Indigenous Microbial Communities in Albertan Sediments are Capable of Anaerobic Benzene Biodegradation Under Methanogenic, Sulfate-reducing, Nitrate-reducing, and Iron-Reducing Redox Conditions

Korris Lee[1]*, Ania Ulrich[1].

1 *Department of Civil and Environmental Engineering, University of Alberta, Alberta, Canada.

Author Note

Correspondence concerning this article should be addressed to Korris Lee, Civil and Environmental Engineering, Natural Resources Environmental Facility, University of Alberta, 9105 116 St, Edmonton, Alberta, Canada, T6G 2W2. Email: korris@ualberta.ca

Abstract

Alberta is a major center for oil and gas production, and correspondingly harbors hundreds of unresolved contamination sites by environmental hazards such as benzene (C_6H_6). Due to its cost-effectiveness, bioremediation has become a promising strategy for C_6H_6 removal. Contamination sites typically take on an anaerobic context, which complicates the energetics of contamination sites and is a subject that is scarcely broached in studies of Albertan sediments. This study examines the innate potential for indigenous microbial communities in Albertan sediments to remove C_6H_6 in a multitude of reduced conditions. Community profiles of these sediments were analyzed by 16S rRNA gene amplicon sequencing, and removal rates and reaction stoichiometries were observed by gas chromatography and ion chromatography. Organisms belonging to known primary degrader taxa were identified, including *Geobacter* (iron-reducing), and *Peptococcaceae* (nitrate-reducing). Furthermore, benzene removal patterns of the cultures were similar to those observed in previously reported microcosms, with lag times between 70-168 days and removal rates between 3.27-12.70 μM d^{-1}. Such information could support a more comprehensive survey of Albertan sediment consortia, which may eventually be utilized in informing future remediation efforts in the province.

Keywords: Benzene, Bioremediation, Anaerobic, Alberta, Canada.

Practitioner Points:

- Clay and sand sediments originating from Northern Alberta could remove benzene under methanogenic, sulfate-reducing, iron-reducing, and nitrate-reducing conditions.
- Degradation profiles were broadly comparable to those of reported cultures from other geographical locales.
- Key degrader taxa observed included *Geobacter* (Fe^{3+}-reducing) and *Peptococcaceae* (NO_3^--reducing).

- Knowledge gained can be the start of a more extensive survey of Albertan sediments. Eventually this collection of information can be used to generate robust C_6H_6-degrading cultures that can be implemented for bioaugmentation, and be implemented in informing remediation strategies in soil and water matrices for priority contamination cases such as leaking underground storage tanks and orphan wells.

Introduction

The province of Alberta in the Canadian Prairies is notable for the ubiquitous presence of the oil and gas energy sector (Economic Dashboard - Oil Production), which is a major revenue stream for the provincial economy (Government of Alberta). Consequently, Alberta is replete with petroleum storage units (Government of Canada et al.), pipelines, and orphan wells (Dachis et al.) that pose an imminent environmental risk. These risks are especially elevated with oil-and-gas-related hydrocarbons such as benzene (C_6H_6), a priority contaminant (Canada and Canadà. Health and Welfare...) associated with both natural fossil fuel deposits and anthropogenic processes, and characterized by its singular stability, mobility, and toxicity (McHale et al. 2012), with long-standing links to acute myeloid leukemia (Fishbein 1985). Currently, there are a total of 1265 BTEX (benzene, toluene, ethylbenzene, xylene)-contaminated sites in soil and/or groundwater in the Federal Canadian Contaminated Sites Inventory, 34% of which are active in Alberta (Government of Canada 1994). C_6H_6 contamination events are therefore an immediate public health concern that will require effective engineering solutions to remedy.

Bioremediation shows promise as an unintrusive, cost-effective alternative to conventional physicochemical removal approaches (Azubuike et al. 2016), and previous scholarship has reported enrichment cultures capable of degrading C_6H_6 (Keller et al. 2018; Rabus et al. 2016; Fuchs et al. 2011; Philipp and Schink 2012). Importantly, although aerobic

pathways have been characterized, field site conditions regularly demonstrate a tendency towards highly-reduced conditions (Weelink et al. 2010), and it is therefore the anaerobic removal of C_6H_6 that deserves particular attention. In contrast to the regular presence of C_6H_6 in Alberta's energy sector, little is known about the indigenous microbiota of Alberta and its ability to remediate C_6H_6. Obtaining natively-derived anaerobic cultures that can attenuate C_6H_6 contaminants has the potential to benefit remediation studies in a province of which C_6H_6 contamination is of critical importance.

Hydrocarbon degradation typically proceeds according to redox potential values in decreasing order and according to the availability of terminal electron acceptors (Abbasian et al. 2015), which can have dramatic effects on microbe population profiles and degradation pathways. Anaerobic benzene degradation has previously been demonstrated in various redox contexts (Rabus et al. 2016; Fuchs et al. 2011; Philipp and Schink 2012), with evidence for multiple pathways of benzene ring activation, including: methylation into toluene, hydroxylation into phenol, and carboxylation into benzoate (Meckenstock et al. 2016). NO_3^--reducing cultures have been reported, with the primary oxidizer being identified as a *Peptococcaceae* (van der Zaan et al. 2012), a *Dechloromonas* (Coates et al. 2001), or an *Azoarcus* (Kasai et al. 2006), although the latter has not been seen to host any canonical degradation genes (Devanadera et al. 2019). A *Peptococcaceae*-containing culture known as the Cartwright culture has previously been seen to have a consistent degradation rate of 5.00-10.00 μM d^{-1} (Luo et al. 2014). Fe^{3+}-reducing cultures have also been reported, with the primary degrader being identified as *Geobacter metallireducens* (Zhang et al. 2012; Abu Laban et al. 2010), with degradation rates of such cultures measured at 6.00 μM d^{-1} (Kunapuli et al. 2007). SO_4^{2-}-reducing cultures exist as well, which have separately demonstrated degradation rates of 36.00 μM d^{-1} (Kleinsteuber et al. 2008) and lag times of about 200 days post-benzene dispersion (Taubert et al. 2012); these

cultures have primary degraders of the *Clostridiales* (Kleinsteuber et al. 2008) and *Cryptanaerobacter/Pelotomaculum* (Taubert et al. 2012) taxa, respectively. Methanogenic cultures have shown primary degraders from the *Deltaproteobacteria* (Luo et al. 2016) and the *Desulfobacterales* and *Coriobacteriaceae* (Luo et al. 2016; Noguchi et al. 2014), with demonstrated degradation rates of 1.40-25.00 μM d^{-1} (Luo et al. 2016). Although understanding of anaerobic benzene biodegradation continues to expand globally, information on benzene-degrading consortia native to Alberta remains limited, in contrast to the ubiquity of the province's oil and gas ventures and their associated contamination risk factors. Such knowledge of native benzene-degrading consortia can help inform engineering remediation strategies, by determining the viability of natural attenuation, predicting degradation rates, suggesting biostimulatory tactics, and opening up the possibility of bioaugmentation with lab-cultivated material. The logistical and technical challenges in acquiring this body of knowledge are many, due to the general metabolic lethargy in anaerobic cultures and the fastidious syntrophy of benzene-degrading mixed cultures (Vogt et al. 2011).

Therefore, this study examines the ability of intrinsic microbial communities in uncontaminated Albertan sediments to initiate C_6H_6 removal, the aim of which is to establish the challenges inherent to anaerobic benzene-degrading culture maintenance and show the history of these cultures as a contribution to the scholarship. This information is intended to initiate a more comprehensive study of Albertan sediments that will eventually allow remediation specialists to more effectively assess and monitor bioremediation of contaminated sites in the province, and to ultimately generate cultures capable of robust biodegradation for bioaugmentation efforts. The sediments under study were collected from a location near an oil-sands mine-lease site in the northeastern Athabasca region (56°58'40.1"N 111°28'56.3"W) in Alberta. Two sediment types were gathered from clay (10 mbgs) and sand (25 mbgs) horizons

of this site and cultured anaerobically as previously described (Holden et al. 2011) with C_6H_6 enrichments and in the presence of CO_2, SO_4^{2-}, NO_3^-, or Fe^{3+} serving as TEAs (terminal electron acceptors). C_6H_6, CH_4, SO_4^{2-}, NO_3^-, NO_2^-, and Fe^{2+} levels were monitored over the course of the study, and microbial consortia were analyzed by NGS (next generation sequencing). As described above, both *Peptococcaceae* and *Geobacter metallireducens* appear to be relatively common primary degraders, and should be expected in Albertan NO_3^- and Fe^{3+}-reducing cultures, respectively. Other redox conditions have more diverse primary degrading taxa, and methanogenic and SO_4^{2-}-reducing cultures are therefore less predictable in regards to metabolic activity and the identity of their primary degrading organisms, although their removal rates should be expected to follow redox potential patterns (Silva et al. 2005; Vogt et al. 2011).

Methodology

Culture Source and Enrichment

Microcosm inoculum material was collected at clay and sand horizons adjacent to the South Tailings Pond in Northern Alberta, at 10 and 25 mbgs, respectively (Holden et al. 2011). These sediments are predominantly composed of illite and kaolinite (Jansen, A.T., Sharma, J.S., Barbour, ...; Holden et al. 2011), with aluminum, iron, manganese, silicon, and strontium detected as trace minerals in groundwater samples at transect monitoring wells on-site (Ulrich, A.C., Donahue, R., Biggar, K....). Clay sediments had a cation exchange capacity of 15.5 meq/100 g, a specific gravity of 2.643 at 20℃, and an organic content fraction of 0.986%. Sand sediments had a cation exchange capacity of 5.59 meq/100 g, and specific gravity of 2.733 at 20℃ (Holden et al. 2011). Sealed sediments were frozen at -20℃ for storage, thawed in an anaerobic chamber (Coy Laboratory Products, Madison, WI) (atmosphere 5/5/90% v/v/v H_2/CO2/N2), transferred to sterile, anaerobically-acclimated 250 mL glass vials, and infused with 150 mL of previously-defined anaerobic mineral media (Edwards and Grbić-Galić 1994).

Microcosms were amended as appropriate to establish methanogenic (n/a), sulfate-reducing (15 mM SO_4^{2-}), iron-reducing (2% v/v insoluble Fh ($(Fe^{3+})_2O_3$•$0.5H_2O$, ferrihydrite)), and nitrate-reducing (3 mM NO_3^-) conditions. A suite of eight microcosms was established: MC, MS, SC, SS, IC, IS, NC, and NS, with bi-letter designations for, firstly, redox conditions (M = methanogenic, S = sulfate-reducing, I = iron-reducing, N = nitrate-reducing) and secondly, sediment type (C = clay, S = sand).

Bottles were sealed with Mininert™ caps and separately amended with neat C_6H_6 via a 10 µL glass syringe (Hamilton Company, USA) to an expected final liquid concentration of 280 µM. (Edwards and Grbić-Galić 1994). A dimensionless Henry's law constant of 0.183 was used to calculate liquid concentrations (Peng and Wan 1997), and equilibrium (280±0.7 µM) was reached within 24 h of C_6H_6 dispersal (Supplemental 1). Concentrations of C_6H_6 and TEA were analyzed regularly and amendments were performed periodically to maintain metabolic activity. All manipulations were performed under anaerobic conditions, and all microcosms were stored in an anaerobic atmosphere with light-exclusion at 25°C. Microcosms were enriched for 260 days over the course of the study.

A separate 50 mL sample of culture, designated as a heat-sterilized control, was transferred to a 125 mL serum bottle, capped with a butyl stopper, and sealed with an aluminum crimp. The serum bottle then underwent heat-sterilization with three separate 90 min liquid autoclave cycles and was supplemented with neat C_6H_6 to a liquid concentration of 280 µM. Equilibrium (280±0.7 µM) was reached within 24 h of C_6H_6 dispersal (Supplemental 1). Over the course of 108 days there was a 5.87 nM d^{-1} change in C_6H_6 in the killed control sample (Table 1) (Supplemental 1). The biletter designation of HK denoted the heat-killed control.

C_6H_6 and CH_4 Analysis

C_6H_6 was quantified by headspace analysis using an Agilent™ 7890A equipped with a flame ionization detector and an HP-1 methylsiloxane (30 m x 320 µm x 0.25 µm) column. The injector was set to 17.735 psi with septum purge flow at 3 mL min^{-1}, the column was set at 350℃, 4.5 mL min^{-1}, the oven was set at 50℃, 4.5 min, and the detector was set at 250℃, 45 mL min^{-1} hydrogen, 450 mL min^{-1} air, and 25 mL min^{-1} helium.

CH_4 was quantified by headspace analysis using an Agilent™ 7890A equipped with a flame ionization detector and an HP-1 methylsiloxane (30 m x 320 µm x 0.25 µm) column. The injector was set to 17.735 psi with septum purge flow at 3 mL min^{-1}, the column was set at 350℃, 4.8 mL min^{-1}, the oven was set at 50℃, 8 min, and the detector was set at 250℃, 40 mL min^{-1} hydrogen, 450 mL min^{-1} air, and 23 mL min^{-1} helium.

Single injections were performed using a 500 µL manual injection volume extracted from the headspace under anaerobic conditions with gas-locking syringes (Sigma-Aldritch™ VICI Series A-2). The error for C_6H_6 concentration measurements was ±6.24%. Calibration was performed with external standards and the detection limit was 0.01 mM C_6H_6 and 0.2% CH_4. Daily removal rates (µM d^{-1}) were determined by calculation of overall removal over days 117-260 of culturing i.e. (total [C_6H_6] removed (µM)/(143 d). Total cumulative C_6H_6 removal was calculated by the summation of removal cycles (defined as the difference in concentrations immediately adjacent to feeding events, and excluding intervening data points). Statistical significance in the differences between removal cycles between experimental samples and controls were estimated with Student's t-test in Excel™ Analysis Toolpak, and deemed significant upon presentation of p-values < 0.05. Sampling was performed approximately once every 30 d for methanogenic cultures and once every 7 d for SO_4^{2-}, Fe^{3+}, and NO_3^--reducing cultures.

TEA and Byproduct Analysis

SO_4^{2-}, NO_3^-, and NO_2^- concentrations were quantified by 100 µL injections of 0.2 µm-filtered, 50X-diluted liquid samples into a Dionex™ ICS-2100 ion chromatographer with an IonPac™ AS18 IC column. The eluent flowrate was 0.25 mL min^{-1} with 10-32 mM KOH, the suppressor was a 2 mm ASRS set at 20 mA, the conductivity detector was set at 35°C, and the background conductance was < 1 µS with a backpressure of ~ 2000 psi. Calibration was performed with external standards and the detection limit was 2.5 mg L^{-1} SO_4^{2-}, NO_3^-, or NO_2^-. Iron was quantified by the ferrozine assay as described previously (Lovley and Phillips 1986), with a detection limit of 0.001 M.

16S rRNA Community Analysis

Regular acquisition of 1 mL culture for TEA analysis coincided with harvesting for DNA extraction at 260 days. Samples were centrifuged at 8000 xg for 3 min, and DNA extraction on collected biomass was performed with FastDNA™ kits. Primary PCR of the V3-V4 region of the 16S rRNA gene was performed by Phusion™ polymerases using the following oligonucleotides, specific for *Archaea* and *Bacteria*: 16S rRNA V3-4 IL-F (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG) and 16S rRNA V3-4 IL-R (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C). PCR cleanup was performed by QIAEX™ kits (Qiagen™), and prepared samples were delivered to the DNA core at the University of British Columbia for Illumina-MiSeq™ analysis. All kit-based techniques were performed as directed by the manufacturer. Raw fastq file reads were processed by the Mothur pipeline for 16S rRNA community analysis, with DNA library preparation and analytic workflows adapted from previously-developed toolkits and protocols (Schloss et al. 2009). A cutoff threshold identity of 97% was used in analyses, and alpha diversity was calculated by Shannon indices. Raw sequence reads were submitted to the NCBI database, and the data are openly available at https://www.ncbi.nlm.nih.gov, Accession No. PRJNA608926. To generate a

phylogenetic analytic of primary degraders, selected sequences (Noguchi et al. 2014; Alfreider and Vogt 2007; Sakai et al. 2009; Luo et al. 2016; Musat and Widdel 2008; Oka et al. 2008; Zhang et al. 2012; Bedard et al. 2007; Abu Laban et al. 2009; van der Zaan et al. 2012; Luo et al. 2014) were imported into Mega7 (Kumar et al. 2016; Gatesy 2002), aligned via the default ClustalW algorithm parameters (Kumar et al. 2016), and input into a maximum-likelihood tree construction tool using the Tamurai-Nei model (Page and Holmes 2009; Kumar et al. 2016; Swofford DL, Olsen GJ, Waddell PJ & H...).

Results and Discussion

Removal rate and stoichiometry

C_6H_6 removal under all redox conditions and sediment types was observed through multiple removal cycles (Table.1.), with the highest rates visible in the Fe^{3+}-reducing (IC/IS), and NO_3^--reducing (NC/NS) microcosms (11.20-12.70 μM d^{-1}), and the lowest rates visible in the methanogenic (MC/MS) and SO_4^{2-}-reducing (SC/SS) microcosms (3.27-8.14 μM d^{-1}). This is in agreement with energetics predicted by redox potentials (Silva et al. 2005; Vogt et al. 2011). The high removal rates of Fe^{3+}-reducing cultures may be due to the high redox energy potential of Fe^{3+} (ΔE^0 = +36 V) (Silva et al. 2005; Lueders 2017) as a terminal electron acceptor, as well as the low toxicity of Fe^{2+}, which would not interfere with further C_6H_6 removal in the closed system except at very high levels. This is in contrast with NO_3^--reducing and SO_4^{2-}-reducing cultures, which can generate NO_2^- and H_2S compounds that can interfere with culture activity (Silva et al. 2005). Previous scholarship suggests a wide range of NO_2^- tolerance in various cultures, but significant inhibition of C_6H_6 metabolism has been reported at levels of 5-7 mM NO_2^- (Gitiafroz 2012).

Compared to cultures elsewhere, the rates of NC and NS (11.32-12.20 μM d^{-1}) (Table.1.) were highly similar to previously reported values of 10.00 μM d^{-1} (Luo et al. 2014), whereas the

rates of IC and IS (11.20-12.70 μM d^{-1}), were about twice as high as those of previously reported cultures (Kunapuli et al. 2007). The IC and IS cultures, however, had been enriched for 260 days, while the microcosms described in previous studies had been enriched for only 120 days (Kunapuli et al. 2007). Enrichment correlates with redox specialization (Winderl et al. 2008) and enhanced removal, so the extra culturing time may account for the difference in activity. This may also explain the low rates of SC/SS when compared to well-studied cultures derived from a German aquifer (Taubert et al. 2012) (Table.1.), which had been under continuous cultivation for multiple years. Both iron-reducing and nitrate-reducing microcosms exhibited removal cycles that were significantly different from the heat-killed control.

Stoichiometry ratios indicated that C_6H_6 oxidation occurred at varying degrees within tested redox conditions. Fe^{3+}-reducing microcosm (IC/IS) stoichiometric values only reached approximately 50-75% that of theoretical stoichiometric values (30:1 mol Fe(II):mol C_6H_6) (Vogt et al. 2011) - this may be due to the low bioavailability of amorphous iron, resulting in concurrent degradation of C_6H_6 by methanogenesis, as well as precipitation of ferrous iron (Villatoro-Monzón et al. 2008). Additionally, methanogenic cultures (MC/MS) demonstrated approximately 37% fidelity to theoretical (3.75:1 mol CH_4:mol C_6H_6) stoichiometries; the cause of this discrepancy is unknown. SO_4^{2-} and NO_3^--reducing redox conditions demonstrated similar stoichiometric comparisons to theoretical values (Table.1.), suggesting that the appropriate TEA in NO_3^- and SO_4^{2-}-reducing microcosms was being consumed during the oxidation of C_6H_6. NO_3^- reduction stoichiometries are particularly notable because their values are intermediate between full and partial denitrification (Vogt et al. 2011). Combined with the presence of low quantities of NO_2^- (Figure 1C, 4C), these observations suggested that incomplete reduction of NO_3^- had taken place in the NC and NS cultures. The conformity of empirical stoichiometry ratios to theoretical values is a helpful indicator for the nature and energetics of reactions occurring both

in vitro and *in situ*. These metrics can predict the generation of chemical byproducts, toxic (NO_2^-/H_2S) or otherwise, and can be used to track or alter the progression and kinetics of benzene degradation at a contamination site.

Lag periods were observed in all microcosms, in which no change in C_6H_6 concentrations occurred post-dispersal. This lag was most prominent in methanogenic and SO_4^{2-}-reducing cultures. This is consistent with the low redox potential of both TEAs, and with the long doubling times of previously-reported methanogenic key degraders like *Deltaproteobacteria* ORM2, estimated to be approximately 34±3.0 d (Luo et al. 2016). The lag period was also similar to previously-described SO_4^{2-}-reducing cultures that exhibited lag times of 200 d (Taubert et al. 2012). The long lag time of these cultures was in contrast to Fe^{3+} and NO_3^--reducing microcosms, both of which have higher redox potentials and exhibited lag times similar to previous Fe^{3+}-reducing cultures, at 87-122 days (Lovley et al. 1994). Additionally, MS had a leak event between 117-163 days (Figure 2) which was not included in degradation and stoichiometry calculations. All microcosms other than the MC/MS cultures exhibited decreased removal rates at 183-203 days; this coincided with a dilution event of 15% v/v anaerobic mineral media that occurred at that time in the IC/IS, NC/NS, and SC/SS cultures, and demonstrated the importance of cell concentrations in maintaining robust C_6H_6 catabolism, and the potential negative consequences of diluting culturing volumes. Remediation doctrine for natural attenuation often assumes that the absence of contaminant removal is evidence for the absence of degradative microorganisms; however, a lack of activity may merely indicate that degrading population concentrations are too low to sustain removal (Meckenstock et al. 2015).

Microbial Community Analysis and Quantification

Members of known anaerobic primary C_6H_6 degrader taxa were discovered in cultivated microcosms that reduced ferric iron and nitrate, with similar Shannon index metrics and

coverage exceeding 99% in all samples (Figure 3, Figure 4, Figure 5). MC and MS microcosms hosted organisms of the *Deltaproteobacteria*, mainly of the *Desulfomicrobiaceae* (4.14%) and *Desulfuromonadaceae* (2.94%) family, respectively. However, these were not the same organisms as those found in previous scholarship (Luo et al. 2016). SC and SS cultures contained members of the *Clostridiales* (0.60 and 4.59% read instances, respectively), but again, these were not the same as those found in previous scholarship (Abu Laban et al. 2009). Another known primary degrader in SO_4^{2-}-reducing conditions is *Pelotomaculum* (Abu Laban et al. 2009), thought to have decarboxylase activity, but this taxa was not present in the SC consortium. IC and IS cultures hosted members of the *Geobacteraceae* (6.61 and 2.02% read instances, respectively), while NC and NS cultures contained *Peptococcaceae* organisms (57.8% and 1.13% read instances, respectively). Those *Peptococcaceae* in NC cultures had identical 16S rRNA sequences as those found in previous scholarship (Luo et al. 2014), although the *Peptococcaceae* in NS cultures did not. The presence of *Peptococcaceae* in multiple cultures described worldwide (Abu Laban et al. 2010) illustrated the extent of the ubiquity of these organisms.

Auxiliary taxa were identified as well. Fe^{3+}-reducing cultures contained known methanogens such as *Methanosarcinaceae* (1.76-5.64%) (Balch et al. 1979) (Figure 3, Figure 4), perhaps indicative of methanogenesis occurring alongside Fe^{3+} reduction. The incongruence between empirical and theoretical stoichiometries (Table.1.) in these microcosms further suggests that methanogenesis may be taking place concurrently within the sediment microniches of these cultures. In addition, the presence of *Hydrogenophilaceae* (2.87-16.56%) in multiple cultures, including non-methanogenic ones, may be an artifact resulting from an infiltration of H_2 from the glovebox atmosphere into the sample bottles. The presence of H_2 in anaerobic chambers is a common practice necessary for the scavenging of micro-quantities of

O_2 over a Pd catalyst, which maintains a strict anaerobic atmosphere. The sand-based cultures IS, NS and SS (Figure 4.) also contained a notable population of another taxon present in previously-reported NO_3^-/SO_4^{2-}-reducing cultures, namely *Anaerolineae* (2.01-2.48%) (Keller et al. 2018), suggesting a sand-specific habitat for this organism, but more analysis remains to be done in order to identify important syntrophic relationships between primary degraders and auxiliary organisms.

The identification of these taxa suggests a worldwide distribution of primary benzene degraders *Geobacter* and *Peptococcaceae* and hints that such organisms can be utilized as biomarker candidates for field sites that can indicate a positive prognosis for natural attenuation or advocate for biostimulatory introductions of certain TEAs like Fe(III) and NO_3^-. Although primary degraders were positively identified in iron-reducing (clay and sand) and nitrate-reducing (clay) cultures, more research has to be done in order to confirm primary degrader identities in other redox conditions and sediments. Furthermore, these data on candidate organisms can be used as a starting point for modeling reaction kinetics, and determining population threshold values and their correlation to removal rates.

Conclusions

Anaerobic culture enrichment was successful in establishing the ability of native Albertan sediments to remove C_6H_6 under methanogenic, SO_4^{2-}-reducing, Fe^{3+}-reducing, and NO_3^--reducing redox conditions, at rates (3.27-12.70 μM d^{-1}) comparable to known C_6H_6-degrading cultures from previously-reported systems (Aburto-Medina and Ball 2015). Lag periods were also determined, ranging from 77-168 d, establishing broad temporal guidelines based on ideal conditions for laboratory cultivators to set timelines required to observe degradation in batch experiments and according to a variety of redox environments.

These redox reactions were confirmed stoichiometrically, particularly in SO_4^{2-}-reducing cultures, and revealed incomplete denitrification reactions in NO_3^--reducing cultures. Consequently, the expected accumulation of H_2S or NO_2^- in these environments may be deleterious for attenuations operating under these redox conditions, and the toxicity of these metabolites may require redress in engineered remediation programs, perhaps by the introduction of ferrous salts to react with sulfide (Firer et al. 2008) or by coincubation with ammonium oxidizers that consume nitrite (Peng et al. 2017). Stoichiometric values of iron-containing cultures also suggested a subset of reactions unrelated to Fe^{3+}-reduction, suggesting that the bioavailability of the excess amorphous iron is limited and that concurrent methanogenic reactions may be taking place. Cultivation plans may therefore need to remain cognizant that benzene-oxidizing reactions reliant on Fe^{3+}-reduction may be complicated by incomplete exploitation of ferric iron, even when the required iron oxides are in excess.

In regards to the microbial community of these sediments, the presence of notable primary degrader taxa was established, including *Geobacter* (Fe^{3+}-reducing) and *Peptococcaceae* (NO_3^--reducing), in both clay and sand-based cultures.

These data can ultimately be valuable for helping to define C_6H_6-contaminated environments in Alberta, a province in which such data are highly relevant. Observed lag times and degradation rates serve as convenient guides for laboratory cultivation in regards to forecasting degradation endpoints from *ex situ* sediments, and observed stoichiometries are useful for researchers to confirm energetics and anticipate byproduct generation for new laboratory cultures undergoing establishment. Observed key degrader taxa (*Geobacter* and *Peptococcaceae*) can be further investigated for their candidacy as biomarker populations, the presence and quantity of which can be used to predict contaminant removal rates. Further research may also include work on co-contaminants which inhibit benzene degradation

(Edwards et al. 1992; Cunningham et al. 2001; Da Silva and Alvarez 2007) such as EtOH, a common additive in gasoline, which can produce a co-solvent effect that enhances benzene solubility in water (Silva et al. 2002; Powers et al. 2001; Ruiz-Aguilar et al. 2002), resulting in complications in remediation due to the increased expanse of the plume.

These studies were constrained by the logistics of anaerobic culturing which allowed for a limited volume of culture. As such, reproducibility is achieved through repeated removal cycles within each culture as opposed to replication of cultures; well-established cultures require years to decades of cultivation(Taubert et al. 2012; Luo et al. 2016; Luo et al. 2014), and the acknowledgement of these timelines compelled sharing of these data, such that researchers and industrial partners can appreciate the complications involved. This work is therefore a starting point for the increased surveying of Albertan sediments; such a body of data can move towards the continued development and enrichment of anaerobic benzene cultures, with an aim to ultimately produce cultures for more active implementation through bioaugmentation.

Acknowledgements

Thanks to NSERC Discovery for financially supporting this research, and to Edwards Lab (University of Toronto) personnel for their assistance in anaerobic microcosm cultivation.

Table 1

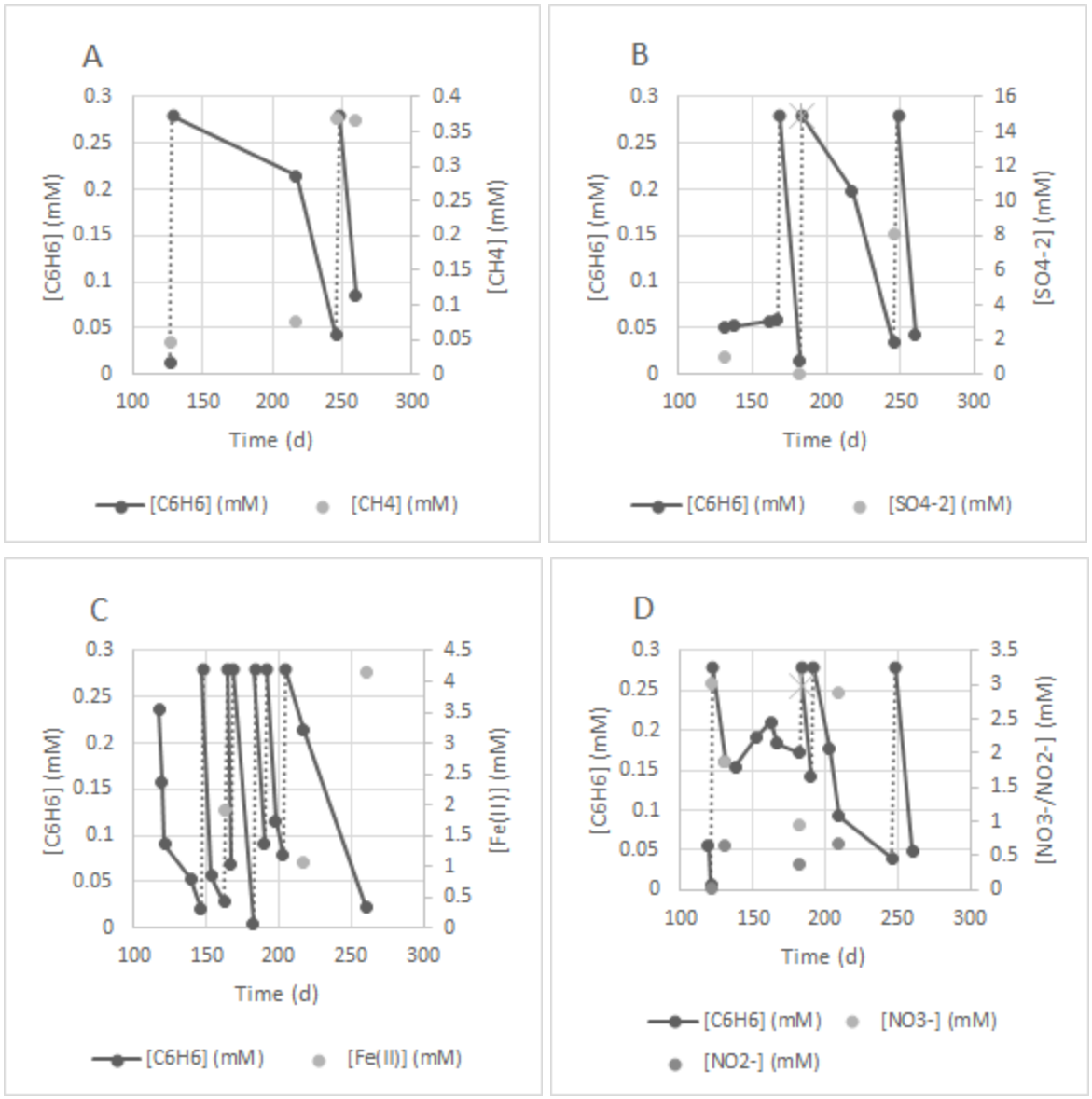
Degradation Rates and Stoichiometry of Enrichment Cultures.

Microcosm	Lag period (d)	Removal Rate (μM d^{-1})		Stoichiometry (mol:mol TEA consumed/byproduct generated:C_6H_6)	
		Literature	Empirical	Theoretical	Empirical
MC	128	1.40-25.00 (Luo et al. 2016)	3.27	3.75:1	1.35:1
MS	147		5.08		1.58:1
SC	168	36.00 (Taubert et al. 2012)	8.14	3.75:1	3.85:1
SS	117		6.55		4.07:1
IC	80	6.00 (Kunapuli et al. 2007)	11.20*	30:1	16.10:1
IS	89		12.70*		22.72:1
NC	80	10.00 (Luo et al. 2014)	11.32*	6/15:1	9.63:1
NS	77		12.20*		7.66:1
HK	n/a	n/a	0.05	n/a	n/a

Rates and stoichiometries were calculated from overall degradation over days 117-260 of culturing i.e. (total [C_6H_6] degraded (μM)/(143 d) (Figure 1-4.) and compared to theoretical values (Vogt et al. 2011). Theoretical ratios for NO_3^--reducing reactions are based on either complete ($NO_3^- \rightarrow N_2$, 6:1 mol:mol NO_3^-:C_6H_6) or incomplete ($NO_3^- \rightarrow NO_2^-$, 15:1 mol:mol NO_3^-:C_6H_6) denitrification (Vogt et al. 2011). * = statistical significance (p-value < 0.05).

Figure 1

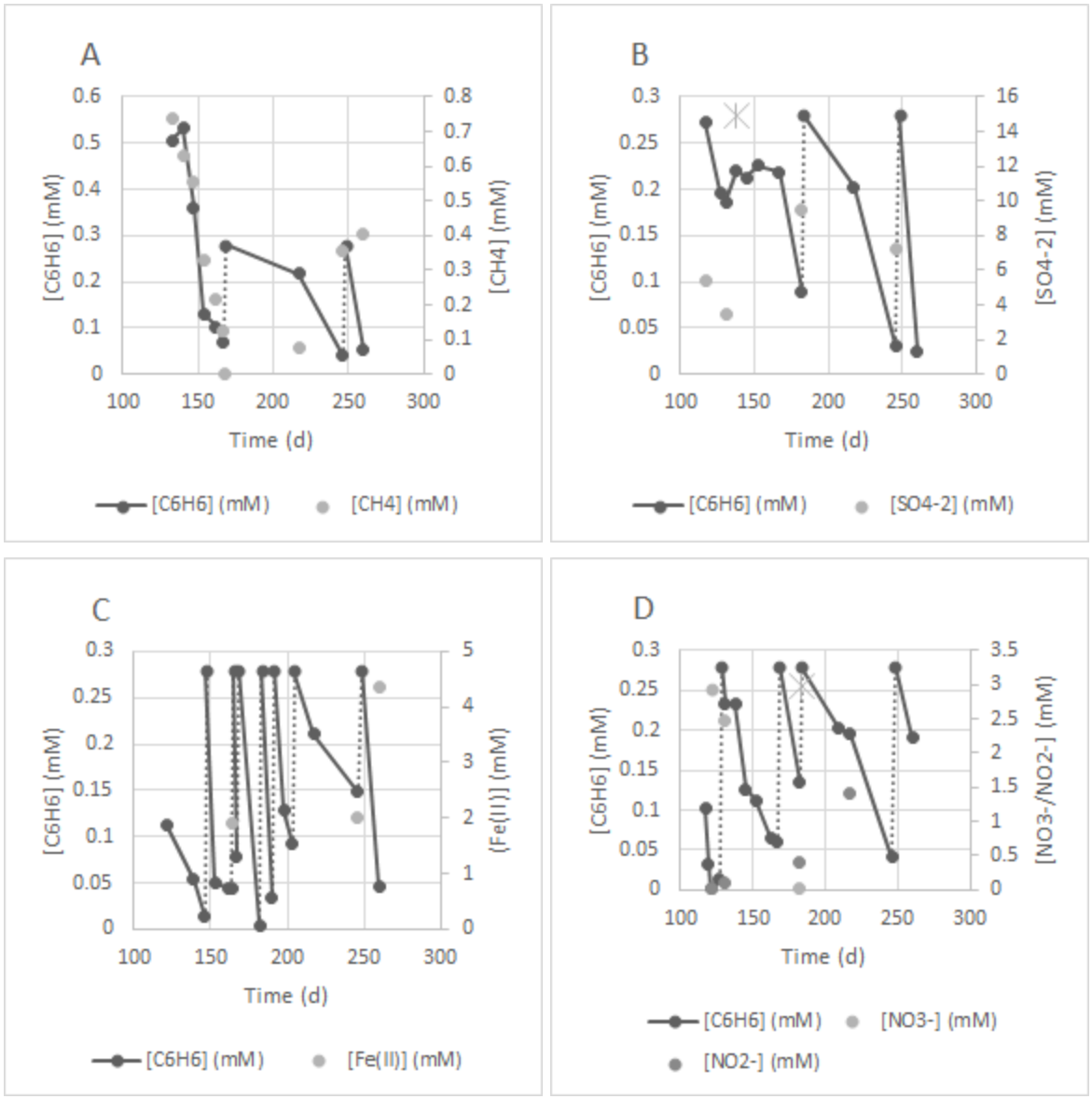
C_6H_6 Degradation and TEA Depletion/byproduct Generation of Clay-based cultures.



Enrichment cultures were maintained in anaerobic conditions, and C_6H_6 and CH_4 concentrations were monitored by headspace GC analysis. TEA depletion was measured by anion-exchange IC, and Fe^{2+} generation was measured by ferrozine assay. A = MC (methanogenic), B = SC (SO_4^{2-}-reducing), C = IC (Fe^{3+}-reducing), , D = NC (NO_3^--reducing). A 15% v/v dilution of anaerobic mineral media was performed at 183-200 days in all cultures except for MC. C_6H_6 feeding events are represented by dotted lines and TEA feeding events are represented by asterisks (*). Data points represent single measurements.

Figure 2

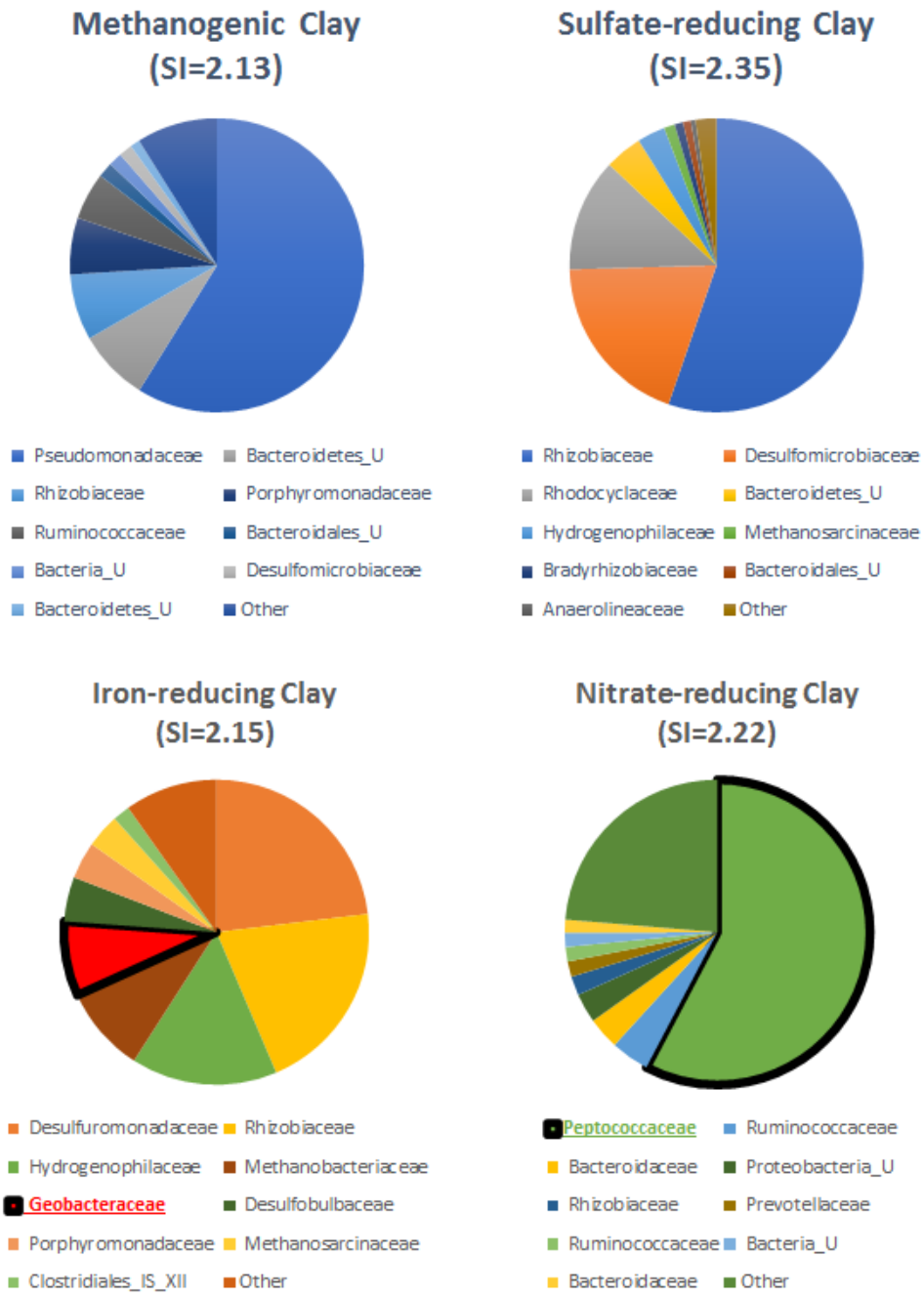
C_6H_6 Degradation and TEA Depletion/byproduct Generation of Sand-based Cultures.



Enrichment cultures were maintained in anaerobic conditions, and C_6H_6 and CH_4 concentrations were monitored by headspace GC analysis. TEA depletion was measured by anion-exchange IC, Fe^{2+} generation was measured by ferrozine assay. A = IS (Fe^{3+}-reducing), B = MS (methanogenic), C = NS (NO_3^--reducing), D = SS (SO_4^{2-}-reducing). A 15% v/v dilution of anaerobic mineral media was performed at 183-200 days in all cultures except for MS. C_6H_6 feeding events are represented by dotted lines and TEA feeding events are represented by asterisks (*). Data points represent single measurements.

Figure 3

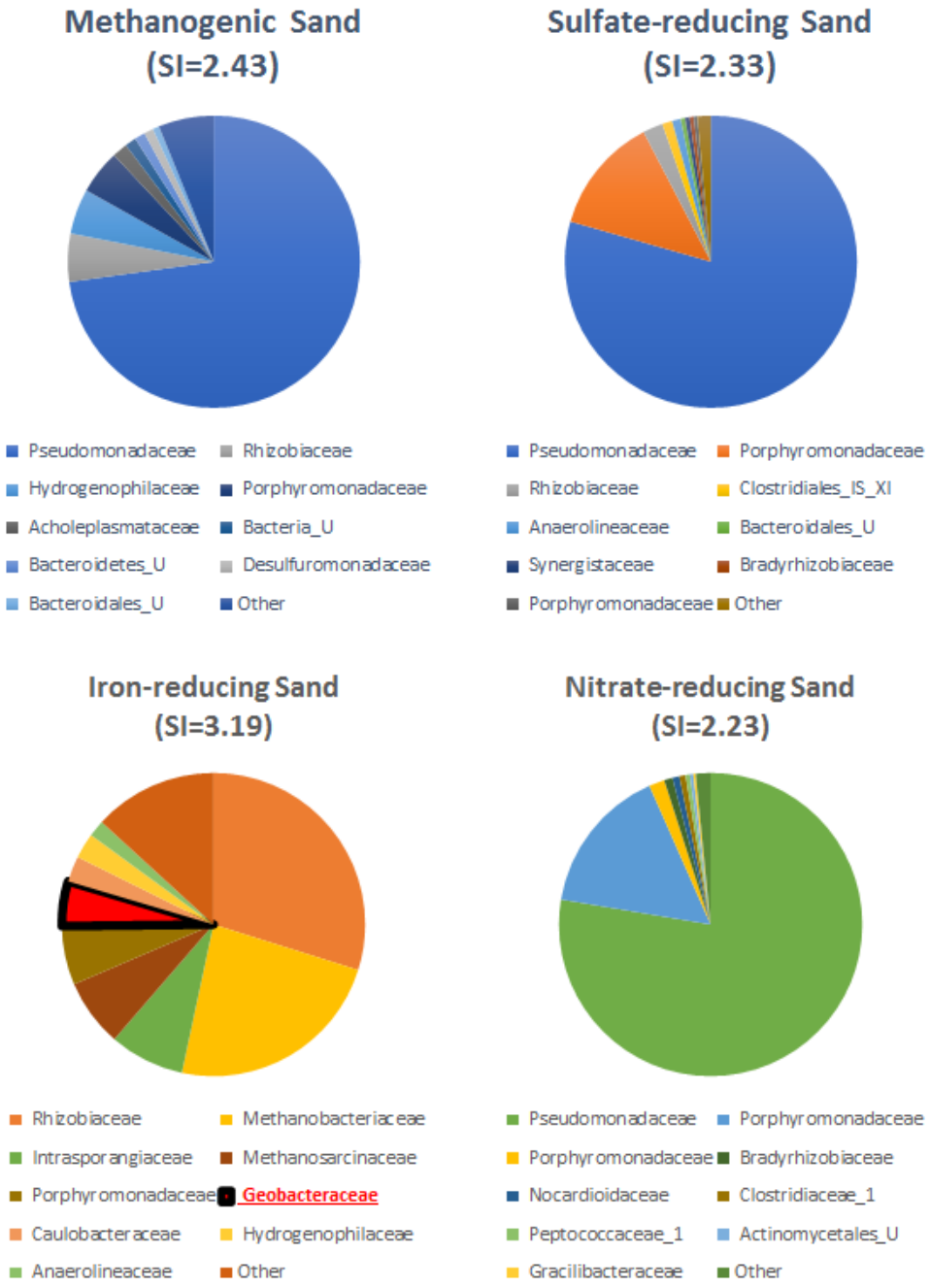
16S rRNA Community of Clay-based Microcosms MC, SC, IC, NC.



The top 9 OTUs (operational taxonomic units) at day 260 are shown in community profile at the family taxon level, in order of abundance with 1° degraders in bold. U = unclassified, SI = Shannon Index.

Figure 4

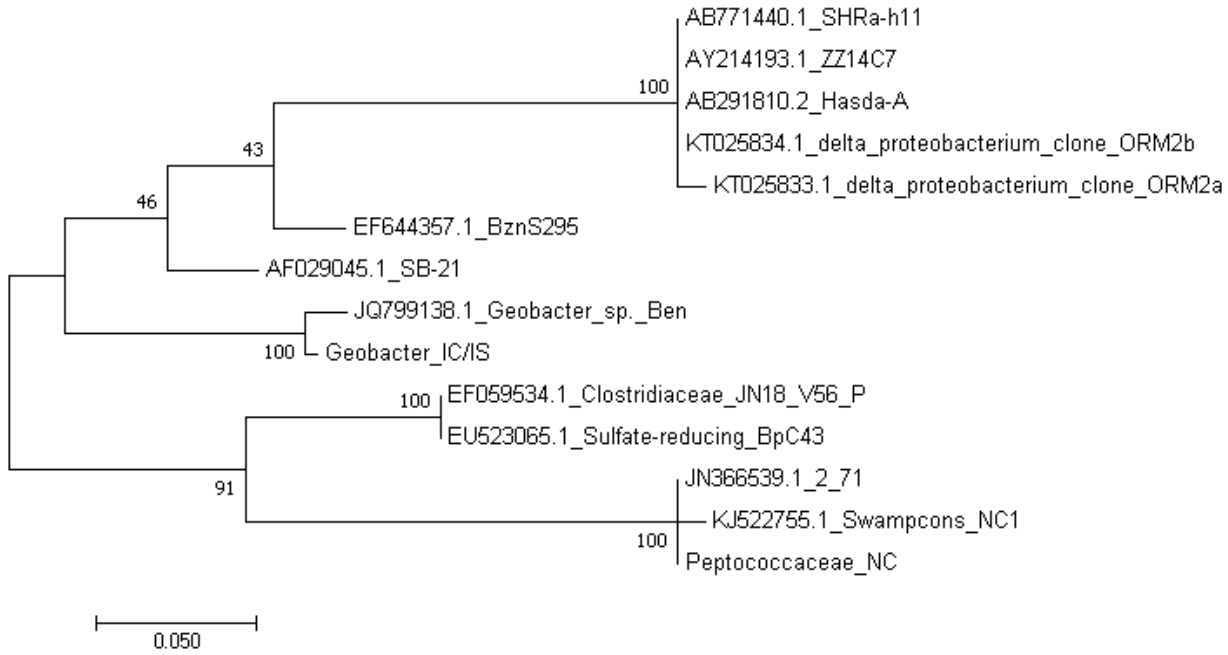
16S rRNA Community of Sand-based Microcosms MS, SS, IS, NS.



The top 9 OTUs (operational taxonomic units) at day 260 are shown in community profile at the family taxon level, in order of abundance with 1° degraders in bold. U = unclassified, SI = Shannon Index.

Figure 5

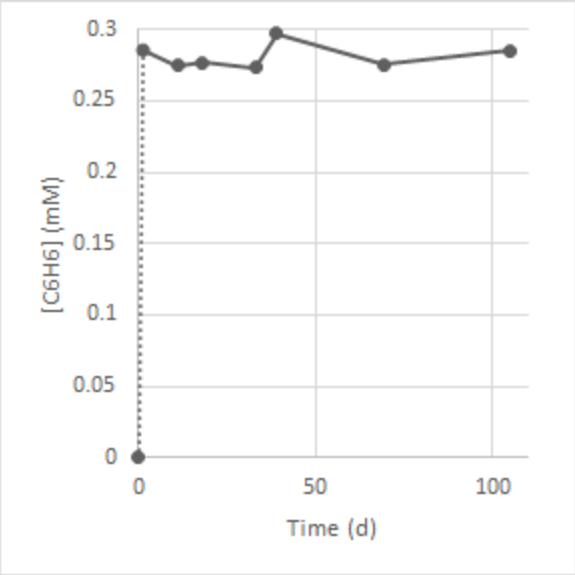
Identification of Primary Benzene Degraders by 16S rRNA Gene Homology



16S rRNA gene amplicon sequences of suspected primary benzene degraders were compared with those of known degraders from literature (Noguchi et al. 2014; Alfreider and Vogt 2007; Sakai et al. 2009; Luo et al. 2016; Musat and Widdel 2008; Oka et al. 2008; Zhang et al. 2012; Bedard et al. 2007; Abu Laban et al. 2009; van der Zaan et al. 2012; Luo et al. 2014), to establish a minimum of 97% sequence homology. Maximum likelihood joining of said sequences was performed to generate a phylogenetic analysis, with branch lengths measured in number of substitutions per site (Page and Holmes 2009; Kumar et al. 2016; Swofford DL, Olsen GJ, Waddell PJ & H...). Accession numbers for Genbank are included for reference. The two primary degraders identified in this study are listed as Geobacter_IC/IS and Peptococcaceae_NC, found in IC/IS and NC cultures, respectively.

Supplemental 1

C_6H_6 removal in Heat-killed Control (HK)



Samples of culture were heat-sterilized with triple 90 min autoclave cycles, amended with C_6H_6, and maintained in anaerobic conditions over 105 days. C_6H_6 concentrations were monitored by headspace GC analysis. Dotted lines represent C_5H_5 feeding events. Data points represent the average of duplicates.

References