9.04±1.49	12.39±0.17	0.018
	Day 159	15.07±0.45	11.83±0.2	
Contaminated site 2	Day 0	12	12	
	Day 38	4.71±0.12	12.97±0.61	0.000
	Day 62	14.67±0.71	13.15±0.77	
	Day 107	5.51±1.61	12.79±0.34	0.002
	Day 159	12.84±0.48	12.08±0.15	

Note: Initial concentrations on Day 0 was not measured but an estimate based on 1,4-dioxane added.

Anaerobic Microcosms

The 1,4-dioxane concentrations in the anaerobic microcosms inoculated with the agricultural soils were measured under all four treatments for approximately 300 days. Significant differences between the samples and abiotic controls were observed for two time points for soil E and F under nitrate-amended conditions and under no electron acceptor amendment (Figure 1). No clear trend was noted for soil G with no electron acceptor, however, a clear decrease in concentrations in the samples compared to the controls was noted for soil G under nitrate amended conditions. Both experimental setups (nitrate and no amendment) produced significant quantities of methane. These samples have been re-spiked with 1,4-dioxane and future research will involve continuing to measure1,4-dioxane concentrations. No consistent trends were noted for the sulfate amended microcosms (data not shown). Depletion of 1,4-dioxane was noted in the iron amended samples as well as the abiotic controls, indicating abiotic 1,4-dioxane removal (data not shown).

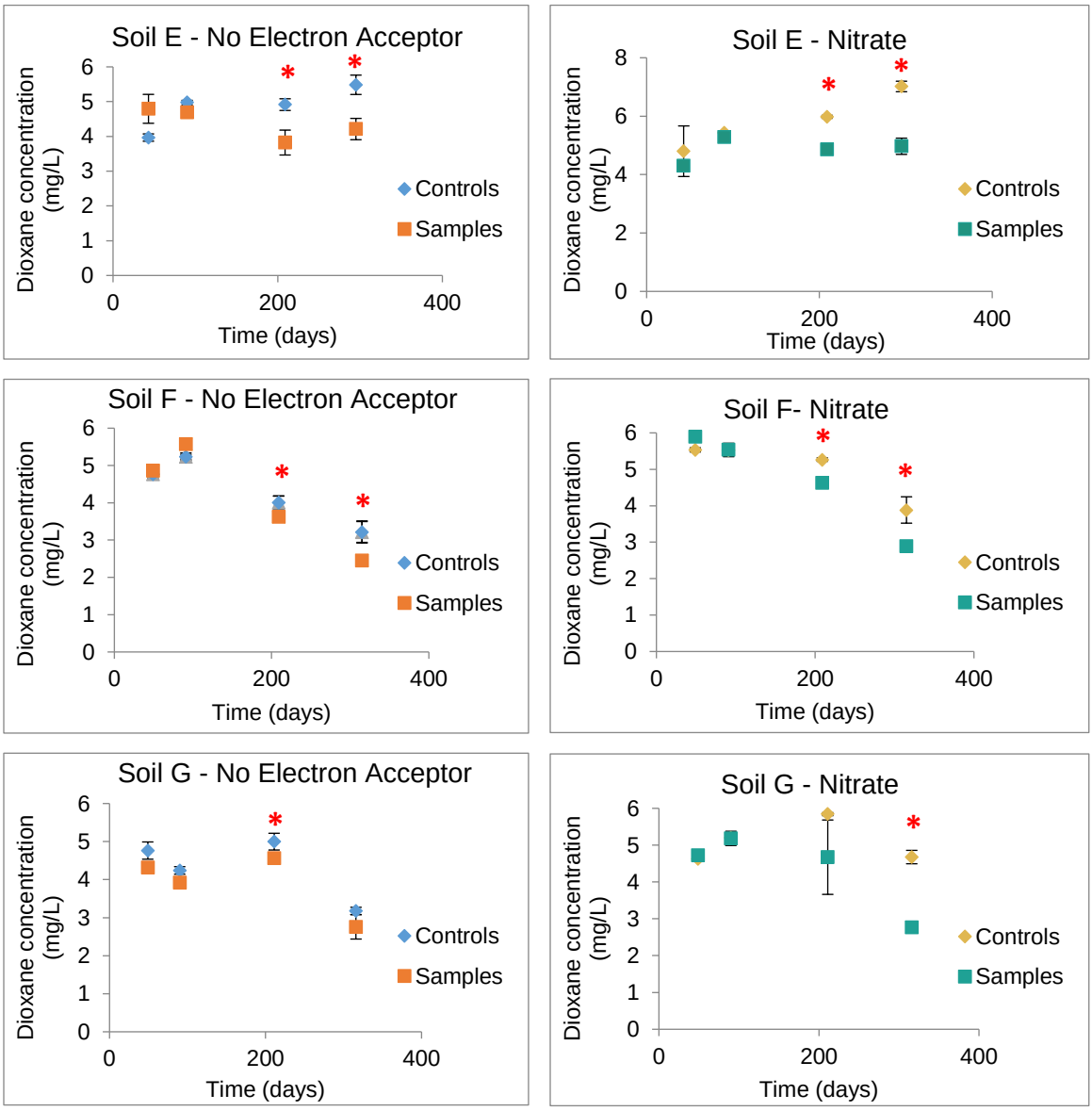

Figure 1. Average 1,4-dioxane concentrations in triplicate microcosms and abiotic controls with inocula from agricultural soils, with no amendment (left) and amended with nitrate (right). The bars represent the standard deviation and the red asterisk indicate significant differences between samples and controls ($p<0.05$).

1,4-Dioxane concentrations in the anaerobic microcosms inoculated with river sediment H were measured for 319 days. A significant decrease ($p<0.05$) was noted in the samples compared to the abiotic controls for the microcosms with no amendments (Figure 2). To date, no clear trends have been observed for the microcosms initially amended with nitrate, iron or sulfate (data not shown). However, future research will continue to monitor these microcosms.

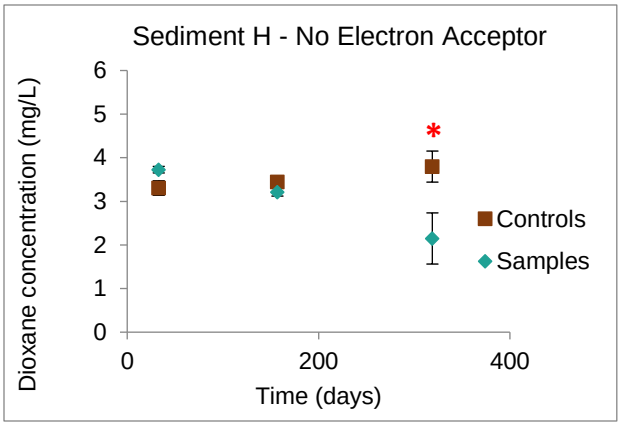

Figure 2. Average 1,4-dioxane concentrations in triplicate microcosms and abiotic controls with inocula from river sediments with no amendment. The bars represent the standard deviation and the red asterisk indicate significant differences between samples and controls.

1,4-Dioxane concentrations in the anaerobic microcosms inoculated with samples from contaminated site 3 (Maine) were measured for approximately 350 days. Both the sulfate amended microcosms and the microcosms with no amendments indicated significant differences in concentrations compared to the controls (Figure 3). Future research will involve continuing to amend these microcosms with 1,4-dioxane to enrich for 1,4-dioxane degraders.

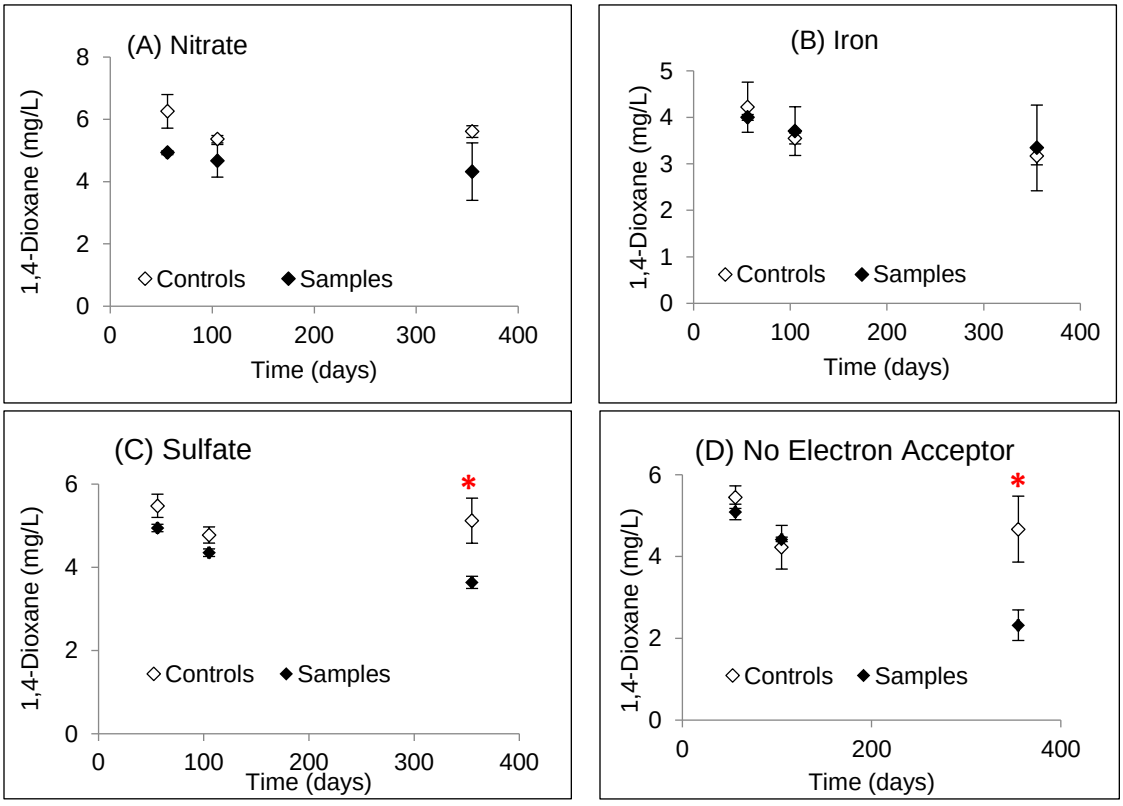

Figure 3. Average 1,4-dioxane concentrations in triplicate microcosms and abiotic controls with inocula from 1,4-dioxane contaminated site 3, amended with nitrate (A), iron (B), sulfate (C) and no amendment (D). The bars represent the standard deviation and the red asterisk indicate significant differences between samples and controls.

The microcosms amended with contaminated site 1 material illustrated smaller reductions in 1,4-dioxane concentrations in the samples compared to the controls compared to the other inoculants (Figure 4). Significant differences in concentrations between the samples and controls were only observed for the last time point in the nitrate amended and no electron acceptor microcosms. Again, we are continuing to monitor these microcosms.

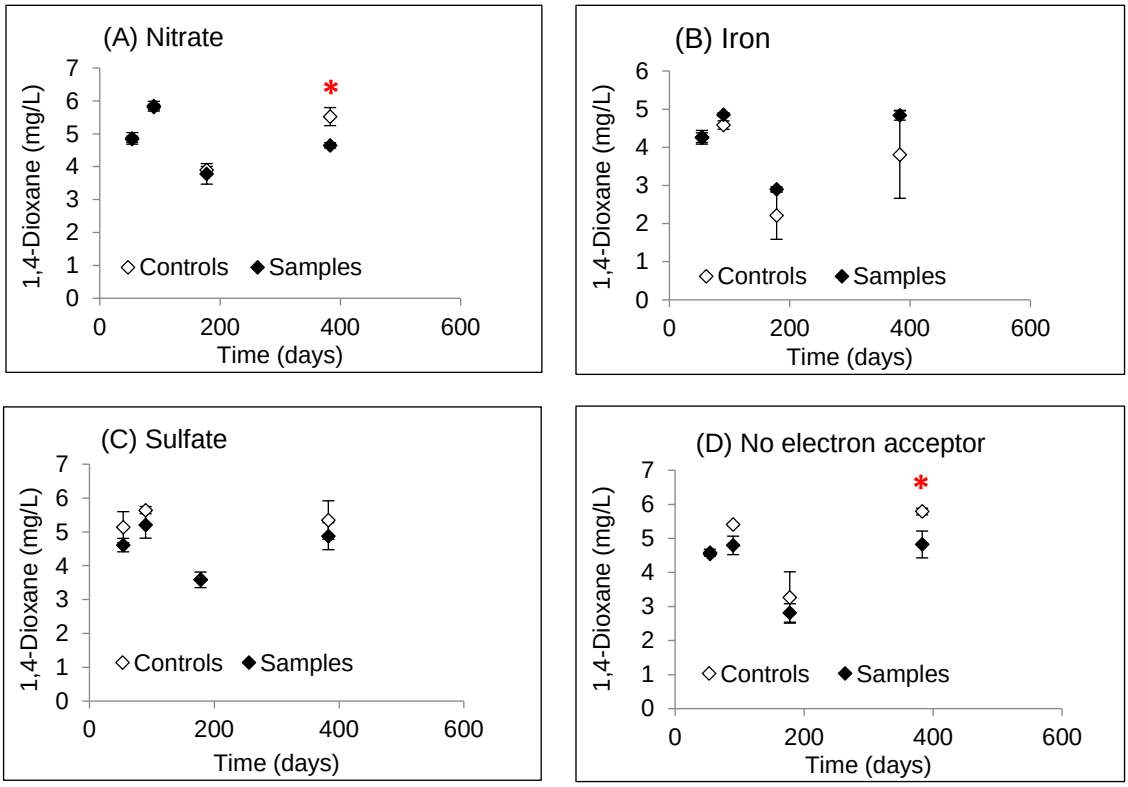

Figure 4. Average 1,4-dioxane concentrations in triplicate microcosms and abiotic controls with inocula from 1,4-dioxane contaminated site 1, amended with nitrate (A), iron (B), sulfate (C) and no amendment (D). The bars represent the standard deviation and the red asterisk indicate significant differences between samples and controls

Compound specific isotope analysis (CSIA) was used to confirm biological degradation in one set of anaerobic 1,4-dioxane degrading microcosms. 1,4-Dioxane degradation should result in more positive $^{13}C/^{12}C$ and $^{2}H/^{1}H$ ratios (or more positive $\delta^{13}C$ and $\delta^{2}H$ values) because bonds involving heavier isotopes are more difficult to break, and so bonds consisting of lighter isotopes are preferentially degraded, causing the residual, non-degraded contaminant to be heavy isotope enriched. Subsamples from three live microcosms and three abiotic controls (at day 450) from the agricultural microcosms (nitrate amended) were sent to UWEIL. This laboratory has already developed the methodology to measure $\delta^{13}C$ and $\delta^{2}H$ values for 1,4-dioxane (Bennett et al., 2018). **As expected, more positive $\delta^{13}C$ and $\delta^{2}H$ values were found in the live samples compared to the controls** (**Figure 5**). The differences were only significant for the $\delta^{13}C$ values. Due to the cost associated with CSIA measurements, future research will focus only on determining $\delta^{13}C$ values

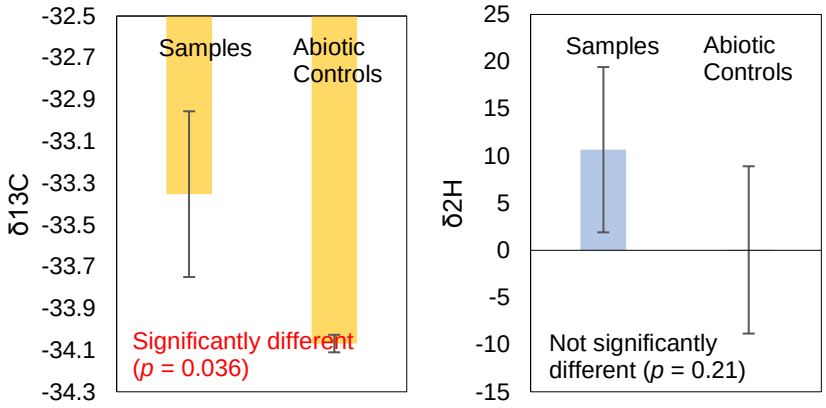

Figure 5. Enrichment of ^{13}C and ^{2}H in agricultural soil F amended with nitrate.

CONCLUSIONS

Significant decreases in 1,4-dioxane concentrations ($p<0.05$) were observed in both aerobic and anaerobic microcosms. The aerobic cultures demonstrate above 50 % removal in most of the agricultural soil microcosms in approximately 40 days. Removal was noted under anaerobic conditions but with longer lag times. The anaerobic removal of 1,4-dioxane was noted past 300 days of incubation in most cases. The CSIA aids in studying the isotopic ratio due to biodegradation since the lighter isotopes ^{12}C gets degraded faster or reacts quicker than the heavier ^{13}C. Hence, biodegradation can cause shifts in the ratio of stable isotopes. Thus, the results from the CSIA analysis further points to anaerobic biodegradation of 1,4-dioxane.

Further anaerobic microcosms have been established under methanogenic conditions with media and sodium lactate to stimulate microbial growth and confirm the biological removal reported here. Also, DNA extraction will be carried out for the aerobic and anaerobic microcosms to identify the dominant microorganisms in the 1,4-dioxane degrading enrichments. Further plans include combining the developed enrichment cultures with the bioaugmentation culture SDC-9 to determine if the chlorinated solvents can be reduced during 1,4-dioxane biodegradation.

Based on these data sets, additional microcosms have been established (no electron acceptor amendment) to provide samples for DNA extraction, high throughput sequencing and microbial community analysis.

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