

Bioaugmentation coupled with activated carbon for the treatment of petroleum hydrocarbons in groundwater systems

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

The experimental designs presented in Chapters 3 and 4 were developed by Dr. Neil R. Thomson, Dr. Elizabeth A. Edwards, Dr. Courtney R.A. Toth, Marianne Vandergriendt, and Andrea Marrocco. The experiments were performed by me and Andrea Marrocco who also reported on data from the KC, KC-PAC, SC, PC-BA, and A-PAC-BA microcosms and the KC-PAC, PC-BA, and A-PAC-BA columns in her PhD thesis (Marrocco, 2023). I analyzed all the data and prepared all the figures presented in Chapters 3 and 4. Dr. Neil R Thomson and William McLaren reviewed and edited contents presented in this thesis.

The experiments described in Chapter 3 were performed at the University of Waterloo in the Ecohydrology Laboratory in the Department of Earth and Environmental Science (Marianne Vandergriendt), and the Hug Research Laboratory in the Department of Biology (Dr. Laura A Hug). The experiments discussed in Chapter 4 were performed in the Soil and Groundwater Remediation Engineering Laboratory at the University of Waterloo in the Department of Civil and Environmental Engineering (Dr. Neil R. Thomson). The aquifer material used in Chapter 3 and 4 was collected from Canadian Forces Base Borden, Ontario, Canada by Bob Ingleton, Paul Johnson and Aaron Vandenhoff from the University of Waterloo, Department of Earth and Environmental Sciences. Petroleum hydrocarbon, carbon dioxide, and methane sample analyses were completed by Shirley Chatten in the Soil and Groundwater Remediation Engineering Laboratory at the University of Waterloo. Molecular analysis was completed at the BioZone Center for Applied Bioscience and Bioengineering at the University of Toronto. Dissolved inorganic carbon sample analysis was completed Richard Elgood at the in the Environmental Geochemistry Laboratory at the University of Waterloo. Sulfate sample analysis was primarily completed by Mark Merlau in the Department of Civil and Environmental Engineering Environmental and Water Resources (EWR) Laboratory at the University of Waterloo.

Abstract

The contamination of groundwater by petroleum hydrocarbons (PHCs) is a global concern with negative human health and environmental impacts. The injection of an activated carbon (AC) particulate amendment to create a permeable reactive barrier (PRB) to prevent additional downgradient migration of a dissolved PHC plume from a source area has gained popularity. In this remedial application the selected AC amendment is strategically injected across a dissolved PHC plume and provides sufficient adsorption capacity to remove dissolved PHCs from the groundwater that flows through the PRB. This concentrated mass of PHCs is considered to serve as a haven for indigenous microorganisms to thrive and results in biodegradation of the PHCs and regeneration of the adsorption capacity of the AC. Often anaerobic conditions develop within the PRB which leads to depleted concentrations of electron acceptors. To overcome this limitation, bacterial cultures are co-injected with the AC to increase the rate of biodegradation since some indigenous microorganisms are perhaps not capable, in low abundance or not present to operate effectively under anaerobic conditions. The overarching objective of this research was to investigate if an AC particulate amendment coupled with bioaugmentation (culture injection) can synergistically enhance the biodegradation of PHCs. The hypothesis postulated was that the combination of powdered AC (PAC) and enriched methanogenic cultures will enhance the biodegradation of benzene, toluene and *o*-xylene (BTX) under anaerobic conditions.

To evaluate this hypothesis, data was collected from microcosm experiments representing static conditions, and from continuous flow column experiments mimicking *in situ* conditions. Both the microcosm and column experiments were conducted in anaerobic environments and included controls and active treatment systems involving combinations of BTX, PAC, and/or bioaugmentation (BA). The PAC utilized was a virgin coconut-based thermally activated product with a mean particle size of 13.1 μm . The methanogenic cultures were enriched from nature and have been shown to completely degrade benzene (DGGTM-B), toluene (DGGTM-T), and *o*-xylene (DGGTM-X). The aquifer material used in the experiments was collected from the University of Waterloo Groundwater Research Facility at Canadian Forces Base (CFB) Borden.

Five different microcosm systems were constructed using artificial groundwater, aquifer material, methanogenic cultures and PAC. A single-compound experiment used toluene only while a multi-compound experiment used BTX. The active systems included microcosms with and without BA and with (A-PAC-BA and A-PAC) and without (A-BA and A) PAC. Control microcosm systems included killed control (autoclaved with biocide), positive control (BA without aquifer material), and starved control (no addition of toluene or BTX). A total of 300 microcosms bottles were assembled and stored on their side in an anerobic glove chamber undistributed except during sampling. Microcosms were sampled at selected timepoints over a period of nearly one year using a repetitive and sacrificial strategy. Dissolved phase samples were used to determine pH, oxidative reductive potential (ORP), dissolved oxygen (DO), BTX concentrations, dissolved inorganic carbon (DIC) content, and sulfate and sulfide concentrations. Gas phase samples collected from the microcosm headspace were analyzed for methane (CH₄) and carbon dioxide (CO₂). Solid phase samples were also collected to determine bulk BTX concentrations, and deoxyribonucleic acid (DNA) extractions were assayed by quantitative polymerase chain reaction (qPCR). Toluene or BTX mass was replenished as needed in the active systems.

There was no evidence of biodegradation in either the killed or starved controls. Depletion of toluene and *o*-xylene in conjunction with consumption of sulfate and production of methane and carbon dioxide indicated biodegradation occurred in the positive control and all active bioaugmented microcosm systems. In all microcosm systems the depletion of benzene was not observed. As expected, the presence of PAC in the active microcosm systems considerably reduced the aqueous concentrations of toluene or BTX. Bulk toluene or BTX concentration data provided evidence for the regeneration of PAC sorption capacity because of biodegradation. qPCR results support the depletion of toluene and *o*-xylene in the active bioaugmented systems with elevated populations of key degraders (*Desulfosporosinus* (DSP) and *Peptococcaceae* (PEP)). During the final ~30 days, a higher temporal resolution sampling strategy was implemented to collect data to estimate compartmental mass distributions and system biodegradation rates. The estimated biodegradation rate for A and A-PAC microcosms (no BA) were not statistically different at the 5% LOS, as well as the estimated biodegradation rate for A-BA and A-PAC-BA microcosms. Based on the data set assembled from the microcosm experiments, there is no evidence that supports the stated hypothesis. Specifically, the presence of PAC by itself or in combination with BA did not increase the mass of toluene and *o*-xylene biodegraded or the rate of biodegradation.

Five different columns systems were used to examine BTX biodegradation under anaerobic conditions. The active systems included columns with and without BA and with (A-PAC-BA and A-PAC) and without (A-BA and A) PAC. A control column (killed control) containing PAC was constructed from autoclaved materials. The columns were packed with aquifer material and a 6-cm long PAC zone (0.5% by wt) was emplaced in the central part of the column to mimic a PRB for some systems. An access port was used to inject cultures into the PAC zone. Anaerobic artificial groundwater augmented with BTX was used as the feed solution. Biocide was added to the feed solution for the killed control column. The columns were run for a one-year acclimation period followed by 9 months of a comprehensive sampling. Dissolved phase samples collected from the influent and effluent were used to determine pH, ORP, DO, DIC content, and BTX, sulfate, CH₄, and CO₂ concentrations. At the termination of the experiment, solid phase subsamples were collected from each active column and used to determine bulk BTX concentrations, and DNA extractions were assayed by qPCR.

There was no evidence of biodegradation in the killed control column. In the absence of BA (A vs A-PAC columns), the PAC zone improved the biodegradation of toluene as supported by the production of CH₄ and CO₂, and increased population of toluene degraders within the PAC zone. While in both BA systems (A-BA and A-PAC-BA) toluene biodegradation was near complete (>95% mass reduction), suggesting that the PAC zone did not enhance the biodegradation capacity of toluene when combined with BA. Biodegradation of benzene (~25% mass reduction) occurred in the A-PAC-BA system, despite a larger population of benzene degrading microbes in the A-BA system. *O*-xylene biodegradation was the highest in the A-BA system (~90% mass reduction), which was supported by the DNA results (>10⁷ copies/g *o*-xylene degraders), along with production of CH₄ and CO₂. Based on the data set assembled, there is evidence that supports the stated hypothesis. Specifically, the presence of a PAC zone in the column by itself improved the biodegradation of toluene (~95% versus ~70% mass reduction) and *o*-xylene (~25% versus <20 % mass reduction) compared to the column with no PAC zone. The combination of PAC and BA increased benzene biodegradation (~25% versus <20 % mass reduction) but decreased *o*-xylene biodegradation (~90% versus ~80% mass reduction) compared to BA but without a PAC zone.

Taken together, experimental findings were not able to prove that PAC and BA work synergistically to enhance the biodegradation of BTX. Nevertheless, PAC did not reduce the biodegradation ability of the systems and can therefore still provide benefits when used for groundwater remediation applications. Specifically, the use of PAC in combination with bioaugmentation in field applications may provide benefits by containing the BTX mass in a more spatially confined area for longer durations (> 20 years), which would provide more time for biodegradation to occur.

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List of Abbreviations

A	active system type
AC	activated carbon
AGW	artificial groundwater
BA	bioaugmented system type
BS	Borden sand
BTX	benzene, toluene, <i>o</i> -xylene
DCM	dichloromethane
DIC	dissolved inorganic carbon
DO	dissolved oxygen
DSP	<i>Desulfosporosinus</i>
FID	flame ionization detector
GC	gas chromatography
IC	ion chromatography
KC	killed control system type
MDL	method detection limit
ORP	oxidation-reduction potential
PV	pore volume
PHC	petroleum hydrocarbon
PAC	powdered activated carbon
PEP	<i>Peptococcaceae</i>
PRB	permeable reactive barriers
PC	positive control system type
PTFE	polytetrafluoroethylene
PV	pore volume
qPCR	quantitative polymerase chain reaction
SC	starved control system type
SS	stainless steel
TCD	thermal conductivity detector
TIC	total inorganic carbon

Chapter 1

Introduction

1.1 Background

Throughout human history, there have been technological and scientific breakthroughs that have led to periods of rapid growth in specific sectors. One such breakthrough in the field of stored energy was the discovery that petroleum products (e.g., gasoline, propane) could provide power for industrial and household applications. During its infancy, the energy industry was ignorant of the safety hazards surrounding the handling and disposal of petroleum products, resulting in large amounts of them being released to the environment. Currently, environmental effects persist from faulty and leaking storage tanks, old airfields, and legacy chemical manufacturing plants that continue to release significant amounts of petroleum products into soil and groundwater, producing dissolved plumes that have been expanding for more than half a century (Andreoni and Gianfreda, 2007). Accidental releases result in the pollution of groundwater sources and pose a significant threat to both human and environmental health (ECCC, 2017). Over the past several decades, significant research on how to mitigate and manage petroleum pollutants in groundwater has been completed.

Technologies that have been used to address petroleum contamination in groundwater include air sparging, groundwater pump-and-treat technology, passive/reactive barriers, bio-slurping, ultraviolet-oxidation treatment, bio-sparging, groundwater circulation wells, horizontal well technology, and natural attenuation (Khan et al., 2004). However, there remain many challenges with the cleanup of petroleum contaminated sites, which has led to significant interest in developing technologies that are rapid and functional in a wide array of subsurface settings (Khan et al., 2004). The application of a remedial technology is dependent on its feasibility and economic viability, which are influenced both by the location of the contamination and the environmental conditions. For example, the depth of contamination will determine the type of treatment needed, with shallow contamination requiring more aerobic treatment and deeper contamination requiring more anaerobic treatment when considering bio-treatment options (Ossai et al., 2020). Furthermore, low permeability groundwater zones, deep aquifers, and sites below existing infrastructure favour *in situ* and anaerobic approaches (Farhadian et al., 2008).

Permeable Reactive Barriers (PRBs) as an *in situ* technology function to bring the contaminants into a reactive zone, where immobilization and destruction can take place. While many different

reactive materials exist such as zero valent iron (ZVI), zeolites, and ash, this project focuses on activated carbon (AC). AC is a carbonaceous material with a chemically heterogeneous surface and moderate to high adsorption capacity that serves to immobilize the contaminant (Obiri-Nyarko et al., 2014). Despite the availability of these technologies, the contamination of groundwater with BTEX remains an ongoing threat as the fate of hydrocarbons in anaerobic conditions is not completely understood (Fowler et al., 2016).

The work presented in this thesis focuses on petroleum hydrocarbons (PHCs), a category of petroleum by-products that includes benzene, toluene, and xylene isomers, together known as 'BTX'. While many remedial technologies are viable options for treating subsurface PHC contamination (i.e., with toluene or xylenes), other PHCs (i.e., benzene) are recalcitrant in anaerobic environments (Toth et al., 2021). The bioaugmentation cultures investigated are DGG™-B, DGG™-T, and DGG™-X (which anaerobically degrade benzene, toluene, and *o*-xylene respectively), named in honor of anaerobic hydrocarbon biodegradation pioneer, Dunja Grbić-Galić. This work links PRBs and natural attenuation (biodegradation/bioremediation) through bioaugmentation.

1.2 Research Objective

This research project aims to assess the effectiveness and improve the use of powdered activated carbon (PAC) (a type of AC) coupled with bioaugmentation as a technology for addressing BTX pollution in anaerobic environments. The specific objective is to measure the effect of PAC on anaerobic biodegradation of BTX with and without bioaugmentation present to test whether PAC and bioaugmentation synergistically enhance degradation.

1.3 Research Hypothesis

The hypothesis this project aims to test is that the presence of PAC will increase the biodegradation of BTX in systems capable of biodegradation both with and without bioaugmentation under anaerobic conditions.

1.4 Project Scope

To adequately address the research objective and test the research hypothesis, two phases of experimentation were used:

1. Evaluation of static system behaviour in soil/PAC microcosms

2. Evaluation of dynamic system behaviour in flow through column experiments mimicking *in situ* conditions

This thesis contains five chapters. Chapter 1 provides the motivation, objective, and scope of this thesis. Chapter 2 provides relevant background knowledge pertaining to this work. Chapter 3 and 4 outline the experimental design, methods, results, and discussion for the microcosms and columns, respectively. Finally, Chapter 5 outlines the conclusions and recommendations from this work.

Chapter 2

Background

2.1 Benzene, Toluene, Ethylbenzene and Xylene (BTEX)

The monoaromatic hydrocarbons benzene, toluene, ethylbenzene, and three isomers of xylene (*m*-, *o*-, *p*-xylene) (collectively known as ‘BTEX’) are a family of petroleum hydrocarbons (PHCs) found in many different fuels (Kao et al., 2006). As a component of gasoline, diesel, and jet fuel, BTEX compounds have found their way into the environment and are common groundwater contaminants (Da Silva and Alvarez, 2002). As of December 2022, there are 2991 known contaminated groundwater sites across Canada. Of these sites, 1499 (~50%) are contaminated with PHCs, of which 392 (~13%) are specifically contaminated with BTEX (Treasury Board of Canada Secretariat, 2022).

Regulatory agencies recognize the dangers of complex mixtures, including BTEX, and their associated effects on human health and the environment (Khan et al., 2004; Vogt et al., 2011). BTEX compounds are toxic aromatics, with most being confirmed or suspected carcinogens (ATSDR, 2007a; ATSDR, 2007b; ATSDR, 2017). Furthermore, research indicates additional negative health effects resulting from the consumption of these compounds including liver lesions, drowsiness, and irritation of organs (Fayemiwo et al., 2017). Benzene is especially dangerous, as it has been classified as a carcinogen with the lowest allowable concentration for a BTEX compound in groundwater in Canada and the United States ($< 5 \text{ } \mu\text{g/L}$) and may persist in subsurface environments for extended periods of time due to its low natural attenuation rate (USEPA, 2004; ATSDR, 2007a).

This project focuses on the subset of BTEX that includes benzene, toluene, and *o*-xylene (BTX), which have chemical properties that make them similar pollutants. Benzene, toluene, and *o*-xylene (BTX) are water soluble (Table 2.1), and as such are common constituents in subsurface PHC plumes. BTX are capable of entering groundwater systems through a non-aqueous phase liquid (NAPL) release proceeded by dissolution, as a result of accidents (Patterson et al., 1993). The aqueous solubility of BTX compounds mean they are a concern for regions that rely on impacted groundwater for their domestic water supply (USEPA, 2006).

Table 2.1: Physical and chemical properties of BTX compounds (National Centre for Biotechnology Information, 2022).

Property	Benzene ¹	Toluene ²	<i>o</i> -Xylene ³
Molecular Formula	C ₆ H ₆	C ₆ H ₅ CH ₃	C ₆ H ₄ (CH ₃) ₂
Molecular Weight (g/mol)	78.11	92.14	106.16
Density (g/cm ³ @ 20°C)	0.876	0.862	0.880
Water Solubility (mg/L @ 25°C)	1790	526	178
Vapor Pressure (Pa @ 25°C)	10000	3800	700
Henry's Law Constant (Pa m ³ /mol @ 25°C)	556	664	518
Organic Carbon-Water Partition Coeff (log K _{oc})	1.92	2.25	2.39

Notes:

1. Compound Summary for Benzene (NCBI, 2022a)
2. Compound Summary for Toluene (NCBI, 2022b)
3. Compound Summary for *o*-Xylene (NCBI, 2022c)

BTX are capable of being degraded in the environment through biodegradation, which is the process whereby microorganisms metabolize organic contaminants into inorganic end products (e.g., carbon dioxide, methane, salts) under natural or engineered conditions (Dott et al., 1995). Although BTX are readily degraded through biological activity in aerobic soil systems, the degradation of other contaminants in the subsurface will result in the depletion of available oxygen. Under anaerobic conditions, the degradation of BTX needs alternative electron acceptors, such as sulfate, to undergo biodegradation (Beller et al., 1992). For example, toluene oxidation when coupled with sulfate reduction is an exergonic reaction that yields energy to bacteria (Beller et al., 1992). The degradation stoichiometry in different anaerobic degradation regimes for benzene, toluene and *o*-xylene are presented in Table 2.2 (Farhadian et al., 2008).

Table 2.2: Monoaromatic properties and degradation stoichiometry of Benzene, Toluene, and *o*-Xylene. Adapted from Farhadian et al. (2008). Reactions do not account for the background degradation of cells or for the use of carbon for microorganism growth.

Compound	Overall Reaction
Benzene (B)	$C_6H_6 + 4.5 H_2O \rightarrow 2.25 CO_2 + 3.75 CH_4$ (methanogenic) $C_6H_6 + 3.75 SO_4^{2-} + 7.5 H^+ \rightarrow 6 CO_2 + 3.75 H_2S + 3 H_2O$ (sulfate reducing)
Toluene (T)	$C_7H_8 + 5 H_2O \rightarrow 2.5 CO_2 + 4.5 CH_4$ (methanogenic) $C_7H_8 + 4.5 SO_4^{2-} + 9 H^+ \rightarrow 7 CO_2 + 4.5 H_2S + 4 H_2O$ (sulfate reducing)
<i>o</i> -Xylene (X)	$C_8H_{10} + 5.5 H_2O \rightarrow 2.75 CO_2 + 5.25 CH_4$ (methanogenic) $C_8H_{10} + 5.25 SO_4^{2-} + 10.5 H^+ \rightarrow 8 CO_2 + 5.25 H_2S + 5 H_2O$ (sulfate reducing)

2.2 Activated Carbon Particulate Amendments

Many treatment technologies have been developed to remediate soil and groundwater contaminated with BTX. Permeable reactive barriers (PRBs) are *in situ* treatment technologies (i.e., treating the water where it resides, rather than removing and treating elsewhere) used to cut off a plume and either immobilize and/or destroy the contaminant (Obiri-Nyarko et al., 2014). One form of PRB utilizes activated carbon (AC) particulates, which serve as sorption sites for contaminants, allowing for their immobilization and/or destruction (Obiri-Nyarko et al., 2014). Some major vendors that use AC for *in-situ* applications include REGENESIS Bioremediation Products (Plumestop™) in California and Remediation Products, Inc. (BOS-200®) in Colorado (Simon, 2015). Table 2.3 provides more information for the most commonly used AC-based remedial products used for *in situ* applications (Fan et al., 2017).

Table 2.3: Summary of the three most commonly used AC-based remedial products for *in situ* applications. Modified from Fan et al. (2017).

Product	Physical Property	Additive	Target Contaminants	Degradation Pathway
BOS-200 [®]	Powdered manufactured by Calgon	Electron acceptors, phosphorus, nitrogen nutrients, CaSO ₄ , Facultative bacteria mix	PHCs	Short-term aerobic and long-term anaerobic bioaugmentation
COGAC [®]	Granular or powdered	Calcium peroxide, sodium persulfate, Nitrate occasionally	PHCs and chlorinated solvents	Chemical oxidation, aerobic and anaerobic biostimulation
PlumeStop [®]	Colloidal-sized AC suspension	Proprietary organic polymer, hydrogen or oxygen release compounds, bacterial strains	PHCs or chlorinated solvents	Anaerobic biodegradation for chlorinated solvents or aerobic biodegradation for PHCs (augmentation or stimulation)

One type of AC particulate is powdered AC (PAC), which consists of particles with an average size of 15-25 μm (Aktaş and Çeçen, 2007). Originally, AC was predominately used for biological oxygen demand (BOD) and chemical oxygen demand (COD) removal in wastewater (Aktaş and Çeçen, 2007), but has since been used in a variety of water treatment applications, including domestic water filtration (e.g., Brita filters), wastewater treatment, and *in situ* treatment of many groundwater contaminants, including BTX compounds.

2.2.1 PAC Mechanisms for Treatment Viability

The ability of PAC to sorb BTX is primarily a function of its highly porous internal structure, which mimics a dendritic network and can be subdivided by diameter: macropore ($> 50 \text{ nm}$), mesopore (between 2 and 50 nm), and micropore ($< 2 \text{ nm}$) (Figure 2.1) (Bansal and Goyal, 2005). The macropores and mesopores function as transport pathways through which BTX can travel by diffusion (Choi et al., 2007; Furuya et al., 1996), while the surface area of the micropores function as major adsorption sites due their diameter being similar to that of BTX molecules (Figure 2.1)

(Li et al., 2002). The size similarity between the pores and contaminant molecules makes adsorption stable, as there are multiple contact points between the contaminant and the pore wall (Li et al., 2002).

Physical adsorption is the dominant adsorption mechanism for BTX under typical subsurface conditions. Physical adsorption is a reversible process characterized by weak van der Waals forces (Bansal and Goyal, 2005). Due to this reversibility, adsorption and desorption are in a dynamic equilibrium that is influenced by environmental conditions (i.e. pH, temperature, concentration) (Fan et al., 2017). This mechanism is in contrast to irreversible adsorption, which is a function of chemisorption (high energy bonding) and oxidative polymerization in the presence of oxygen (Aktaş and Çeçen, 2007), and is not the primary adsorption mechanism for BTEX.

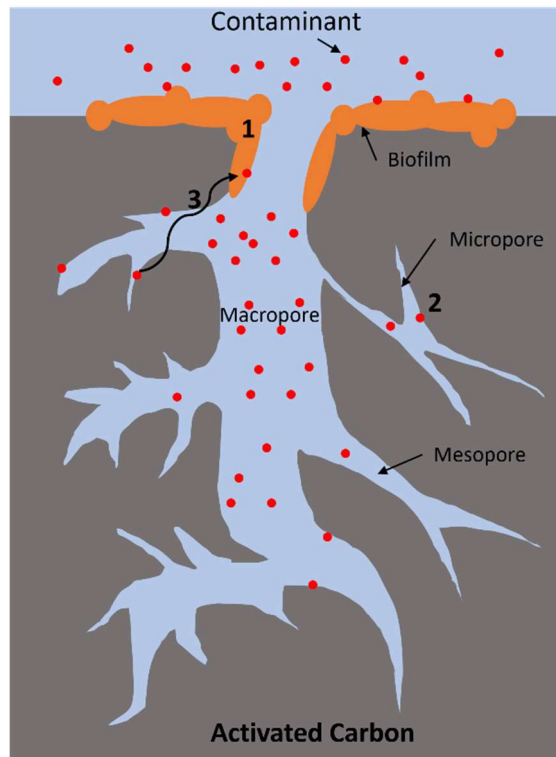


Figure 2.1: Diagram of AC pores and the removal of contaminants. (1) direct adsorption and degradation by microorganisms; (2) micropore adsorption; (3) desorption, concentration gradient driven diffusion, followed by degradation in microbial biofilm. Modified from Fan et al. (2017). Figure 2.1 illustrates the sorption and biodegradation processes in PAC for the case of BTX compounds. Long-term biodegradation occurs due to microbial attachment and colonization, which results in the formation of a biofilm on the surface of the AC. The porous nature of PAC (high surface area) promotes biodegradation processes by allowing for relatively large quantities

of BTX compounds to be held in close proximity (Simpson, 2008). As biofilm development cannot take place directly adjacent to sorbed mass located in the micro- and mesopores due to the size of the microorganisms, contaminant degradation occurs either before the contaminant reaches the micropores (location 1 on Figure 2.1), or after desorption and diffusion out of the micropores (location 3 on Figure 2.1) (Fan et al., 2017). Fan et al. (2017) suggest that degradation that occurs before the contaminant reaches the micropores is dominant when degradation rates surpass the contaminant influx, which takes place after the biofilm has matured and an ample acclimation period has been met. Conversely, desorption and diffusion out of the micropores is dominant when the degradation rate is slower than contaminant influx. This further illustrates that contaminant removal in the presence of PAC and biofilm is a function of the dynamic equilibrium between adsorption, desorption, and degradation processes.

PAC and biodegradation work synergistically to provide enhanced contaminant remediation. Compared to unassisted biodegradation (without PAC), sorption to AC provides a couple of benefits: a retardation to contaminant migration and a mitigation of fluctuations in contaminant aqueous concentrations (Fan et al., 2017). Additional benefits are the extension of the contact time between the contaminants and degraders, and a higher contaminant concentration inside the pore matrix (several orders of magnitude) in relation to the bulk aqueous phase (Çeçen and Aktas, 2011). Furthermore, biodegradation extends the lifetime of PAC beyond the point where its capacity would have been exhausted in the absence of biodegradation due to regeneration of sorption sites (Tsuno et al., 2006). Research suggests that the formation of biofilms is promoted by the presence of AC (Figure 2.1) and that this is the main factor behind enhanced biodegradation (Weber et al., 1978). Biofilms provide numerous advantages for microbial communities, including the modification of microbial composition, increased nutrient concentration, and the resistance to toxic substances, predators, and changes in environmental conditions (Simpson, 2008). Therefore, when present in PAC applications, adsorption and biodegradation is proposed to be more effective than solely biodegradation (Fan et al., 2017).

2.2.2 PAC Treatment Efficacy

There are multiple factors that can affect the *in situ* efficacy of groundwater treatment using PAC. Changes to the physicochemical properties of PAC occurs through varying environmental factors and processes. For example, dissolved organic matter (DOM) will readily adsorb to the PAC and

compete for sorption sites. As DOM has a range of sizes, it can compete for sorption sites present in the micropores or can completely block entrances to the micropore network by lodging/sorbing to larger diameter pores (Fan et al., 2017). Biological materials may also lead to a decrease in PAC sorption capacity as byproducts of microorganism biodegradation pathways have similar effects as DOM (Aktaş and Çeçen, 2007).

Another important property of PAC is that sorption processes are concentration dependent. The number of available PAC sorption sites (i.e., remaining adsorptive capacity) declines with increasing aqueous concentration so that the PAC either needs to eventually be replaced (not feasible *in situ* without extensive disruption) or regenerated in some fashion (Aktaş and Çeçen, 2007). Isotherm data collected for single compound systems containing benzene, toluene, or *o*-xylene and coconut-based PAC from Calgon Carbon (WPC®, < 325 US mesh) confirm the dependency of limited PAC sorption sites at increasing aqueous concentration (Marrocco, 2023). For example, Figure 2.2 illustrates how as aqueous benzene concentration increases, the concentration sorbed to PAC increases rapidly at lower concentrations and then more slowly at higher concentrations (Marrocco, 2023).

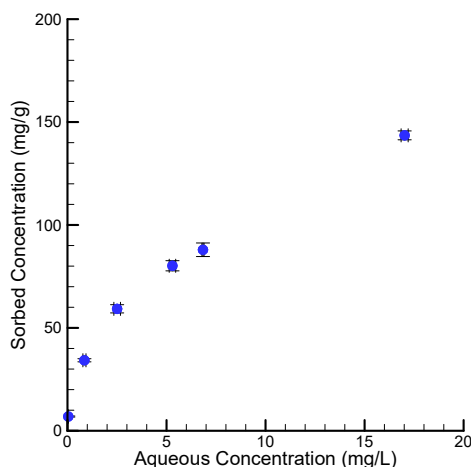


Figure 2.2: Isotherm data for benzene on WPC® PAC. Data from Marrocco (2023). Error bars represent $\pm$ one standard deviation.

In a multiple compound system (such as BTX), there is also competition for the limited sorption sites on AC, with the sorbed concentrations of benzene, toluene, and *o*-xylene being dependent on the strength of adsorption of the solutes (ΔG), their individual aqueous concentrations, and AC properties (Çeçen and Aktaş, 2011). In a competitive system, benzene, toluene, and *o*-xylene will

have lower individual sorbed concentrations for a given aqueous concentration than they would in a non-competitive system due to the occupation of limited sorption sites by other compounds present in the bulk solution (Aktaş and Çeçen, 2007; Yonge and Keinath, 1986). PAC expresses unique adsorption affinities when exposed to BTX compounds. From lowest to highest, the adsorption affinity for BTX is in the order of benzene < toluene < *o*- xylene, following increasing molecular weight and decreasing solubility (left to right) (Table 1.1) (Daifullah and Girgis, 2003).

2.3 Bioremediation and Bioaugmentation

Bioremediation is the processes by which biological degradation is intentionally used to break down contaminant pollution. *In situ* bioremediation has advantages over other forms of *in situ* treatment due to its relative simplicity, low cost, and potential for persistent BTX removal (Dott et al., 1995). As BTX is completely metabolized to carbon dioxide, methane, and salts, there is no secondary waste that would need to be addressed during the process of bioremediation, aside from the metabolites and degradation byproducts (macromolecules secreted during biodegradation) (Dott et al., 1995).

Unassisted bioremediation, known as intrinsic or natural attenuation, is the process where naturally occurring subsurface systems biodegrade contaminants (e.g., BTX) without any alteration or human intervention (Farhadian et al., 2008). While natural attenuation can handle small contaminant loadings (BTX), they need to be augmented to improve degradation rates when concentrations are high. This challenge has led to the development of engineered *in situ* bioremediation techniques to increase the rate of organic contaminant degradation. Enhancing intrinsic degradation pathways (anaerobically) have been successful in some contaminated sites with oil products (Farhadian et al., 2008).

Bioaugmentation is a type of bioremediation strategy where biocatalysts (generally microorganisms) are added into the groundwater system to degrade a target contaminant. These biocatalysts consist of mixed bacteria that have been cultured from natural environments and show elevated levels of biodegradation for target contaminants. Bioaugmentation can either increase the population of existing organisms which treat contaminants of concern or introduce them into the system where there was no prior potential for degradation (Lyon and Vogel, 2013).

Bioaugmentation is beneficial with low to no operations and maintenance costs once in place, in comparison to biostimulation, which relies upon stimulating organisms that are already present at the site with nutrients or electron acceptors. Historically, bioaugmentation as a treatment avenue was considered in the 1980s to early 1990s, once PHC bioremediation strategies were being accepted as feasible methods. To make these bioremediation strategies more robust, vendors started to offer bioaugmentation in the form of microbial inoculants.

2.3.1 Bioregeneration of PAC

As mentioned previously, a limitation of PAC is that the number of available PAC sorption sites declines with increasing aqueous concentration (Aktaş and Çeçen, 2007). The combination of PAC with indigenous microorganisms or bioaugmentation are an engineered *in situ* bioremediation approach that enhances the regeneration of PAC through microbial activity, or ‘bioregeneration’. Bioregeneration is when microorganisms consume contaminants (e.g., BTX) that have desorbed off PAC, thus regenerating the available sorption capacity to allow for further contaminant mass sequestration from the aqueous phase.

There are some limitations in the ability of bioregeneration to enhance PAC performance. One example is that desorption of contaminants is a prerequisite to PAC bioregeneration, so that any condition that affects the desorption negatively also affects bioregeneration capabilities (Aktaş and Çeçen, 2007). Desorption from AC acts in response to operational condition changes such as a decrease in aqueous phase concentration, or displacement of adsorbed solutes due to competitive sorption (Thacker et al., 1983). As desorption is concentration dependent, the aqueous concentration must be decreased around the PAC to promote desorption.

There are also several challenges with identifying evidence of biodegradation for *in situ* applications. Adsorption may result in the removal of both the parent compound and degradation byproducts (i.e., evidence of degradation), and adsorption rates are typically much faster than degradation rates, obscuring the evidence of biodegradation (Fan et al., 2017).

Further expanding on bioregeneration, it is impacted by degradation by-products and metabolites that occupy sorption sites, resulting in the deterioration of PAC adsorption capacity over time as biodegradation proceeds (Aktas, and Çeçen, 2007; de León, 2021). The decrease in the adsorption

capacity of PAC has been further linked to the presence of the biofilm itself, which can clog PAC pores (de León, 2021).

As desorption of BTX is a prerequisite step for bioregeneration, any decrease in AC adsorption capacity suggests a decrease in the available sorption sites to be bioregenerated. Over time, with the coupling of biofilm and PAC, biofilm will continue to create degradation byproducts, further decreasing PAC sorption capacity. However, this does not necessarily mean the treatment technology will not have adequate performance; as long as degradation rates can be maintained, bioregeneration remains the primary mass removal mechanism (sequestration) (Fan et al., 2017). Even with significant biofilm establishment and biodegradation as the primary mass removal mechanism (destruction), adsorption can be effective at dealing with sudden environmental shocks (e.g., an increase in mass flux beyond biodegradation rates) (Voice et al., 1992).

Chapter 3

Microcosm Experiments

This chapter describes the methodology and results of the microcosm experiments, which were conducted to evaluate the interaction between PAC and biodegradation. Microcosms contained various combinations of aquifer material, PAC, bioaugmentation cultures, and artificial groundwater (AGW). They were maintained under anaerobic conditions and only mixed before sampling. The aquifer material used was Borden Sand (BS) with an indigenous microbe community, and the activated carbon was a commercial coconut-based PAC. Bioaugmentation cultures used were DGG™-B, DGG™-T, and DGG™-X, which anaerobically degrade benzene, toluene, and *o*-xylene, respectively (Toth et al., 2021).

3.1 Materials and Methods

Two sets of microcosm experiments were conducted using different PHCs. The single-compound experiments used toluene only (identified as ‘T-only’) along with the DGG™-T culture and represented a system with no competition between hydrocarbons as a substrate or sorbate. The multi-compound experiment included benzene, toluene, and *o*-xylene (BTX) and aimed to represent a more realistic system that included competition. This multi-compound experiment used the DGG™-B, DGG™-T, and DGG™-X cultures.

The design of the two microcosm experiments is summarized in Table 3.1. Each experiment included five treatment types that were further subdivided into “series” based on the experimental conditions as follows:

1. ***Killed Control (KC)***: Used autoclaved BS and contained biocide which served to assess abiotic degradation. The KC type had three series: BS only (KC), BS with a bioaugmentation culture addition (KC-BA), and BS with PAC addition (KC-PAC).
2. ***Starved Control (SC)***: Represented background degradation from the indigenous microbes without an electron donor present (i.e., T or BTX) and had two series: BS only (SC), and BS with a bioaugmentation culture addition (SC-BA).

3. **Positive Control (PC):** Used to monitor the effect of bioaugmentation without the presence of BS (PC-BA).
4. **Active (A):** Identical to the KC types but without biocide and autoclaved BS. The A type included two series: without bioaugmentation (A) and with a bioaugmentation culture (A-BA).
5. **Active with PAC (PAC):** Same as the active type but with the addition of ~15 mg of PAC and included two series: active with PAC (A-PAC), and an active with PAC and bioaugmentation culture (A-PAC-BA).

Table 3.1: Microcosm experimental design, for the T-only and BTX experiments. ✓ indicates presence, -- indicates absence.

Type	Series	Borden Sand (BS)	PAC	DGG TM inoculum ⁶	T-only	BTX	Number of Bottles
Killed Control (KC) ¹	KC	✓	--	--	✓	✓	6 (2x trip) ⁷
	KC-BA	✓	--	✓	✓	✓	6 (2x trip)
	KC-PAC	✓	✓	--	✓	✓	6 (2x trip)
Starved Control (SC) ²	SC	✓	--	--	--	--	6 (2x trip)
	SC-BA	✓	--	✓	--	--	6 (2x trip)
Positive Control (PC) ³	PC-BA	--	--	✓	✓	✓	24 (8x trip)
Active (A) ⁴	A	✓	--	--	✓	✓	24 (8x trip)
	A-BA	✓	--	✓	✓	✓	24 (8x trip)
Active with PAC (A-PAC) ⁵	A-PAC	✓	✓	--	✓	✓	24 (8x trip)
	A-PAC-BA	✓	✓	✓	✓	✓	24 (8x trip)

Notes :

1. This type accounts for abiotic degradation, and sorption to BS or PAC in the absence of microbes (BS autoclaved).
2. This type accounts for any background degradation.
3. This type accounts for biodegradation through DGGTM inoculum only, without BS. In T-only system 1 mL of DGGTM-T added, and in BTX system 1 mL of DGGTM-B, DGGTM-T, and DGGTM-X added (total of 3 mL).
4. This type accounts for any biotic degradation without PAC present.
5. This type accounts for biotic degradation with PAC present.
6. BA = bioaugmented with DGGTM. In T-only system 1 mL of DGGTM-T added, and in BTX system 1 mL of DGGTM-B, DGGTM-T, and DGGTM-X added (total of 3 mL).
7. Triplicate set (3 bottles)

3.1.1 Materials

Toluene (purity > 99%, EMD Millipore, Darmstadt, DEN), benzene (purity 95%, EMD Millipore, China), and *o*-xylene (purity 99%, VWR Chemicals BDH®, Poole, ENG) were all analytical grade.

The sorbent material used was a coconut-based PAC product (WPC®, < 325 US mesh) used as received from Calgon Carbon Corporation (Pittsburgh, PA, USA). Table 3.2 outlines the typical physical and chemical properties (Marrocco, 2023; Calgon Carbon Corporation, 2015).

Table 3.2: Physical and chemical properties of WPC PAC.

Properties	Value
Ash Content	18 wt. % (max)
Moisture Content	8 wt. % (max)
<325 US mesh (0.045 mm)	90 wt. % (min)
<100 US mesh (0.150 mm)	99 wt. % (min)

The DGG™ cultures were obtained from SiREM (Guelph, ON, CAN) on an as-needed basis in 250 mL bottles with a Teflon lined septa. The cultures were stored in a dark environment at room temperature in a 4 L glass container sealed with a rubber stopper and anaerobic glove box tape. All cultures were fed their respective substrate on a weekly basis until use.

The aquifer material (BS) was obtained from cores taken from the University of Waterloo Groundwater Research Facility at Canadian Forces Base (CFB) Borden in Ontario, Canada. To maintain anaerobic conditions, the cores were encased in polyvinyl chloride (PVC) core sleeves capped at the ends. All cores were stored in the dark at the University of Waterloo at 4°C for 274 and 232 days prior to use in T-only and BTX microcosm experiments, respectively (Marrocco, 2023). Each core was cut into 0.3-meter sections to allow for handling within an anaerobic chamber with a N₂/H₂ atmosphere (Coy, Grass Lake, MI, USA). The open ends of each 0.3-meter section of core were immediately capped to preserve anaerobicity and transported into the anaerobic chamber. As an added measure to ensure material used for experiments was anaerobic, ~ 2.5 cm on each end was discarded. The remaining BS was then loaded into 0.6 m × 0.91 m sterile polypropylene autoclave bags (VWR, Padnor, PA, USA), and all BS (51.6 kg) was homogenized in preparation for microcosm and column experiments (Marrocco, 2023).

The BS composed of fine to medium grain sand (0.070 – 0.69 mm with a median grain size of 0.15 mm) (Mackay et al., 1986). The aquifer material was extracted from a shallow (1.5 to 4.5 m below ground surface (bgs)), unconfined, sandy aquifer located in a sandpit area with existing PHC contamination (BTX concentrations between ~ 0.15 and 1.3 mg/L). The groundwater contains background concentrations of iron (between ~ 22 and 28 mg/L) and sulfate (between ~ 10 and 29 mg/L) associated with a historical leachate plume. The aquifer material contains native anaerobic microbial communities of sulfate reducers and potentially methanogens that degrade TX compounds (Wei et al. 2018; Bartlett et al. 2019) that varies depending on the influx of organic contaminants, such as anthropogenic soluble gasoline compounds (Butler et al., 1997).

Artificial groundwater (AGW) adapted from Middledorp et al. (1998) was used in all microcosms (Table 3.3). Milli-Q water was obtained from a Millipore Milli-Q® water system (18.2 MΩ·cm at 25 °C).

Table 3.3: Composition of the artificial groundwater.

Chemical	Concentration (Source)
Salts	
NH ₄ Cl	0.93 mM (98%, Merck KGaA, Darmstadt, Germany)
MgCl ₂	0.05 mM (98%, Sigma-Aldrich, St. Louis, USA)
MnCl ₂ •4H ₂ O	0.02 mM (99%, Sigma-Aldrich, St. Louis, USA)
NaCl	0.12 mM (99%, Merck KGaA, Darmstadt, Germany)
CaCl ₂ •2H ₂ O	1 mM (99%, Merck KGaA, Darmstadt, Germany)
Na ₂ SO ₄	0.06 mM (99%, Anachemia Canada Inc, Montreal, Canada)
Sodium Bicarbonate	
NaHCO ₃	1.2 mM (99%, Sigma-Aldrich, St. Louis, USA)
Trace Minerals	
H ₃ BO ₃	4.85 µM (99.5+%, Sigma-Aldrich, St. Louis, USA)
ZnCl	1 µM (98+%, Alfa-Aesar, Ward Hill, USA)
NiCl ₂ •6H ₂ O	3.16 µM (98%, Alfa Aesar, Ward Hill, USA)
MnCl ₂ •4H ₂ O	5.05 µM (98%, Sigma-Aldrich, St. Louis, USA)
CuCl ₂ •2H ₂ O	0.59 µM (99+%, Alfa Aesar, Ward Hill, USA)
CoCl ₂ •6H ₂ O	6.3 µM (99%, Sigma-Aldrich, St. Louis, USA)
Na ₂ SeO ₃	0.12 µM (99.75%, Alfa Aesar, Ward Hill, USA)
Al ₂ (SO ₄) ₃ •18H ₂ O	0.15 µM (97%, Sigma-Aldrich, St. Louis, USA)
H ₂ SO ₄	0.1 µM (95%, Sigma Aldrich, St. Louis, USA)
Phosphate Buffer	
KH ₂ PO ₄	0.2 mM (99%, Sigma-Aldrich, St. Louis, USA)
K ₂ HPO ₄	0.2 mM (99%, Sigma-Aldrich, St. Louis, USA)
Ferrous Sulfide	
(NH ₄) ₂ Fe(SO ₄) ₂ •6H ₂ O	0.3 mM
Na ₂ S•9H ₂ O	0.3 mM
Resazurin	

3.1.2 Bottle Setup

Each microcosm experiment required 150 bottles (Table 3.1). A representation of the bottle setup for the different microcosm types is shown in Figure 3.1, and the gaseous, aqueous, and solid components for each series of microcosm bottle are listed in Table 3.4. The materials were loaded into sterile screw cap Boston round clear glass bottles (250 mL) (Systems Plus, New Hamburg, ON, Canada) sealed with Mininert® valve screw caps that served as the microcosm vessels. The microcosm vessel assembly was done inside an anaerobic glove chamber (COY Laboratory Products Inc.; Grass Lake, MI, USA) shown in Figure 3.2.

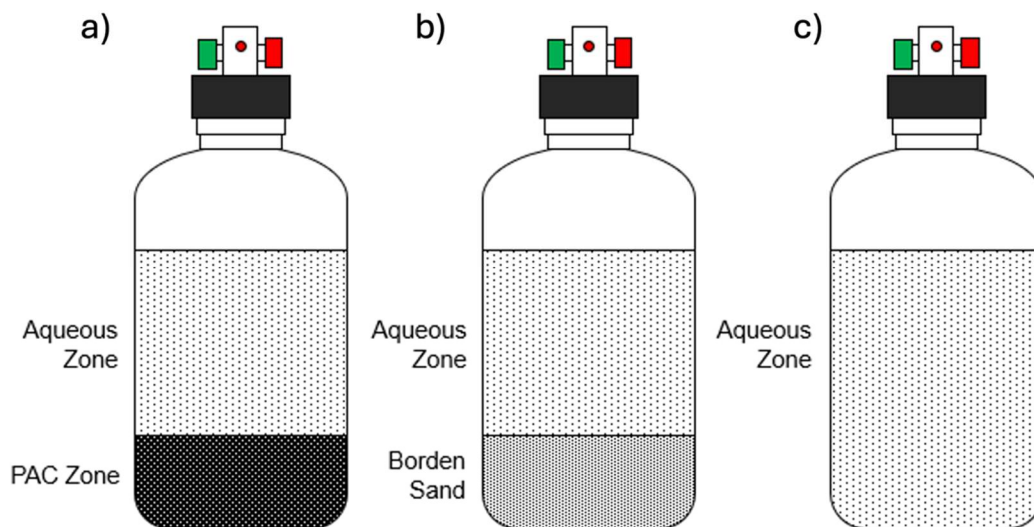


Figure 3.1: Microcosm schematic of the bottles for the a) PAC, b) KC, SC, A, and c) PC bottle types.

Table 3.4: Composition of microcosm bottles.

Phase	Component	Component volume	Component mass
Gaseous	Headspace ¹	30 mL	
	Artificial Groundwater ²	175 mL or 220 mL	
Aqueous	Toluene ³	4.2 µL	
	BTX ³	12.4 µL (4.1 µL benzene, 4.2 µL toluene, 4.1 µL <i>o</i> -xylene)	
	Biocide ⁴	2.5 mL mercuric chloride 0.45 mL sodium azide	
	Resazurin ⁵	0.1 mL	
	DGG TM Inoculum ⁶	1 mL (DGG-T only) 3 mL (DGG-B,T,X mixture)	
Solid	BS ⁷	45 mL	81.5 g
	PAC ⁸		15 mg

Notes :

1. Required for gas production of carbon dioxide and methane.
2. Aqueous volume designed in tandem with other major component additions. Volume depletion from sampling was designed to be < 10-20% of the initial aqueous volume.
3. Addition of pure phase compound(s).
4. Addition of 2.71 g/100 mL mercuric chloride and 5 g/100 mL sodium azide.
5. Colour indicator to ensure solution is sufficiently reduced and oxygen-free; addition to one bottle in a triplicate set.
6. In T-only system 1 mL DGGTM-T added, and in BTX system 1 mL of DGGTM-B, DGGTM-T, and DGGTM-X added (total of 3 mL).
7. BS from CFB Borden, bulk density of 1.81 g/cm³.
8. Coconut-based PAC (WPC®, < 325 US mesh) from Calgon Carbon Corporation.

**Figure 3.2:** Anaerobic glove chamber (Coy, Grass Lake, MI, USA) used in the microcosm experiments. Photograph taken by A. Marrocco, 2021.

3.1.3 Sampling Strategy

The T-only and BTX experiments followed the same sampling strategy with different sampling intervals for the different microcosm types. The bioaugmented microcosms were monitored periodically over the experimental period and any bottles under pressure from CO₂ or CH₄ accumulation (from any biodegradation) were degassed. Degassing was done using a 20 mL syringe with a Luer-Lok fitting attached to a 22-gauge stainless steel (SS) needle and allowing any gas under high pressure to be passively released into the syringe.

Samples were collected from the KC and SC bottles on Day 2 and ~ Day 7 following assembly, then every ~30 days for ~360 days (Figure 3.3). The first triplicate set from each of these microcosm series was repetitively sampled five times for aqueous analysis before being sacrificially sampled (i.e., the bottle is no longer used after sampling) to gather solid phase information. The second triplicate set was repetitively sampled seven times before being sacrificially sampled.

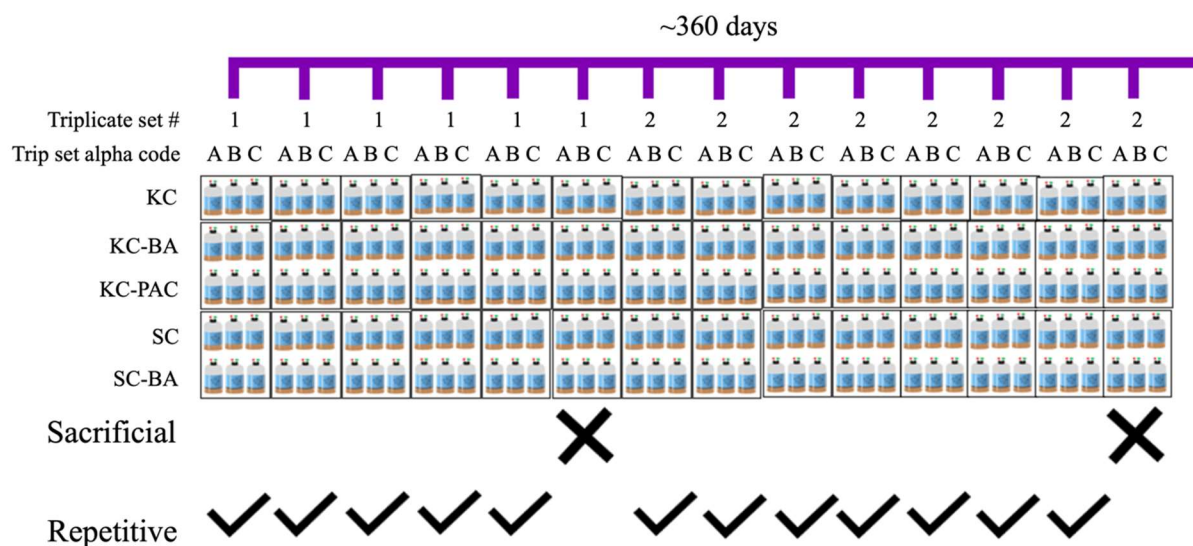


Figure 3.3: Visual representation of the sampling frequency for the KC type (KC, KC-BA, KC-PAC) and SC type (SC, SC-BA) microcosm bottles.

Similarly, the PC-BA, A, and A-PAC series were sampled at 30-day intervals for ~360 days with the first two sacrificial samples collected on Day 2 and ~Day 7 (Figure 3.4). The remaining six triplicate sets were repetitively sampled once before being sacrificially sampled.

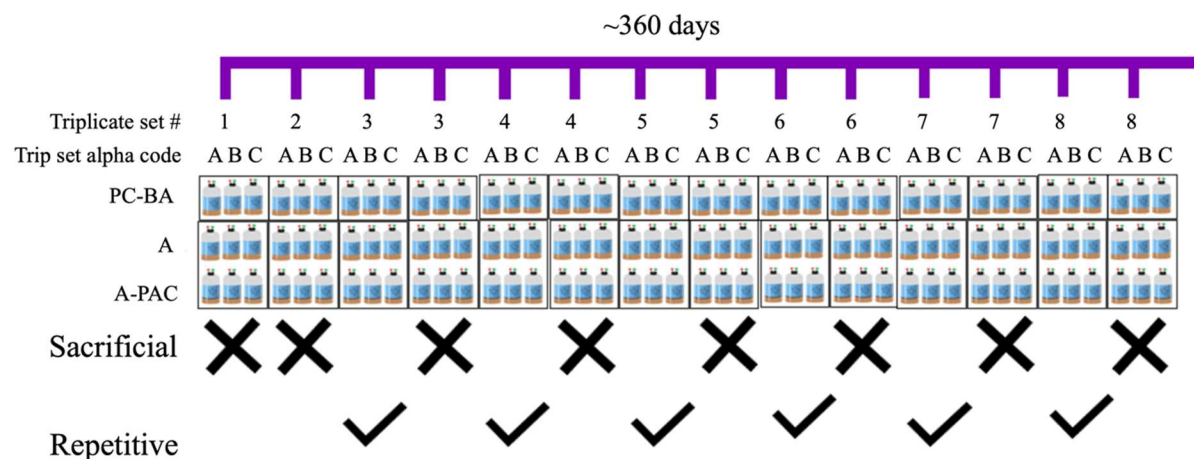
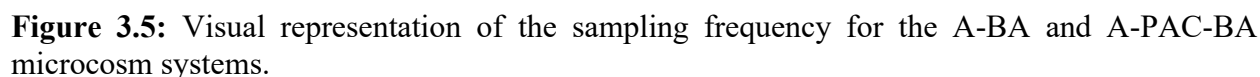


Figure 3.4: Visual representation of the sampling frequency for the PC-BA, A, and A-PAC type microcosm systems.

Figure 3.5 provides a visual representation of the sampling frequency for the A-BA and A-PAC-BA microcosm systems. The A-BA and A-PAC-BA series were sampled at 30-day intervals for ~270 days for the T-only and 310 days for the BTX experiments. The first two sacrificial samples were collected on Day 2 and ~Day 7. To investigate the biodegradation rates of the two systems, a higher temporal resolution sampling strategy was implemented during the final ~30 days of each experiment (Figure 3.5, Table 3.5). For these sampling events, one bottle from the triplicate set was sacrificially sampled every 5 days for solid phase T or BTX analyses, and the aqueous phase in the remaining bottles were sampled. This strategy ensured that the samples from each microcosm system could be compared and that the solution volume remained relatively constant. Due to the decreasing number of bottles sampled over time, the ability to capture experimental variance was reduced. A DNA sample was obtained during the 8B sacrificial sampling episode, where the the solid phase slurry was split for the DNA sample and the solid phase T or BTX sample.



Triplicate Set	Bottle ¹	Event 1	Event 2	Event 3	Event 4	Event 5	Event 6
7	7A	sacrificial					
	7B	repetitive	sacrificial				
	7C	repetitive	repetitive	sacrificial			
8	8A	repetitive	repetitive	repetitive	sacrificial		
	8B ²	repetitive	repetitive	repetitive	repetitive	sacrificial	
	8C	repetitive	repetitive	repetitive	repetitive	repetitive	sacrificial

1. The numerical value associated with the bottle is from the parent triplicate set, and the letter order is the specific sample from the triplicate within the parent set.
2. Sacrificial sampling included DNA sample.

This section provides the details of the sample collection and analytical methods for the microcosm bottles. All sampling was done inside an anaerobic chamber. The bottles to be sampled were manually shaken for several minutes one day prior to sampling the triplicate sets. During each repetitive sampling event, samples were collected to measure the following parameters:

- 22

Additionally, dissolved inorganic carbon (DIC) was sampled during the first repetitive sampling event of the KC, KC-PAC and SC microcosms to establish baseline values.

During each sacrificial sampling event, samples were collected to measure the following parameters:

1. pH, ORP, DO, and sulfate (5 mL)
2. $\text{BTX}_{(\text{aq})}$ (2 mL)
3. Solid phase BTX in the BS and PAC ($\text{BTX}_{(\text{s})}$)
4. $\text{CO}_{2(\text{g})}$ and $\text{CH}_{4(\text{g})}$ (5 mL)
5. DIC (3.5 mL)

Solid phase samples were also collected during the last sacrificial sampling event (Table 3.5, timepoint 8B) and were split in half for BTX and DNA analysis.

pH, ORP, DO

The sample was taken directly from the microcosm bottles using a 10 mL glass syringe (Micro-MateR, Popper and Sons Inc.; New Hyde Park, NY, USA) and transferred to a 5 mL disposable ion chromatography (IC) autosampler vial (Thermo Scientific, Waltham, MA, USA) inside the anaerobic chamber. pH was measured using an Oakton pHTestr® 50S Spear-Top Waterproof Pocket Tester (Oakton Instruments, Vernon Hills, IL, USA) that was calibrated before each sampling using three pH standards: 4.0, 7.0, and 10. ORP was measured using a Thermo Scientific Orion™ Star A325 meter (Thermo Scientific; Waltham, MA, USA) that was calibrated prior to each sampling event according to the manufacturer instructions using Zobell's solution. Finally, DO was measured using a Thermo Scientific Orion™ 5Star- 083005MD probe (Thermo Scientific; Waltham, MA, USA) that was calibrated before each sampling event according to the manufacturer's instructions using a 100% humidity calibration.

Sulfate

Following measurement of pH, ORP, and DO, the sample contained in a 5 mL disposable ion chromatography (IC) autosampler vial was removed from the glove chamber and diluted with Milli-Q water up to 5 mL for sulfate analysis (repetitive samples had a dilution factor of 5, sacrificial samples were undiluted). Sulfate samples were then analyzed on a Dionex ICS-1100 IC

equipped with a Dionex AS-DV Autosampler (Dionex Corporation, Sunnyvale, CA, USA). 0.025 mL of the sample was injected into an AG4A-SC (4 × 50mm) guard column and AS4A-SC (4 × 250mm) column. Operating conditions included: 1.8 mM Na₂CO₃/1.7 mM NaHCO₃ eluent concentration, 2.0 mL/min flow rate, ambient temperature, suppressed conductivity detection using a Dionex Anion Self-Regenerating Suppressor 43 (Dionex ADRS 600 4mm) in Auto Suppression Recycle Mode, and 32 mA applied current (method detection limit (MDL) = 0.1 mg/L) (Marrocco, 2023).

BTX

Aqueous BTX samples were collected by taking 2 mL from the aqueous phase of the bottles using a 10 L glass syringe (Micro-MateR, Popper and Sons Inc.; New Hyde Park, NY, USA). The samples were then injected into a 4.5 mL glass vial with a PTFE Teflon lined septa that was preloaded with 1 mL of dichloromethane (DCM) that contained internal standards of *m*-fluorotoluene and 2-fluorobiphenyl (25 mg/L) (Henderson et al., 1976; Freitas and Barker, 2008). The 4.5 mL glass vial was then taken out of the glove chamber and put on a shaker table (arranged horizontally) and shaken for 15 minutes at 300 rpm. The vial was then oriented vertically for 30 minutes to allow for separation of the solvent from the remaining aqueous phase. An aliquot (> 0.7 mL) of the solvent (DCM with internal standards) was removed using a 1 mL glass syringe and transferred into a 2 mL glass autosampler vial.

During sacrificial sampling, the solid phase BTX was isolated by decanting the remaining aqueous phase from the 250 mL microcosm bottle with a 100 mL glass syringe (Micro-MateR, Popper and Sons Inc., New Hyde Park, NY, USA) attached to an 18-gauge SS needle (Ace Glass, Vineland, NJ, USA). An attempt was made to disturb the solids as little as possible during this process. 20 mL of DCM containing internal standards (*m*-fluorotoluene and 2-fluorobiphenyl (25 mg/L) was then added to saturate the PAC and BS. As the final sacrificial sample was split in half for solid phase BTX and DNA analysis, only 10 mL of DCM was used for those samples. The bottle was shaken by hand for several minutes and vented before being sealed with Teflon lined silicon septa screw caps. An aliquot was then extracted and processed using the same method as described for the aqueous samples and transferred into a 2 mL glass autosampler vial.

Both the aqueous and solid phase samples were analyzed on an Agilent 7890A gas chromatography (GC) equipped with a flame ionization detector (FID). An injection volume of 3 μ L was run on splitless mode (purge on 30 seconds, purge off 10 minutes) onto a 0.25 mm by 30 m DB5 capillary column with a stationary phase film thickness of 0.25 μ m. Specific GC conditions were: port temperature of 275°C, initial column temperature of 35°C held for 0.5 min, heating rate of 15°C/min, final temperature of 300°C held for 2 min, detector temperature of 325°C, and column flow rate of 1 mL/min with helium as the carrier. Calibration was carried out with external standards of methanolic stock of pure compounds at five different concentrations (Henderson et al., 1976; Freitas and Barker, 2008). The MDL for the aqueous B, T, and X were 0.108 mg/L, 0.049 mg/L, and 0.045 mg/L respectively. The MDL for the solid phase concentration was 0.08 μ g/g for benzene, toluene, and *o*-xylene (Marrocco, 2023)

CO₂ and CH₄

Samples for CO_{2(g)} and CH_{4(g)} were collected directly from the gas phase of microcosm bottles using a 10 mL gas tight sampling syringe (Micro-MateR, Popper and Sons Inc., New Hyde Park, NY, USA) with a two-way stop valve. The syringe was equipped with a 1-way polycarbonate stopcock and 22-gauge disposable needle. The stopcock was closed, the syringe was taken out of the glove chamber, and the sample was diluted with purified helium if necessary before analysis. Samples were run immediately in a Shimadzu CG-2014 GC for greenhouse gas analysis (Shimadzu, Kyoto, Japan). 5 mL of the sample was injected into the sample loop and passed on a 1.5 m by 3.2 mm mesh SS column (HayeSep N 80/100) that traps and flushes water vapor. The retained was then passed through a 5 m by 3.2 mm mesh SS column (HayeSep D 80/100) to separate the CO₂ and CH₄. The gas was measured by a thermal conductivity detector (TCD) or FID with a methanizer run for 14 minutes. Operation conditions were: an oven temperature of 80°C, a split temperature of 100°C, an FID temperature of 250°C, FID flame with 50 kPa air and 66 kPa hydrogen, a TDC temperature of 100°C and 125 mA, a TDC reference of 57.5 kPa, a methanizer temperature of 380°C, and a valve temperature of 80°C. The MDL was: 0.005 mg/L for CH₄ and 0.1 mg/L for CO₂.

DIC

DIC samples were taken directly from the aqueous phase of the microcosm bottles using a 10 mL glass syringe (Micro-MateR, Popper and Sons Inc.; New Hyde Park, NY, USA) and a two-way stop valve. An aliquot of the sample was passed through a 0.2 μm membrane filter then transferred to a 4.5 mL glass vial with a butyl rubber cap with PTFE Teflon line septa with no headspace. The 4.5 mL glass vial was preloaded with 1.4% of mercuric chloride stock solution (2.71 g/ 100 mL). DIC samples were analyzed on a Dohrmann DC-190 (Rosemount Analytical Inc., Shakopee, MN, USA). Manual injection of 0.2 mL of sample was sent into the instrument at a flow rate of $200 \pm 5 \text{ cm}^3/\text{min}$ and acidified with 20% H_3PO_4 (Sigma-Aldrich, St. Louis, MO, USA) at ambient temperature. The sample was then loaded on a catalyst bed and held at an elevated temperature until the carbon in the sample was converted to CO_2 through combustion. The CO_2 was then measured by a non-dispersive infrared detector (NDIR) (Marrocco, 2023).

DNA Samples

DNA samples were extracted from the solid phase at Day 0 for baseline characterization and at timepoint 8B (Table 3.5) of both the T-only and BTX microcosms. Samples were collected by removing half of the solid sample mass (BS and plus PAC if present) from the bottle, and placing it into an 50 mL plastic screw top tube (Eppendorf, Hamburg, DE). The samples were then stored immediately at -80°C until being shipped to the BioZone Centre for Applied Bioscience and Bioengineering (University of Toronto) for molecular analyses (Marrocco, 2023). DNA samples were assayed by quantitative polymerase chain reaction (qPCR) using the primer pairs listed in Table B.4 and reaction conditions summarized by Edwards et al., (2021). Reaction mixtures (20 μL) contained 500 nM of each forward and reverse primer, 2 μL of template DNA, 10 μL $2 \times$ SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and UltraPure DNase/Rnase-Free Distilled Water (Invitrogen, Carlsbad, CA, USA). Serial dilutions of plasmids containing corresponding targeted gene fragments were used to generate standard curves. Each qPCR reaction was performed in duplicate on a Bio-Rad CFX96 real time PCR machine. Thermal cycling conditions included: 98°C initial denaturation step temperature (2 min), 40 cycles of denaturation (5 sec, 98°C) and annealing/extension (10 sec, see Table B.4 for annealing temperature), and 65 to 95°C melt curve analysis temperature with an increase of 0.5°C every 10

sec. qPCR results were analyzed using Bio-Rad CFX Manager software [154]. The amplification efficiency for each qPCR run batch ranged as follows (with R^2 values of > 0.996) (Marrocco, 2023):

- Total bacteria: 93.6 - 101.5%
- Total archaea: 87.2 - 104.1%
- Deltaproteobacteria ORM2: 93.7 - 99.8%
- Desulfosporosinus (DSP): 92.8 - 98.2%
- Peptococcaceae (PEP): 86.3 - 94.3%

3.1.5 Data Analyses

Mass Balance

To estimate biodegradation rates of BTX in the A-BA and A-PAC-BA columns, the high resolution sampling data was used to assess how much BTX mass was present in each phase (gaseous, aqueous, solid) through compartmental mass distributions. The total mass of solute i is the sum of the mass of that solute in each phase:

$$m_i = m_{i(g)} + m_{i(aq)} + m_{i(s)} \quad (3.1)$$

where $m_{i(g)}$ = the mass in the gaseous phase, $m_{i(aq)}$ = the mass in the aqueous phase, and $m_{i(s)}$ = the measured mass in the solid phase.

The mass in the aqueous phase was calculated using:

$$m_{i(aq)} = C_{i(aq)} \times V_{aq} \quad (3.2)$$

where $C_{i(aq)}$ = the measured aqueous phase concentration of solute i (mg/L), and V_{aq} = the volume of the aqueous phase (L).

The mass in the gaseous phase was estimated based on Henry's Law and the discrete results from the aqueous sampling:

$$m_{i(g)} = k_{h,i} \times C_{i(aq)} \times V_g \quad (3.3)$$

where $k_{h,i}$ = dimensionless Henry solubility constant of solute i (mg/L), and V_g = the volume of the gas phase (L). The $k_{h,i}$ values used for BTX were 0.225, 0.274, 0.221, respectively (Sander, 2015).

The total measured solid phase mass ($m_{i(s)}$) was assumed to be the sum of the mass residing in the different solid phase components:

$$m_{i(s)} = m_{i(BS)} + m_{i(pore)} + m_{i(PAC)} \quad (3.4)$$

where $m_{i(BS)}$ = the mass sorbed to the BS, $m_{i(pore)}$ = the aqueous mass in the pore water, and $m_{i(PAC)}$ = the mass sorbed to PAC if present (in the A-BA bottles, $m_{i(PAC)} = 0$).

The total volume the water in the pores (V_{pore}) was calculated from the known mass of BS (m_{BS}) in the system and from weighing the bulk BS sample (m_T):

$$V_{pore} = \frac{m_{pore}}{\rho_w} = \frac{m_T - m_{BS}}{\rho_w} \quad (3.5)$$

where m_{pore} = the mass of water in the pores, and ρ_w = the density of water (1 g/mL). An average of the six bottles from the high resolution sampling was used to determine V_{pore} , which was used with the aqueous concentration to determine the BTX mass in this compartment:

$$m_{i(pore)} = C_{i(aq)} \times V_{pore} \quad (3.6)$$

For the A-BA systems, the remaining BTX mass was attributed to the BS (i.e., $m_{i(BS)} = m_{i(s)} - m_{i(pore)}$). For the A-PAC-BA bottles, however, the remaining BTX resided in both the BS and the PAC. In this case, $m_{i(BS)}$ was calculated using the calculated soil adsorption coefficient, $K_{d,i}$, for each solvent:

$$m_{BS,i} = K_{d,i} \times C_{i(aq)} \times m_{BS} \quad (3.7)$$

Values for $K_{d,i}$ are defined as:

$$K_{d,i} = K_{oc,i} \times f_{oc} \quad (3.8)$$

where $K_{oc,i}$ = the organic carbon partition coefficient of solvent i (in L/kg), and f_{oc} = the fraction of organic content sorbed to sand, which was taken as 0.00024 from Xu and Thomson (2008). The empirical relationship for $K_{oc,i}$ was then used (Doucette, 2003; Karickhoff et al., 1979):

$$K_{oc,i} = 10^{\log K_{ow,i} - 0.21} \quad (3.9)$$

where $K_{ow,i}$ = the octanol-water partition coefficient of solvent i (in L/kg). Values for $\log K_{ow,i}$ were directly sourced from tabulated values (USEPA, 1996). A summary of the $\log K_{ow,i}$ values used and the calculated $K_{oc,i}$ and $K_{d,i}$ values are presented in Table 3.6

Table 3.6. Summary of partition coefficients used.

	$\log K_{ow}^1$	K_{oc} (L/kg)	K_d (L/kg)
Benzene	2.13	83.18	0.020
Toluene	2.75	346.74	0.083
<i>o</i> -Xylene	3.13	831.76	0.200

Notes :

1. Octanol-water partition coefficients were retrieved from Appendix K of the USEPA Superfund Soil Screening Guidance document (USEPA, 1996).

Finally, $m_{i(PAC)}$ was calculated as the remaining portion of BTX mass:

$$m_{i(PAC)} = m_{i(s)} - m_{i(BS)} - m_{i(pore)} \quad (3.10)$$

Theoretical TIC and methane estimates

Calculation of the theoretical TIC and methane generation at each sampling timepoint was completed for the PC-BA, A-BA, and A-PAC-BA systems. The stoichiometry used for benzene, toluene, and *o*-xylene biodegradation through both sulfate reducing and methanogenic pathways used for the theoretical mass estimates is taken from Farhadian (2008) and shown in Table 2.2. It is important to note that the theoretical TIC and methane production calculations do not account for the background degradation of cells or for the use of carbon for microorganism growth. Additionally, the calculations only used aqueous BTX concentrations and not solid phase concentrations. For PAC bottles, this means earlier timepoints in a biodegradation cycle would have higher TIC and methane production estimates as sorption to the PAC leads to a decrease in

aqueous concentrations. By the end of each biodegradation cycle, estimates would be reflective of complete biodegradation.

If sulfate was present, the method of biodegradation was assumed to be completely due to sulfate reduction pathways, which generate carbon dioxide but no methane. Carbon dioxide mass was calculated using the decrease in BTX mass at each timepoint and the associated stoichiometric relationship. The estimated CO₂ mass was added to the initial TIC after a conversion from CO₂ mass to as-carbon mass (mg-C). The as-carbon mass values calculated at each timestep were cumulatively added over the course of the microcosm system experiment. The initial value of TIC was taken from a concentration measurement on experiment Day 2. Finally, the standard deviation of the theoretical TIC mass estimates was calculated following the method described in Appendix A1.

Once sulfate was sufficiently reduced to the point where methanogenic activity would dominate, the methanogenic pathway was used to estimate the mass of methane and carbon dioxide generated. Similar to the TIC calculations, theoretical methane generation estimates were calculated from decreasing BTX concentrations at each sampling time. The standard deviation of the theoretical methane values was calculated following the method described in Appendix A1.

3.2 Results and Discussion

The methods outlined in Section 3.1 were executed as designed with no deviations. Degassing events occurred on Day 170 and Day 205 for the toluene-only systems, and on day 63 for the BTX systems. Some microcosms required re-addition of toluene or BTX to reach a nominal 20 mg/L due to depletion of solutes through biodegradation.

This section presents the results of each microcosm system type for both the T-only and BTX experiments to investigate differences in the biodegradation rates of BTX compounds among the microcosm system types. Aqueous and solid phase concentrations of toluene (for the T-only microcosms), BTX (for the BTX microcosms), and their biodegradation by-products (carbon dioxide and methane) are presented. qPCR results that highlight population dynamics of certain fermenters of BTX, total counts of archaea and bacteria are discussed. Finally, theoretical estimates of TIC and methane are compared to experimental results, and compartmental mass balance in the high-resolution sampling period are used to aid in determining the differences in biodegradation rates between bioaugmented systems.

3.2.1 Toluene-only Microcosms

Killed-Control Systems

The time series of aqueous and solid phase toluene concentrations, TIC concentration, and methane mass for the three killed control microcosm systems are shown in Figure 3.6. These microcosms contained biocide and represented abiotic degradation only. Microcosm series included KC (Figure 3.6a), KC-BA (Figure 3.6b), and KC-PAC (Figure 3.6c). Results from all three killed control microcosm systems suggest that no biodegradation took place, as methane production was not evident ($< \text{MDL}$) and there were relatively stable aqueous phase toluene concentrations (Figure 3.6). The aqueous phase toluene concentration in the KC and KC-BA systems steadily decreased from $19.6 \pm 0.8 \text{ mg/L}$ and $20.8 \pm 0.4 \text{ mg/L}$ on Day 0 to $14.8 \pm 1.2 \text{ mg/L}$ and $13.9 \pm 0.7 \text{ mg/L}$ on Day 366, respectively. The declining concentration trend is perhaps due to the sampling methodology where repetitive sampling results in a small loss of toluene mass during each sampling episode. The aqueous toluene concentration in the KC-PAC system had an abrupt decrease at Day 7 from $18.0 \pm 1.0 \text{ mg/L}$ to $6.38 \pm 0.22 \text{ mg/L}$ after which it remained relatively

stable at ~6.5 mg/L for the remainder of the experiment (Figure 3.6c). The sharp decrease in aqueous toluene concentration coincides with the first sampling episode following the addition of PAC, demonstrating a shift in mass which primarily resided in the aqueous phase towards a substantial solid phase toluene concentration.

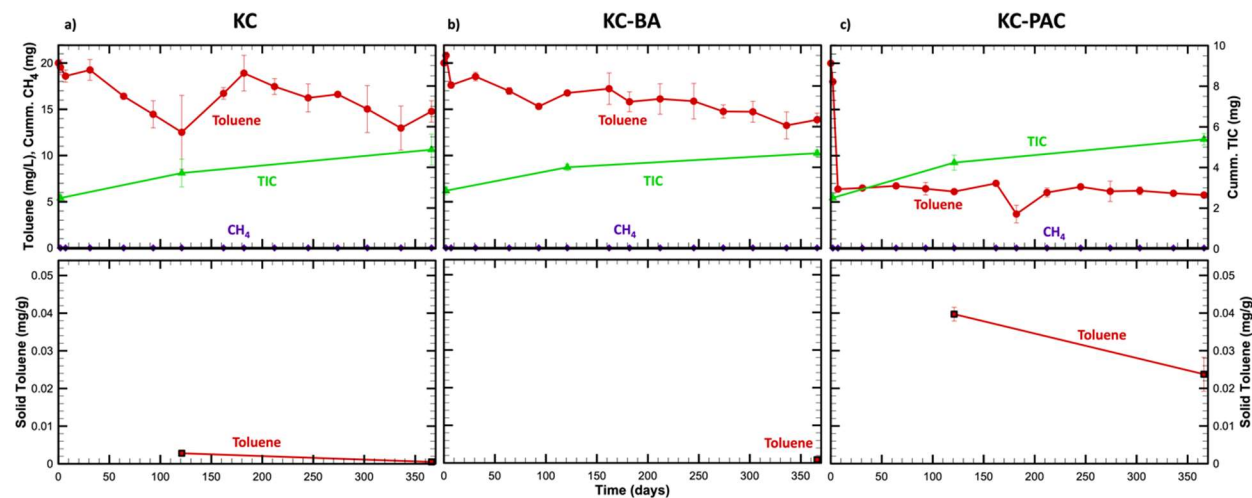


Figure 3.6: a) Aqueous phase toluene concentration, TIC mass, methane, and b) solid phase toluene concentration for the (left to right) KC, KC-BA, and KC-PAC toluene microcosm systems. Error bars represent $\pm$ one standard deviation.

On Day 366, the solid phase toluene concentrations were very low in the KC ($0.505 \pm 0.210 \mu\text{g/g}$) and KC-BA ($0.877 \pm 0.342 \mu\text{g/g}$) microcosm systems compared to the KC-PAC microcosm system ($23.8 \pm 4.5 \mu\text{g/g}$) (Figure 3.6). In the KC systems without PAC, 250 – 300 % more of the toluene mass resided in the aqueous phase compared to the KC-PAC system as sorption to BS is low. In the KC-PAC system, the solid phase toluene concentration declined from $39.7 \pm 1.8 \mu\text{g/g}$ on Day 121 to $23.8 \pm 4.5 \mu\text{g/g}$ on Day 366 (Figure 3.6c). The lack of methane generation and stable aqueous toluene concentrations indicate that no biodegradation occurred. The TIC mass in the system increased from $2.51 \pm 0.19 \text{ mg-C}$ on Day 2 to $5.39 \pm 0.28 \text{ mg-C}$ on Day 366. This increase in TIC was caused by the biocide in the system (HgCl_2), which underwent HgCl_2 acidification (Clayer et al., 2023). The KC, KC-BA, and KC-PAC microcosm systems demonstrate baseline system behaviour without biodegradation present and serve as a reference for the active microcosm experiments.

Starved Control Systems

The time series of methane and TIC mass for the two starved control microcosm systems are shown in Figure 3.7a. This system had aqueous toluene concentrations < MDL, therefore no biodegradation was expected as supported by the constant TIC mass and the absence of methane production. The lack of evidence of toluene biodegradation in the starved control systems confirms that the BS had no background concentrations of toluene. In both SC systems, sulfate decreased throughout the experimental period (see Appendix A1, Figure A.2), which suggests the presence of native sulfate-reducing microorganisms degrading other compounds in the BS that were not monitored.

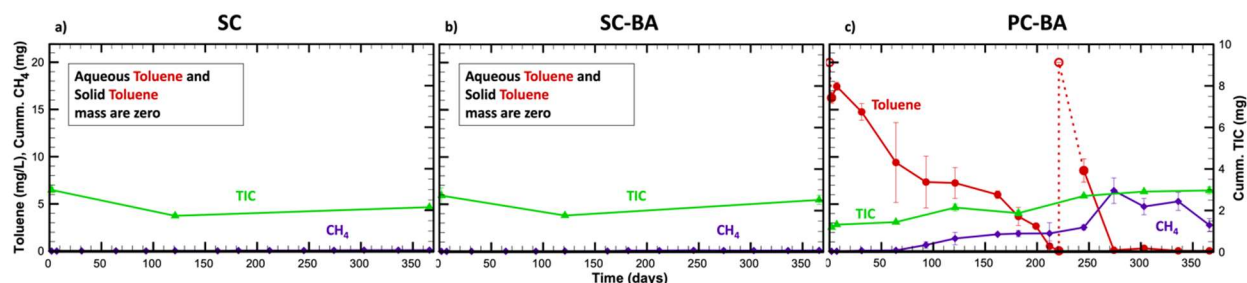


Figure 3.7: Aqueous phase toluene concentration, TIC mass, and methane for the a) SC, b) SC-BA, and c) PC-BA toluene microcosm systems. Error bars represent $\pm$ one standard deviation. Open circle points on the aqueous phase toluene component are theoretical, based on the target dosage of toluene into the system. The dashed line connecting the open circle symbols are assumed trends.

Positive Control System

Figure 3.7(c) shows time series of aqueous and solid phase toluene concentrations, and TIC and methane mass, for the positive control (PC-BA) microcosm system. In this system, no BS was present, so biodegradation resulted only from the added DGGTM-T culture. The DGGTM-T culture contains sulfate-reducing organisms which utilized the sulfate present. The aqueous phase toluene concentration decreased at a rate of 0.143 ± 0.176 mg/L-d from Day 7 to Day 64. Since no methane was produced during this period, biodegradation was caused by sulfate reduction, which is supported by sulfate depletion (8.34 ± 0.46 mg/L on Day 7 to 0.24 ± 0.03 mg/L on Day 121) (see Appendix A1, Figure A.3). Following sulfate depletion, the biodegradation rate declined to 0.032 ± 0.036 mg/L-d between Day 64 to Day 162, before increasing to 0.103 ± 0.050 mg/L-d from Day

162 to Day 212 as a result of a shift towards a more methanogenic microbial population in the presence of toluene due to the significant decrease of sulfate (Edwards and Grbic-Galic, 1994). Following the re-addition of toluene on Day 221, the biodegradation rate increased to 0.372 ± 0.673 mg/L-d from Day 221 to Day 274 (Figure 3.7c).

The biodegradation of toluene was accompanied by a consistent increase in both TIC and methane (Figure 3.7c). Through the first degradation cycle (initial 20 mg/L toluene to < MDL, from Day 2 to Day 162), the slow increase in TIC (0.00442 ± 0.00499 mg-C/d up to Day 182) and methane production (0.0108 ± 0.00257 mg/d to Day 212), was consistent with the slower toluene biodegradation rate during this period. During the second degradation cycle, there was a similar increase in TIC (0.00573 ± 0.00887 mg-C/L from Day 182 to Day 366) and a rapid increase in methane (0.0293 ± 0.0484 mg-C/d from Day 212 to Day 336) production as a result of the accelerated biodegradation rate of toluene.

Active Systems

Aqueous and solid phase toluene concentrations, and TIC and methane mass in the A and A-PAC microcosm systems are shown in Figure 3.8. These systems are identical to the KC systems except no biocide was added and the BS was not autoclaved. Both microcosm systems had stable aqueous phase toluene concentrations after an initial period (< 50 days) of slight decline (Figure 3.8). This behaviour is suspected to be caused by sulfate reducing organisms native to the BS utilizing the initial sulfate present, which is supported by the rapid decrease in sulfate in the microcosms (see Appendix A, Figure A.4). The end of this initial period of toluene reduction coincides with depletion of background sulfate in the system (Day ~100), after which a near stable toluene concentration was observed (Figure 3.8). Similar to the KC-PAC microcosm system, the addition of PAC led to a rapid decline in aqueous phase toluene in the A-PAC system (Figure 3.8b). For both systems, there was a slight increase in methane mass (Figure 3.8), which was caused by methanogens in BS using available toluene to undergo methanogenesis once sulfate had been reduced (Toth et al., 2021). TIC mass remained relatively constant (~2 mg-C), further supporting that minimal biodegradation was occurring. Similar to the KC systems, 250 – 300 % more mass resided in the solid phase in the A-PAC system versus the A system due to the presence of the

PAC. The solid phase toluene concentration was also relatively constant once the background sulfate was depleted (~Day 30) and remained at ~0.03 mg/g.

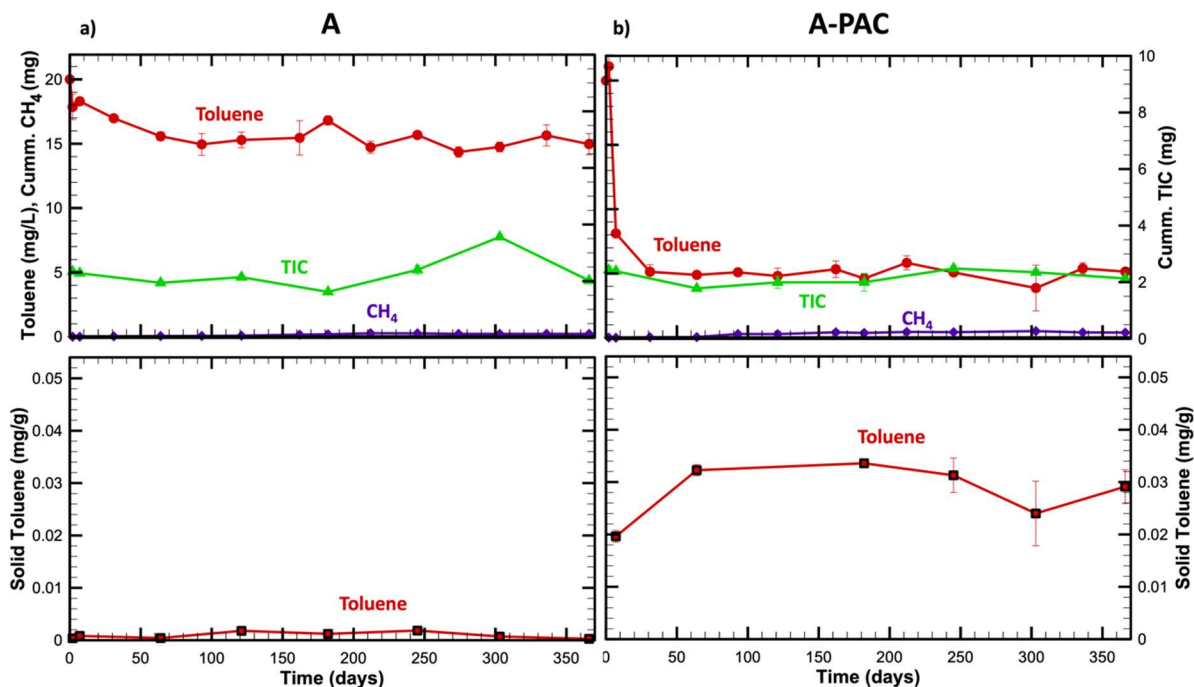


Figure 3.8: Aqueous phase toluene concentration, TIC mass, and methane (top), and solid phase toluene concentration (bottom) for the a) A and b) A-PAC toluene microcosm systems. Error bars represent $\pm$ one standard deviation. Open circle points on the aqueous phase toluene component are theoretical, based on the target dosage of toluene into the system.

Bioaugmented Systems

Figure 3.9 shows the aqueous and solid phase toluene concentrations, and TIC and methane mass for the two active bioaugmented microcosm systems (A-BA, and A-PAC-BA) that contained the DGGTM-T culture. In comparison to the previously discussed microcosm systems, these two systems were the most bio-active due to presence of BS and bioaugmentation cultures, and required toluene re-additions on five occasions (Day 77, 149, 199, 221, and 267) resulting in six degradation cycles. The first degradation cycle of toluene from 20 mg/L to < MDL occurred over ~75 days in both systems. By the last degradation cycle, aqueous phase toluene decreased to < MDL within the ~30-day sampling frequency following each successive re-addition of toluene. The slower biodegradation of aqueous toluene in the first degradation cycle was also present in the PC-BA system (Figure 3.7). Previous work has shown that increasing rates of biodegradation

are due to increasing microbial populations, culture acclimation, and biofilm development (Edwards and Grbic-Galic, 1994; de León, 2021; Toth et al, 2021). The A-BA system degraded more toluene than the PC-BA system because of the presence native microbial populations and larger surface area to allow for biofilm development (de León, 2021). The difference between the A-BA and PC-BA systems may also be due to small variations in the amount of culture addition leading to higher microbial populations in the A-BA system during injection.

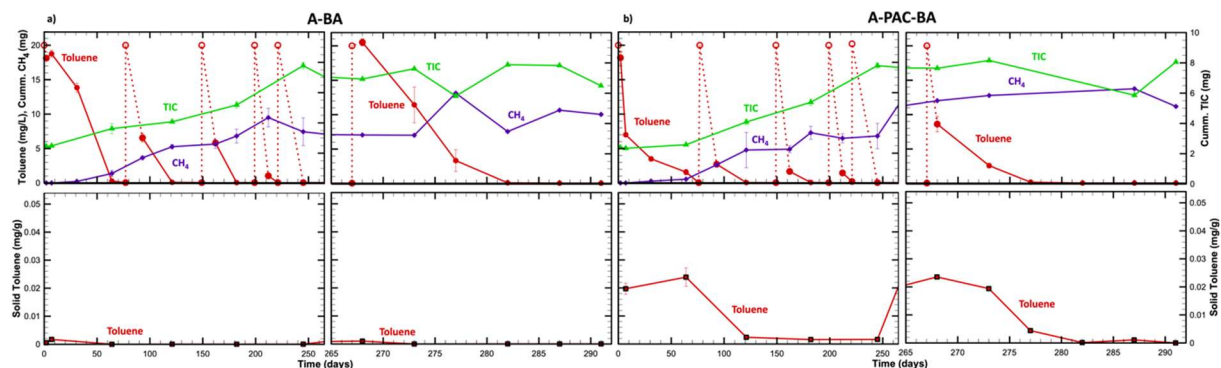


Figure 3.9 Aqueous phase toluene concentration, TIC mass, and methane (top), and solid phase toluene concentration (bottom) for the a) A-BA and b) A-PAC-BA microcosm systems from experiment Days 0-265, and the high-resolution toluene sampling days from Days 265- 300. Error bars represent $\pm$ one standard deviation. Open circle points on the aqueous phase toluene component are theoretical, based on the target dosage of toluene into the system. The dashed line connecting the open circle symbols are assumed trends.

Both the A-BA and A-PAC-BA systems demonstrated similar biodegradation rates and the same amount of toluene mass biodegraded over six biodegradation cycles to $< \text{MDL}$ (Figure 3.9). The aqueous concentration of toluene was biodegraded from 20 mg/L to $< \text{MDL}$ in 75, 44, 33, 22, 24, and 14 days for each biodegradation cycle, respectively. As expected, the A-PAC-BA system had more solid phase toluene mass than the A-BA system at timepoints where aqueous toluene was $< \text{MDL}$ (Day 121 = 0.00241 ± 0.00013 mg/g, Day 182 = 0.00159 ± 0.00020 mg/g, and Day 245 = 0.00164 ± 0.00006 mg/g). This suggests that the biodegradation of toluene was incomplete ($\sim 96\%$ complete to $< \text{MDL}$) before re-addition of toluene in the A-PAC-BA system.

The TIC mass in both systems increased from ~ 2 to ~ 8 mg-C by the end of the experiment. Methane production increased from $< \text{MDL}$ to just over 10 mg by end of the experiment for both systems. For the A-BA system, methane production slowed during two separate biodegradation cycles (Day ~ 220 to 273), which is not supported by the other lines of evidence. Since sulfate

concentrations were < 0.3 mg/L over this period (see Appendix A1, Figure A.5) methanogenesis was the only viable biodegradation pathway. This apparent stall in methane production could be due to experimental upsets (e.g., bottle leaks caused by improper storage in the anaerobic glove chamber, and over-punctured/faulty septa).

qPCR results for the active toluene microcosm systems show the total bacteria, total archaea, and *Desulfosporosinus* (DSP) community populations (see Figure 3.10). Total bacteria for all microcosm systems ranged from 5×10^6 to 10^7 copies/g. Total archaea was lowest in the time zero sample in the DGGTM-T culture vessel (4×10^4 copies/g) compared to the microcosm systems (A and A-PAC are within 5×10^5 - 10^6 copies/g; A-BA and A-PAC-BA are within 5×10^6 - 10^7 copies/g). The higher archaea populations in the BA microcosms reflect increased methanogenesis in these systems (Toth et al., 2021). DSP populations were lower in the A and A-PAC microcosms ($< 10^4$ copies/g) than in the time zero sample ($\sim 7 \times 10^4$ copies/g) and were highest in the A-BA and A-PAC-BA microcosms (10^6 - 10^7 copies/g). As DSP is the qPCR primer target in the DGGTM-T culture (Toth et al., 2022), the elevated DSP values present in the two BA systems support the higher degree of biodegradation observed. The lower DSP populations in the A and A-PAC microcosm were due to initial sulfate depletion followed by a slow shift to methanogenesis to degrade toluene. It is likely that toluene biodegradation would have continued if sulfate was replenished in the system.

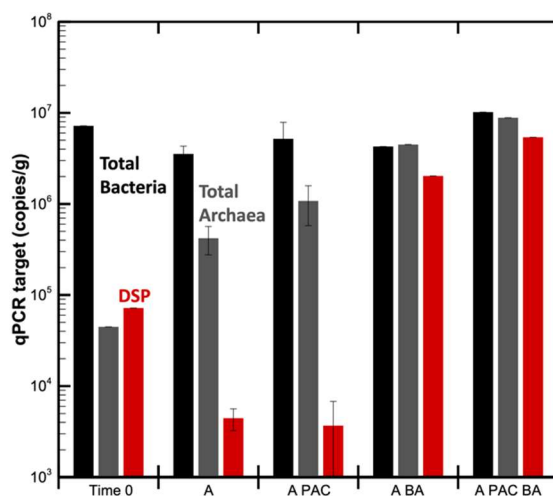


Figure 3.10: qPCR analysis results of the total bacteria, total archaea, and DSP populations for DGGTM-T (time zero), and microcosm samples taken from the active microcosm systems at the end of the T-only experiment.

The time series of the observed cumulative TIC and methane mass for all microcosm systems that contained the DGGTM-T culture along with their associated theoretical values are shown on Figure 3.11. For the PC-BA microcosm system (Figure 3.11a), the theoretical methane mass was consistently lower than the observed mass until Day 366, while the theoretical TIC mass was consistently higher than the observed mass. The theoretical TIC mass assumes that all carbon dioxide generated from the toluene reducing reactions will remain as TIC from both biodegradation pathways (sulfate reduction and methanogenesis); however, some carbon is used for cell growth and hence the theoretical TIC will overestimate actual values (Manzoni et al., 2012). On Day 366, the TIC theoretical overestimation was 1.01 ± 0.51 mg, and the theoretical methane overestimation was 1.27 ± 1.14 mg.

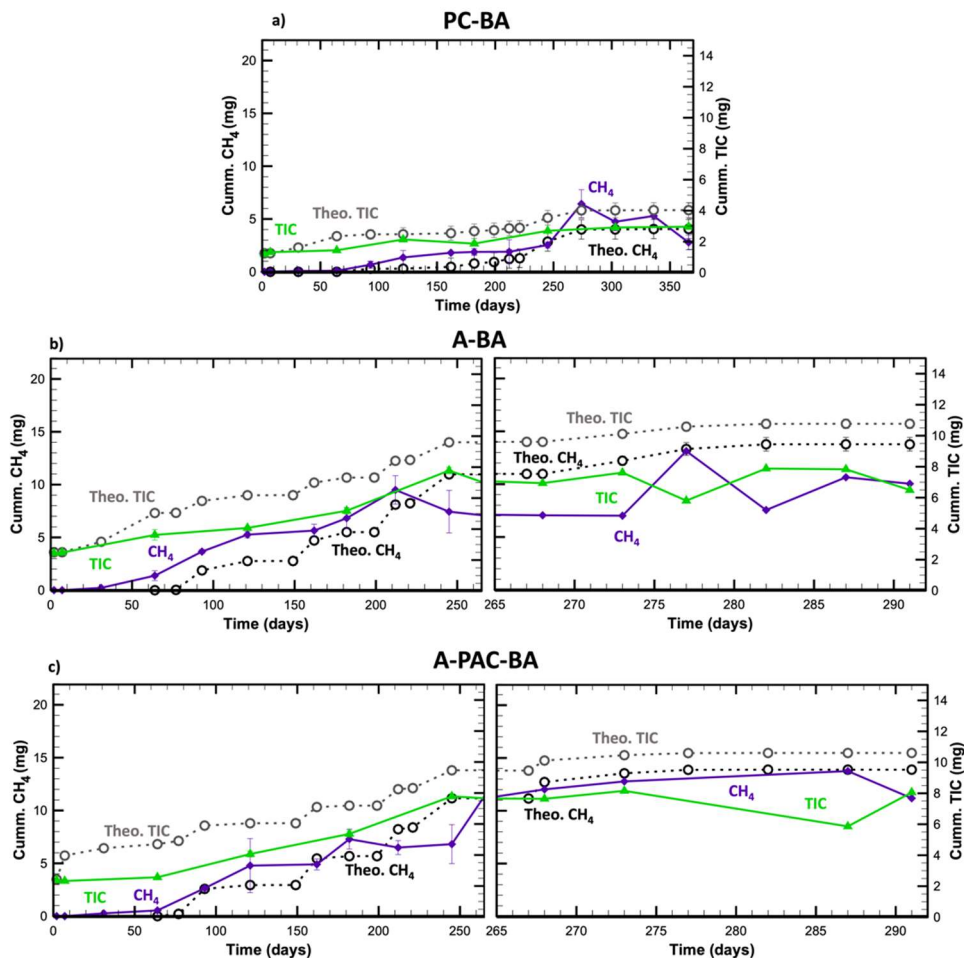


Figure 3.11: Theoretical methane production and theoretical TIC mass alongside the measured methane production and experimental TIC mass for the a) PC-BA, b) A-BA, and c) A-PAC-BA toluene microcosm system. Error bars represent $\pm$ one standard deviation.

In both the A-BA (Figure 3.11b) and A-PAC-BA (Figure 3.11c) systems, the theoretical cumulative TIC mass was also higher than the observed values. The upward trend in theoretical methane mass is similar to the trend in the measured methane mass early in the experiments, but values were consistently ~1 to 3 mg below the observed mass in both systems. In both systems, observations indicate that methane was produced as early as Day ~25, while the theoretical methane production only attributes degradation to methane production beginning on Day 64 (coincident with sulfate depletion). However, the theoretical values become larger than the observed values on Day ~225 for the A-BA system and Day ~210 for the A-PAC-BA system, which coincides with the second degassing event on Day 205. Although degassing should not alter concentrations in the gas phase, operational error may have resulted in some changes to the concentrations. The A-PAC-BA system had measured values close to the theoretical estimates on Day 268, 273, and 287 with the differences of 0.696 ± 0.169 mg, 0.768 ± 0.235 mg, and 0.135 ± 0.240 mg, respectively. Measured methane masses that were close to the theoretically determined values show that in some bottle sets, the estimates were adequate to describe the system, which further supports that some bottles had some deviations from the ideal behaviour.

The compartmental mass distribution of toluene in the A-BA and A-PAC-BA systems during the high-resolution sampling period is shown in Figure 3.12. Total toluene mass rapidly decreased by Day 282 (3.65 ± 0.28 mg to 0.02 ± 0.01 mg) in the A-BA system and by Day 277 (3.29 ± 0.26 mg to 0.03 ± 0.01 mg) in the A-PAC-BA system. In the A-BA system, most of the toluene mass was present in the aqueous phase (~92%) (Figure 3.12a). Conversely, in the A-PAC-BA system, toluene mass was divided between the aqueous phase (~50%) and PAC (~48%) on Day 268 (Figure 3.12b). The gas phase mass was lower in the PAC system since the gas phase concentration is assumed to be in equilibrium with the aqueous phase concentration.

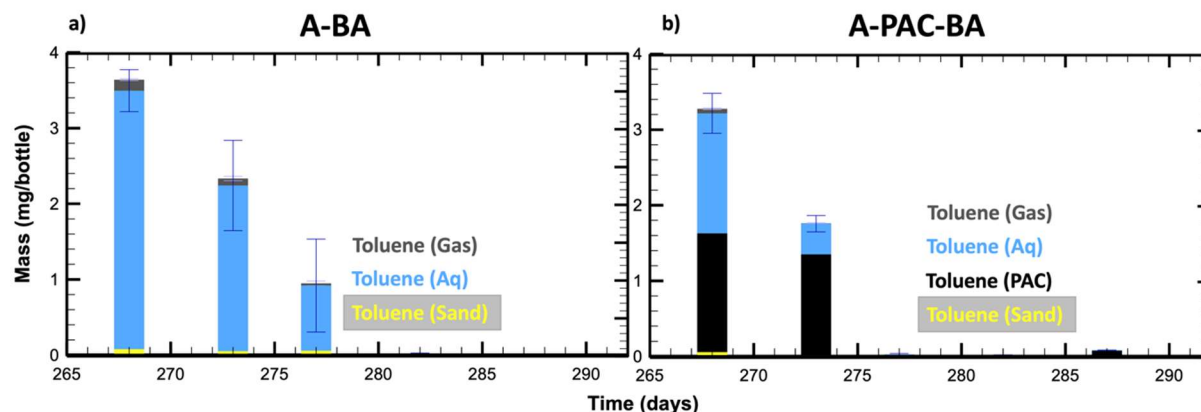


Figure 3.12: Compartmental mass distribution for a) A-BA and b) A-PAC-BA toluene microcosm systems during the high-resolution sampling period.

A linear model was deemed an adequate fit to the data to determine that the biodegradation rates in the A-BA and A-PAC-BA bottles was 0.26 ± 0.08 mg/day and 0.30 ± 0.03 mg/day, respectively. A t-test comparing the biodegradation rates (i.e., the slopes of the fitted model) of the A-BA and A-PAC-BA systems revealed that the rates are equal at the 95% confidence level (see Appendix A2 for statistical test details). Therefore, the toluene biodegradation rates are not statistically different in the A-BA and A-PAC-BA systems, suggesting that the presence of PAC did not significantly increase the biodegradation rate.

3.2.2 BTX Microcosms

Killed Control Systems

Similar to the toluene-only KC microcosms, the three BTX KC systems had a slight decline of aqueous BTX concentrations throughout the experimental period, likely due to the sampling design and general losses (sampling removes mass which allows for re-partitioning into the headspace, which reduces the aqueous concentrations) (Figure 3.13). The relatively constant aqueous phase BTX concentrations and lack of methane generation indicate that no significant biodegradation occurred. The increase in BTX concentrations in the KC-BA system observed on Day 120 is from sampling a new triplicate bottle set (as the previous sampling point was sacrificial (Figure 3.3)), and thus yielded higher values. As in the toluene-only KC-PAC system, the BTX KC-PAC system had decreasing aqueous phase BTX concentrations once PAC was added (Figure 3.13c). The decrease in aqueous phase concentrations is a result of sorption of BTX onto the PAC. The KC

and KC-BA system showed very low mass stored in the solid phase, whereas the KC-PAC system had a much higher solid phase concentration (Figure 3.13). The sorption affinity of the three compounds was also evident from the data from highest to lowest affinity in the order of: *o*-xylene, toluene, benzene (Daifullah and Girgis, 2003; Marrocco, 2023) (Figure 3.13c). The TIC mass increase in the KC systems was previously described for the toluene-only KC systems (Section 3.2.1).

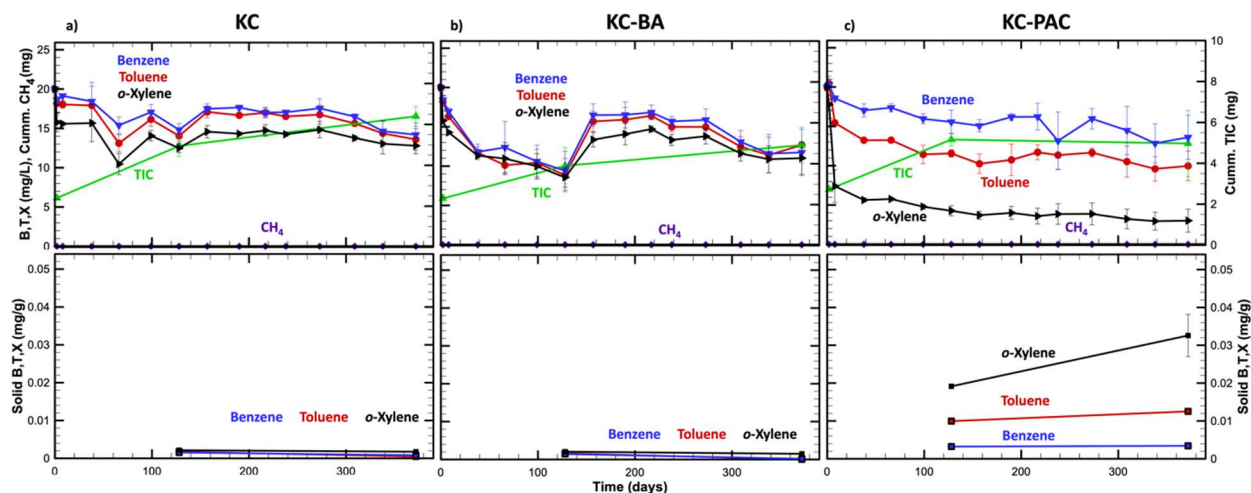


Figure 3.13: Aqueous phase BTX concentrations, TIC mass, and methane (top), and solid phase BTX concentrations (bottom) for the a) KC, b) KC-BA, and c) KC-PAC BTX microcosm systems. Error bars represent $\pm$ one standard deviation. Open points on the aqueous phase component are theoretical, based on the target dosage of BTX into the system.

Starved Control Systems

Figures 3.14a and 3.14b show the time series of methane production and TIC mass in the two starved control microcosms systems that did not have BTX present. The aqueous BTX concentrations were therefore $<$ MDL and show the expected behaviour of a starved, active system with no methane production and a steady TIC mass.

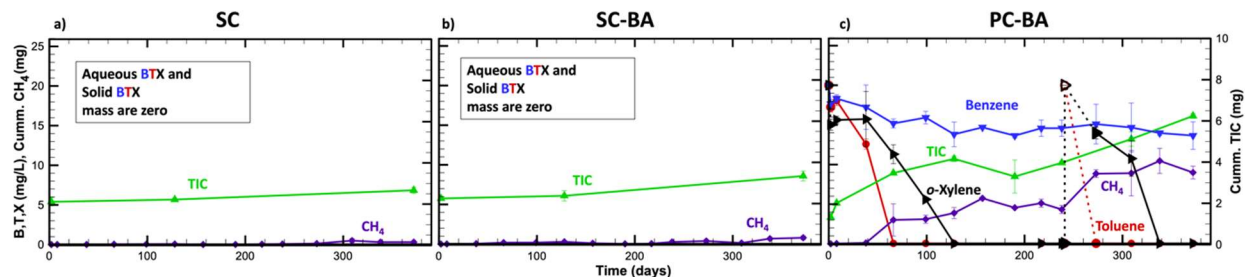


Figure 3.14: Aqueous phase BTX concentrations, TIC mass, and methane for the a) SC, b) SC-BA, and c) PC-BA BTX microcosm system. Error bars represent $\pm$ one standard deviation. Open points on the aqueous phase toluene component are theoretical, based on the target dosage of BTX into the system. The dashed line connecting the open circle symbols are assumed trends.

Positive Control System

The time series of aqueous and solid phase BTX concentrations, TIC mass, and methane for the positive control (PC-BA) microcosm system is shown in Figure 3.14c. As no BS was present in this system, the only potential for biodegradation beyond any native populations was through the addition of DGGTM-Plus cultures. The biodegradation that took place until Day 38 was primarily through reduction of the initial sulfate present in the system, as supported by the absence of methane production (Figure 3.14c). Complete biodegradation of aqueous phase toluene took place by Day ~68, followed by complete *o*-xylene biodegradation by Day ~128 (Figure 3.14c). This order of biodegradation has been well documented and is affected by reaction thermodynamics, presence of microbes that can facilitate the reactions, competitive effects between microbial populations and inhibition of certain biodegradation pathways by contaminants (Thornton et al., 2011) and molecule configurations (Foght, 2008). Sulfate reduction on Day 38 to 1.30 ± 0.21 mg/L from the starting concentration of 6.97 ± 0.03 mg/L coincides with the shift from sulfate reduction to methanogenic activity, supported by the increase in methane to 5.13 ± 0.51 mg (Figure 3.14c).

Following the initial degradation cycle, the re-addition of toluene and *o*-xylene was delayed for 113 days (Day 128 to Day 241) to support potential benzene degradation. As there was no evidence of benzene biodegradation during this period (indicated by a constant aqueous phase benzene concentration and no increase in methane production), the system was replenished with toluene and *o*-xylene only. Complete biodegradation after the second re-addition took 31 days and 97 days for toluene and *o*-xylene respectively (Figure 3.14c). This is 37 and 31 fewer days than the time needed to biodegrade toluene and *o*-xylene, respectively. The second biodegradation cycle of

toluene and *o*-xylene had an increase in the methane mass from 4.33 ± 0.52 mg to 10.46 ± 1.59 mg. The TIC mass similarly increased from 1.32 ± 0.01 mg-C to 3.97 ± 0.08 mg-C during the first degradation cycle, then from 3.97 ± 0.08 mg-C to 6.24 ± 0.12 mg-C during the second cycle.

Active Systems

The time series of aqueous and solid phase BTX concentrations, TIC mass, and methane production are shown in Figure 3.15 for the two active microcosms. Both microcosm systems had stable aqueous phase BTX concentrations after an initial period (< 100 days) of slight decline (Figure 3.15). This behaviour is suspected to be caused by sulfate reducing organisms native to the BS utilizing the initial sulfate present, supported by the rapid decrease in sulfate in the microcosms (see Appendix A, Figure A.9). The end of this initial period of toluene reduction coincides with depletion of background sulfate in the system (Day ~ 100), after which a near stable toluene concentration was observed (Figure 3.15). Both systems showed minimal evidence of biodegradation, having a stable TIC mass (2-3 mg-C) and minimal methane production. The slight increase in methane is suspected to be the same mechanism as that in the toluene-only systems: methanogens using available toluene in BS once sulfate had been reduced (Toth et al., 2021).

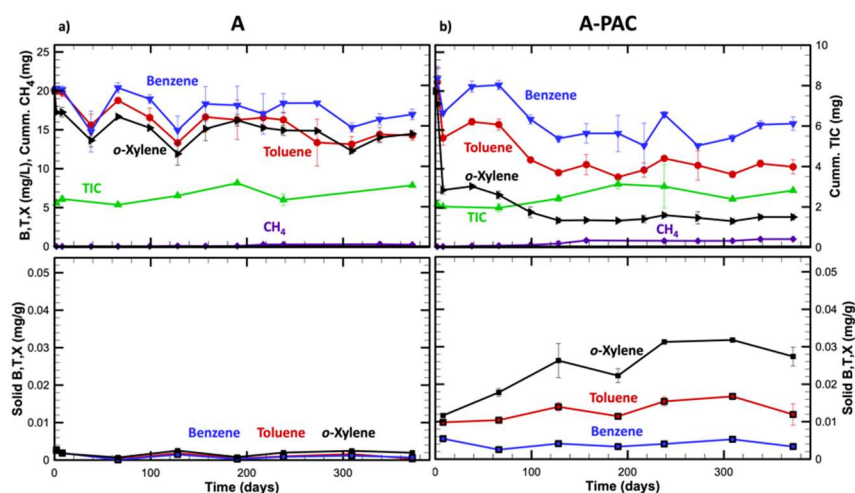


Figure 3.15: Aqueous phase BTX concentrations, TIC mass, and methane (top), and solid phase BTX concentrations (bottom) for the a) A and b) A-PAC BTX microcosm systems. Error bars represent $\pm$ one standard deviation. Open points on the aqueous phase toluene component are theoretical, based on the target dosage of BTX into the system.

The solid phase BTX concentrations in the A system remained low (< 0.004 mg/g) throughout the experimental period, as there was no PAC present. Conversely, solid phase benzene and toluene

concentrations in the A-PAC system were consistent at ~ 0.005 mg/g and ~ 0.014 mg/g, respectively. *o*-Xylene solid phase concentrations increased from ~ 0.012 mg/g to ~ 0.03 mg/g by Day 128, after which time the concentration remained constant. This initial period of increase in solid phase concentration coincided with the depletion of sulfate (Figure A.9) and a decrease in aqueous phase concentration, suggesting the solid phase concentration increase was a result of sorption to PAC. The larger increase in *o*-xylene solid phase concentrations compared to benzene and toluene is due to competitive sorption, as *o*-xylene has the highest sorption affinity of the three solutes.

Bioaugmented Systems

Time series of aqueous and solid phase toluene concentrations, TIC mass, and methane production are shown in Figure 3.16 for the active microcosm systems with bioaugmentation culture (A-BA and A-PAC-BA). In both systems, aqueous phase toluene and *o*-xylene biodegradation took ~ 68 days and ~ 128 days, respectively (Figure 3.16). The length of this initial biodegradation cycle closely mimics that of the PC-BA system (Figure 3.14c). During the first biodegradation cycle, the initial 38 days were predominantly sulfate reducing, as sulfate was present in the system, after which time the system shifted to methanogenesis once sulfate was depleted (Figure 3.16). The second re-addition of BTX was delayed ~ 65 days (until Day 193) to support any potential benzene biodegradation. However, the stable aqueous benzene concentration as well as the methane and TIC mass during this period (Figure 3.16) showed no evidence of benzene biodegradation. The decrease in the aqueous phase benzene concentration in the A-PAC-BA system was due to increased sorption to PAC once toluene and *o*-xylene were depleted from the system. Following the second re-addition of toluene and *o*-xylene on Day 193, the A-BA system biodegraded both the toluene and *o*-xylene mass over the next ~ 80 days (Day 193 to Day 270) (Figure 3.16a). In the A-PAC-BA system, both aqueous and solid phase toluene and *o*-xylene was reduced to $< \text{MDL}$ over ~ 45 days (Days 193-238), which allowed for a third re-addition on Day 241 (Figure 3.16b). These differences observed in the biodegradation between the A-BA and A-PAC-BA systems may be due to PAC locally increasing aqueous BTX concentrations leading to the formation of biofilm that supports locally higher biodegradation rates (de León, 2021). Unlike the T-only systems, the A-BA BTX system showed more similarities to the PC-BA system in terms of BTX

biodegradation, which may be due to more similar amounts of microbial populations being introduced at the time of culture addition.

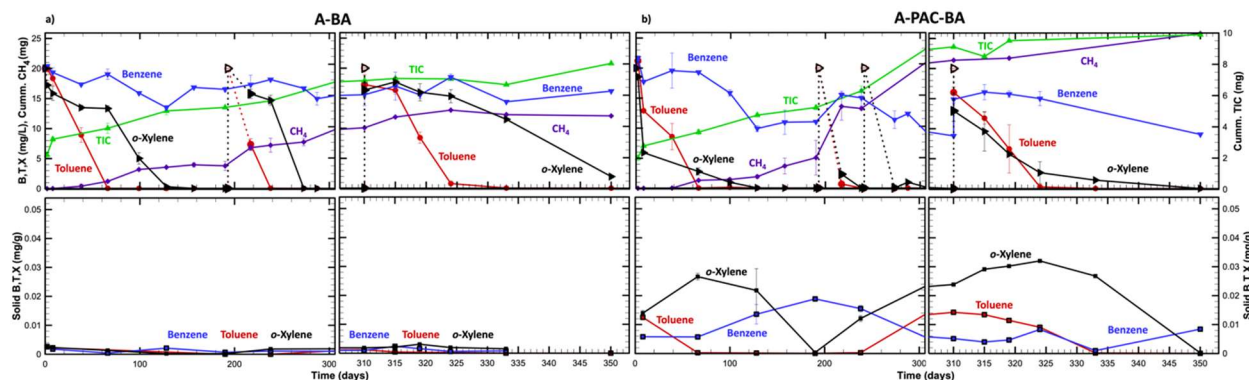


Figure 3.16: Aqueous phase BTX concentrations, TIC mass, and methane (top), and solid phase BTX concentrations (bottom) for the a) A-BA and b) A-PAC-BA BTX microcosm systems from experiment Days 0-310, and the high-resolution toluene sampling days from Days 310- 355. Error bars represent $\pm$ one standard deviation. Open points on the aqueous phase toluene component are theoretical, based on the target dosage of BTX into the system. The dashed line connecting the open circle symbols are assumed trends.

Methane production coincided with the degradation of toluene and *o*-xylene in both systems, supporting methanogenic biodegradation activity (Figure 3.16). The A-PAC-BA system generated more methane due to the extra addition of toluene and *o*-xylene. Both systems generated TIC mass providing further evidence of biodegradation (1.97 ± 0.18 to 9.89 mg-C for A-PAC-BA, and 2.20 ± 0.17 to 8.03 mg-C for A-BA).

The total bacteria, total archaea, Deltaproteobacteria ORM2, DSP, and Peptococcaceae (PEP) populations of the DGGTM cultures (time zero), and samples taken at the end of the experiment are shown in Figure 3.17. The total bacteria populations were relatively consistent across all systems, ranging between 2×10^6 - 2×10^7 copies/g ($> 10^7$ supports viable populations (Toth et al, 2021)). Total archaea populations were lowest in the time zero sample ($\sim 3 \times 10^4$ copies/g), and highest in the two BA systems (between 10^7 - 2×10^7 copies/g), which is indicative of methanogenesis in these systems (Toth et al, 2021). ORM2, DSP, and PEP were present in higher populations in the two BA systems ($\sim 10^5$ copies/g, 6×10^5 - 4×10^6 copies/g, and 10^6 - 3×10^6 copies/g, respectively) compared to the A and A-PAC systems ($< 2 \times 10^4$ copies/g for all three tracers) due to the inoculation of DGGTM cultures. ORM2 was not present in A and A-PAC systems because it is not native to the BS. The increases of these populations (DSP, PEP) supports that they were likely

responsible for the toluene and *o*-xylene biodegradation that occurred in the BA systems. Benzene degradation was not adequately supported throughout these experiments in both bioaugmentation systems, which is posited to be due to the level of ORM2 ($\sim 10^5$ copies/g) being too low to lead to significant degradation in the system. Toth et al. (2021) observed benzene biodegradation in microcosms augmented with DGGTM-B at ORM2 counts of $> 10^6$ copies/mL.

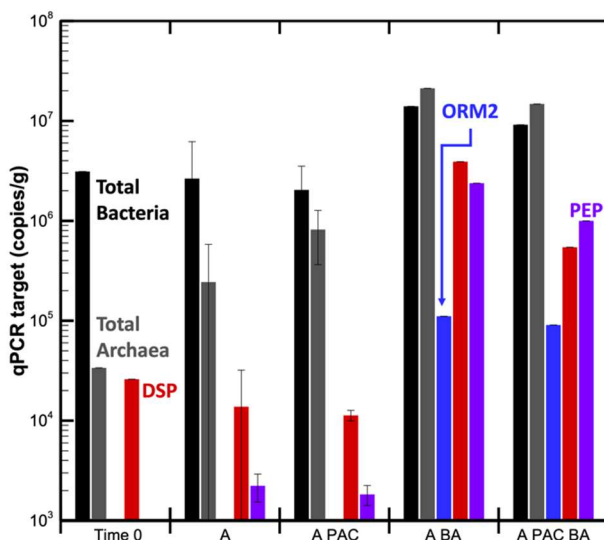


Figure 3.17: qPCR analysis results of the total bacteria, total archaea, ORM2, DSP, and PEP populations for DGGTM Plus (time zero), and microcosm samples taken at the end of the BTX experiment.

Figure 3.18a shows the time series of observed TIC mass and methane mass with their associated theoretical values for the PC-BA microcosm experiments. The theoretical methane concentration closely follows the same increasing trend as the observations but are lower by as much as 2 mg. Compared to the PC-BA toluene-only system (Figure 3.11), the measured and theoretical values in the PC-BA BTX system are much closer to each other. Between Day 0 and Day 160 TIC experimental values are consistently higher than the theoretical, after which the two series fluctuate around each other. As the theoretical estimate assumes complete carbon dioxide conversion with no carbon losses to cell growth, it is expected that the theoretical values are higher than the observed values (Manzoni et al., 2012).

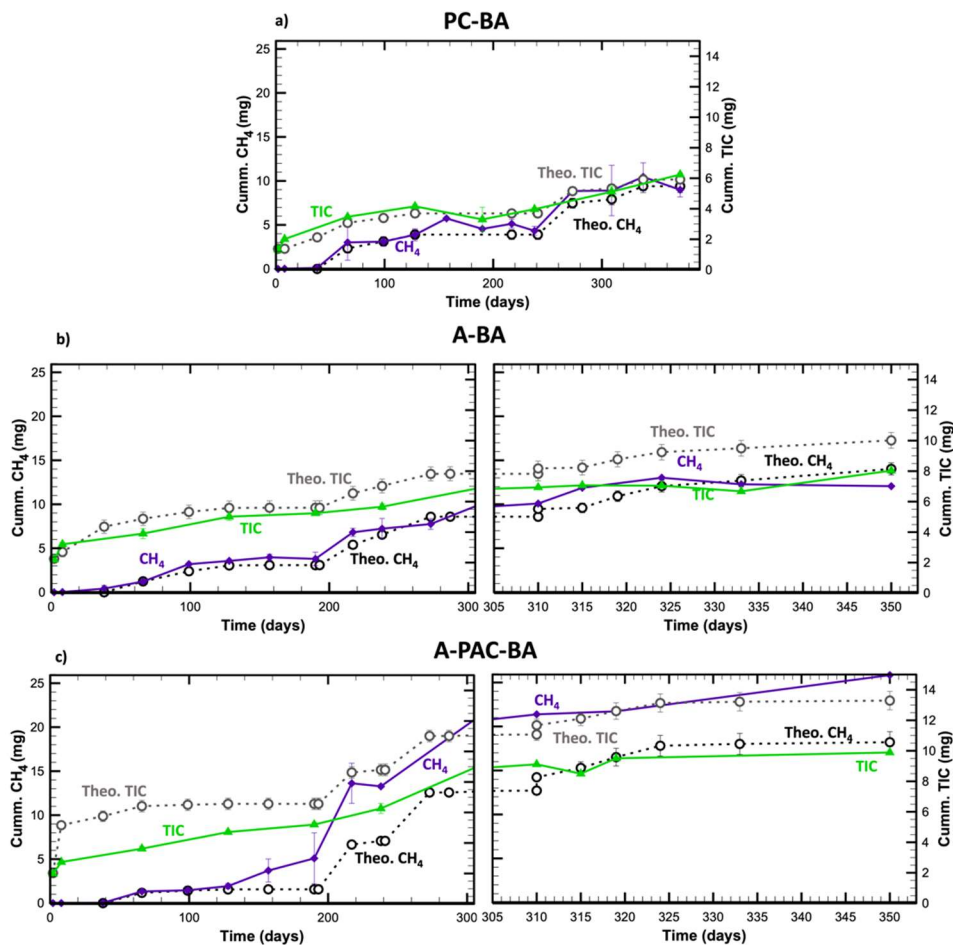


Figure 3.18: Theoretical methane production and theoretical TIC mass alongside the measured methane production and experimental TIC mass for the a) PC-BA, b) A-BA, and c) A-PAC-BA BTX microcosm system. Error bars represent $\pm$ one standard deviation.

The time series of observed TIC and methane mass are shown with their associated theoretical estimates in Figure 3.18 for the A-BA (Figure 3.18b) and A-PAC-BA (Figure 3.18c) microcosm systems. The theoretical TIC mass for the A-BA system is slightly higher than what was observed, while the theoretical methane mass is within ~ 1 mg of the observed mass for most of the experiment, which further supports methanogenic biodegradation (Figure 3.18b). The theoretical TIC mass estimates for the A-PAC-BA system (Figure 3.18c) is higher than what was observed throughout the experiment. Theoretical methane mass tracks very closely the observed mass until Day 175, after which the observed values increase above the theoretical estimates. The difference between the observed and theoretical values is greatest at on Day 350, when it is 7.63 ± 1.20 mg. This difference cannot be explained by any experimental losses, or complete degradation of benzene alongside toluene and *o*-xylene.

The compartmental distributions of benzene, toluene, and *o*-xylene mass in the A-BA and A-PAC-BA systems during the high-resolution sampling period are shown in Figure 3.19. During this period, samples were taken on Day 310, 315, 319, 324, 333, and 350. In both systems, benzene mass was generally constant, at 2.62 ± 0.03 mg to 3.24 ± 0.45 mg for the A-BA system, and 2.85 ± 1.54 mg to 3.24 ± 0.21 mg until Day 333 for the A-PAC-BA system. During this period, the benzene mass remained predominantly in the aqueous phase ($\sim 93\%$ for A-BA, and $\sim 83\%$ for A-PAC-BA) (Figure 3.19a and Figure 3.19d). The total toluene mass rapidly decreased to $< \text{MDL}$ on Day 333 in both the A-BA (Figure 3.19b) and A-PAC-BA (Figure 3.19e) systems. The total *o*-xylene mass decreased in both the A-BA (Figure 3.19c) and A-PAC-BA (Figure 3.19f) systems throughout the experiment to < 0.4 mg on Day 350.

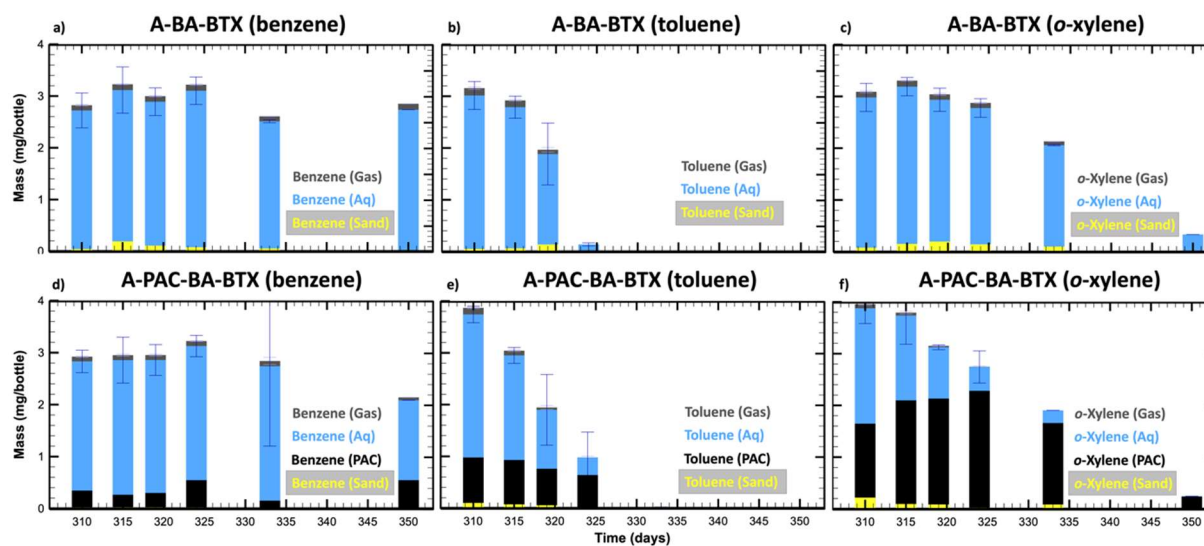


Figure 3.19: Compartmental mass distribution for a) A-BA (benzene only), b) A-BA (toluene only), c) A-BA (*o*-xylene only), d) A-PAC-BA (benzene only), e) A-PAC-BA (toluene only), f) A-PAC-BA (*o*-xylene only). Error bars represent $\pm$ one standard deviation.

In the A-PAC-BA system, benzene had the smallest amount of mass sorbed on the PAC, while *o*-xylene had the most. The amount of mass of the different solutes sorbed on the PAC followed to the sorption affinity of these compounds, reflecting competitive sorption (Daifullah and Girgis, 2003; Marrocco, 2023). The mass of benzene sorbed to the PAC was consistently < 0.6 mg during the high-resolution sampling period. Toluene mass sorbed to the PAC decreased from 0.98 mg to 0.64 mg from Day 310 to Day 324, after which it remained $< \text{MDL}$ from Day 333 to Day 350. The decrease in toluene mass sorbed to the PAC is a function of biodegradation and the ability to regenerate PAC sorption sites. *o*-Xylene mass sorbed to the PAC increased from 1.43 mg to 2.26

mg from Day 310 to Day 324 as toluene mass in the PAC zone decreased, allowing these sorption sites to be taken up by *o*-xylene. Then, *o*-xylene decreased to 0.24 mg on Day 350 due to biodegradation. The ability of *o*-xylene to occupy available sorption sites as toluene mass decreased is a result of regeneration of the PAC zone.

A linear model was fit to the data to determine the biodegradation rates of toluene and *o*-xylene in the A-BA and A-PAC-BA bottles. The biodegradation rate of toluene was 0.22 ± 0.03 mg/day and 0.21 ± 0.02 mg/day in the A-BA and A-PAC-BA bottles, respectively. The biodegradation rate of *o*-xylene was 0.090 ± 0.005 mg/day and 0.099 ± 0.006 mg/day in the A-BA and A-PAC-BA bottles, respectively. A t-test comparing the biodegradation rates (i.e., the slopes of the fitted model) of the A-BA and A-PAC-BA systems revealed that the rates are equal at the 95% confidence level (see Appendix A2 for statistical test details). Therefore, the biodegradation rates for both toluene and *o*-xylene are not statistically different between the A-BA and A-PAC-BA bottles, suggesting the presence of PAC did not significantly increase the biodegradation rates for this experiment.

3.3 Summary

The objective of the research described in this chapter was to assess how PAC coupled with bioaugmentation affects the biodegradation rate of BTX compounds in anaerobic environments under static conditions. The hypothesis was that the presence of PAC will increase the biodegradation rate of BTX in systems with and without bioaugmentation. Once sulfate in the systems was depleted through sulfate reduction, the only remaining pathway for BTX biodegradation was methanogenesis. In the systems that did not receive bioaugmentation (A, A-PAC), there was decreased capability of the system to degrade BTX compounds once sulfate was depleted. The experimental timeframe was likely insufficient to observe a potentially viable methanogenic population to grow in these bottles and to assess whether the presence of PAC significantly impacted biodegradation. Multiple lines of evidence from the systems that contained active bioaugmentation cultures (PC-BA, A-BA) supported biodegradation through methanogenesis occurred. However, the results from the systems with both bioaugmentation cultures and PAC (A-PAC-BA) did not yield evidence from both the monthly sampling and high-resolution sampling periods to support the hypothesis that PAC increases the biodegradation rates

of BTX. The high-resolution sampling regime also revealed no significant difference between the biodegradation rates for systems with and without PAC at the 95% confidence level. Therefore, the microcosms experiments did not support that PAC significantly increases biodegradation rates either by itself or in combination with bioaugmentation in static anaerobic systems. In practical applications with flow through, PAC is used to sequester mass and slow the movement against the groundwater flow and the stagnant nature of the microcosms does not capture this effect.

Chapter 4

Column Experiments

This chapter describes the methodology and presents and discusses the results of column experiments conducted to evaluate the efficacy of linking a carbon based PRB and bioaugmentation. The same aquifer material (BS) and bioaugmentation cultures (DGG Plus™) used in microcosm experiments were employed, but these experiments are more representative of *in situ* conditions. Systems with and without a PAC zone in the central part of the column were explored, as well as with and without bioaugmentation cultures added. Following a nine-month acclimation period, the columns were run for 14 months and the influent PHC concentrations were increased in a stepwise manner. Both the influent and effluent were monitored, and solid phase samples were collected at the end of the experiment to characterise BTX and DNA.

4.1 Materials and Methods

Column experiments were conducted for a multi-compound condition (BTX) using the DGG Plus™ culture. The design of the experiments is summarized in Table 4.1. Three treatment types were examined:

1. ***Killed Control (KC)***: The KC column contained autoclaved BS and a PAC zone, and the biocide was added to the influent solution. The purpose was to capture abiotic degradation and understand sorption behavior.
2. ***Active (A-BA, and A)***: The active control columns were identical to the KC columns but without biocide and included a column system with bioaugmentation culture (A-BA) and a system without bioaugmentation (A).
3. ***Active with PAC (A-PAC-BA, and A-PAC)***: These column systems were similar to the active control columns but with a PAC zone and included an active system with PAC (A-PAC) and an active system with PAC and bioaugmentation (A-PAC-BA).

Table 4.1: Summary of the column experimental design (✓ indicates presence, -- indicates not present).

Type	Series	PAC	DGG TM culture
Killed Control (KC) ¹	KC-PAC	✓	--
Active (A) ²	A	--	--
	A-BA ⁴	--	✓
Active with PAC (A-PAC) ³	A-PAC	✓	--
	A-PAC-BA	✓	✓

Notes :

1. Accounts for abiotic degradation, and sorption to BS and PAC in the absence biodegradation.
2. Accounts for biotic degradation without PAC present.
3. Accounts for biotic degradation with PAC present.
4. BA = bioaugmented with DGGTM. 6.66 mL of DGGTM-B, DGGTM-T, and DGGTM-X were injected (total of ~20 mL).

4.1.1 Materials

The BTX compounds, PAC, BS, and DGGTM cultures used in these column experiments were the same as those used in the microcosm experiments (see Section 3.1.1). The AGW used followed the same recipe as that used in the microcosms but without the amorphous ferrous sulfide (FES) to prevent precipitates. The BS was collected from the same shallow, unconfined, sandy aquifer as the material used for the microcosm experiments (additional details in Section 3.1.1). The BS was stored at 4° C in the dark for approximately 330 days before use.

4.1.2 Column System Design

The columns were constructed from 3.75 cm inner diameter acrylic pipe and were ~36 cm long (minor variation resulted from the tube cutting). To assist with flow distribution at the ends of each column, a network of incisions was made in the end caps starting from the centre and radiating outwards over the entire surface area. To retain fines, a layer of glass wool was placed between two ~3.7 cm diameter stainless steel (SS) screens (Sigma-Aldrich, St. Louis, MO, USA) placed against each end cap. The SS screen on the interior side of the column (i.e., against the BS material) had a U.S. mesh size 80, and the screen adjacent to the end cap had a U.S. mesh size 20.

Four different column packing configurations were used (Figure 4.1). The A, A-BA, A-PAC, and A-PAC-BA columns were packed with 875.3 ± 35.8 g of moist BS inside an anaerobic environment to minimize oxygen exposure. Each column was packed in one-centimeter lifts that were

compacted using a 2.54 cm-diameter acrylic rod. The AGW level was raised as the packing proceeded, lagging 2 cm below the lift to limit entrapped gas and aid in consolidation. The aquifer material for the KC-PAC column was autoclaved at 121°C for 15 minutes three times on three consecutive days before use. The mass of water that was removed during autoclaving (16% volumetric water content) was re-added to the BS in an anaerobic glove bag using deoxygenated Milli-Q water. The KC-PAC column was then packed with 862.7 ± 35.8 g of the sterile, moist BS following the same method as described above.

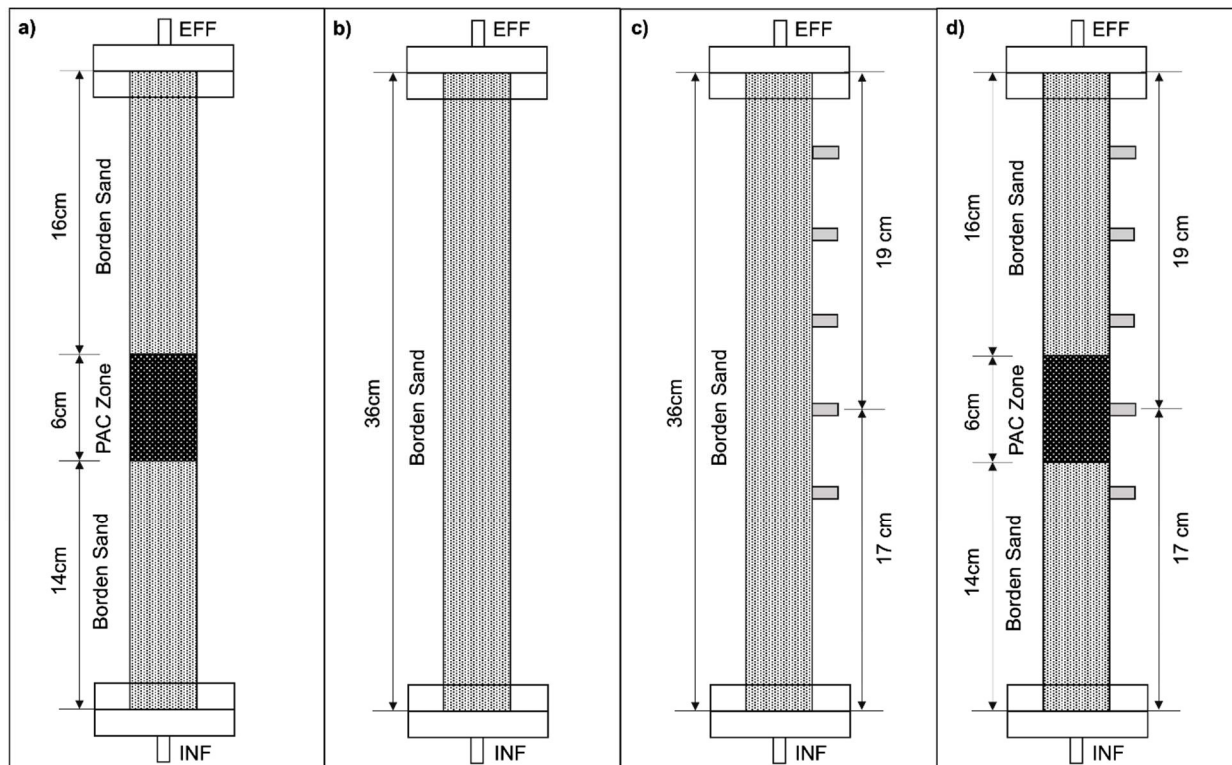


Figure 4.1: Column schematic showing the BS and PAC zone for a) KC-PAC and A-PAC columns, b) A column, c) A-BA column, and d) A-PAC-BA column. INF = influent; EFF = effluent. The small solid grey rectangles extending from the A-BA and A-PAC-BA columns (5 per column) represent the location of sampling ports. Only the sampling port located 17 cm from the influent end was used.

For the columns that included a PAC zone (KC-PAC, A-PAC, and A-PAC-BA), a 6-cm long zone was placed 14 to 20 cm from the influent end (Figure 4.1). This PAC zone was placed 1 cm closer to the influent end to ensure that the bulk of the PAC mass remained near the centre of the column if there was some PAC migration. The material for the PAC zone was made by mixing moist BS with 0.5% by weight of PAC in anaerobic conditions. The length of the PAC zone and mass composition were based on estimated ideal PHC breakthrough times to ensure the systems would

achieve breakthrough within a reasonable timeframe but still allow for appreciable BTX sorption in the PAC zone (see Appendix B1 for additional details). The column systems with bioaugmentation (A-BA and A-PAC-BA) required the addition of a port to allow for the BA culture addition. While five ports were added, only the port located 17 cm from the influent end of the column was used for BA culture addition (Figures 4.1 and 4.2). Each injection port consisted of Swagelok tube fittings (SS0200-1-OR) attached to a two-ferrule tube fitting (316 Swagelok) with a septum inside. The ports were affixed with an 18-gauge SS needle with the tip positioned in the centre of the column. Each needle had a Luer-Lok end cap to minimize entry of oxygen.

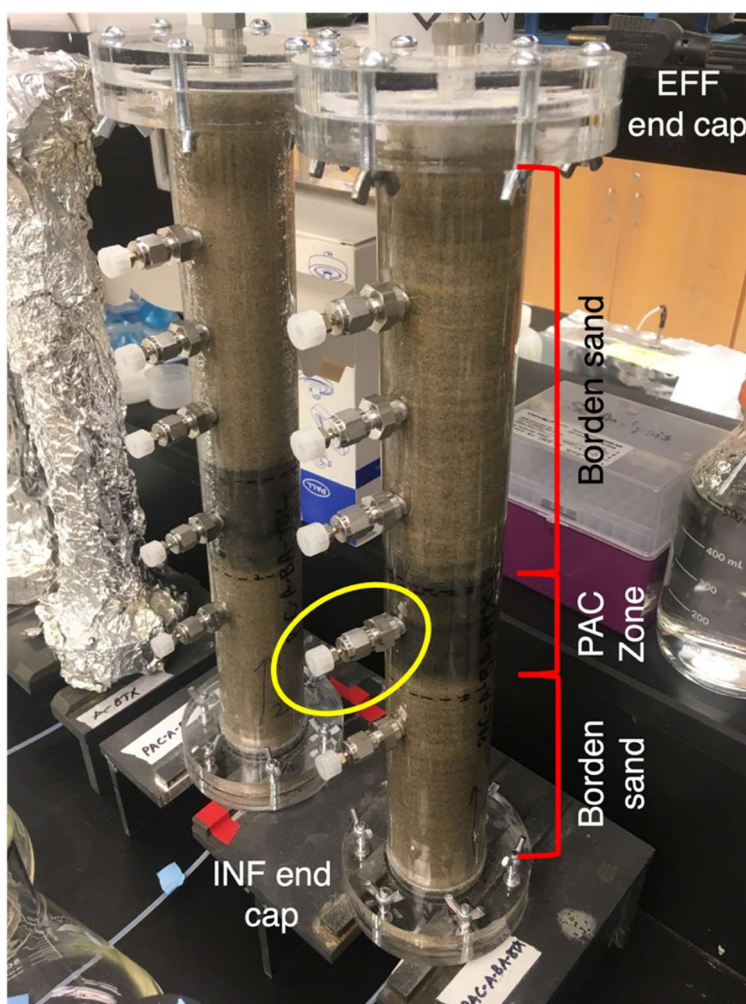


Figure 4.2: Photograph of the A-PAC (left) and PAC-BA (right) columns after packing showing the INF and EFF end caps and the five sampling ports. Five sampling ports are shown; however, only the circled port was used for culture addition.

Once each column was packed, the effluent end was affixed with end caps containing glass wool and SS screens identical to the influent end. The influent and effluent end caps were fit with 316 Swagelok fittings (Swagelok SS-200-1-OR) (Swagelok, Solon, OH, USA) and fastened to 1.6 mm inner diameter SS tubing (10 cm long at the influent end; 80 cm long at the effluent end) with SS ferrules. This SS tubing was non-transparent to avoid light exposure and limit microbial growth inside the tubing. To prevent oxygen from entering the columns and limit light exposure, the SS tubing at the influent and effluent ends were temporarily sealed and the columns were wrapped in aluminum foil.

Once packed, the columns were attached to the remaining column system components (Figure 4.3). The influent AGW and BTX mixture was contained in a sterile 10 L Tedlar sampling bag (SKC Inc., Eighty Four, PA, USA) with a push/pull valve and a stainless-steel septum (Restek, Bellefonte, PA, USA) (Figure 4.3a component 1). The SS fitting on the bag was connected to an 80 cm long section of SS tubing. The other end of the SS tubing was connected to a 25 mm SS microsyringe filter holder (MilliporeSigma, Burlington, MA, USA) through an inlet Luer-lok connection (Figure 4.3a component 2). The filter contained (in order from the inlet to the outlet): a polytetrafluoroethylene (PTFE) O-ring, a 0.2 μm nylon filter (Ezflow, Fox Life Sciences, Londonderry, NH, USA), a SS support screen, and a PTFE flat gasket. The purpose of this filter was to limit the upgradient migration of microorganisms (sulfate reducing bacteria or DGG cultures) from the column into the Tedlar bag. The outlet of the filter was a SS Luer-slip base connection which was connected to a Masterflex L/S® peristaltic pump (Cole-Palmer, Vernon Hills, IL, USA) (Figure 4.3a component 3). The Masterflex L/S Viton pump tubing (RK-96428-14) at the outlet of the pump was attached to a 170 cm long section of SS tubing that led to a 3-way SS ball valve (Swagelok, Solon, OH, USA) (Figure 4.3a component 4). Influent sampling was performed through a sampling port in the ball valve with a 316 Swagelok fitting and an internal rubber septum. The outlet of the ball valve was connected to another SS filter (Figure 4.3a component 5) through a 30 cm long section of SS tubing. Finally, a 3 cm long section of 1.42 mm diameter non-transparent flexible Viton tubing (Cole-Palmer, Vernon Hills, IL, USA) connected the outlet of the SS filter to the SS tubing attached to the influent end of the column.

The 80 cm SS tubing connected to the effluent of the column was attached to another SS filter. The filter was connected to another 3-way SS ball valve using 1.42 mm diameter non-transparent

flexible Viton tubing (Figure 4.3a component 7). This valve was also equipped with a sampling port and served as the effluent sampling point. The outlet of the valve connected to a 70 cm length of SS tubing that led to a final SS filter (Figure 4.3a component 8). The purpose of the two SS filters placed between the column and the effluent flask was to limit the migration of microorganisms upstream from the effluent into the column. Finally, the outlet of the filter was connected to a 10 cm length of SS tubing that led to 1 L Erlenmeyer glass flask with a rubber stopper (Corning Life Sciences, Corning, NY, USA) where the effluent waste was collected (Figure 4.3a component 9).

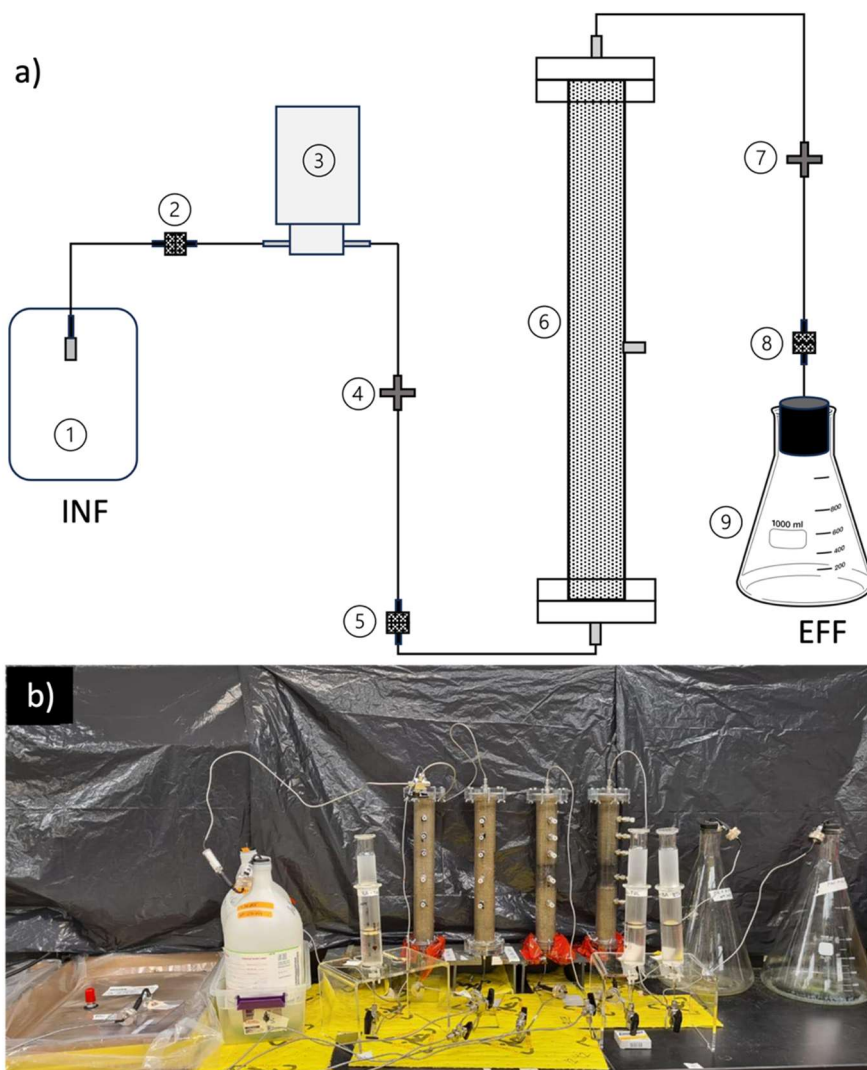


Figure 4.3: a) Column component layout in ascending order which follows the AGW flow direction. Components are labelled as: 1. Tedlar PHC bag, 2,5,8. Stainless-steel filters, 3. Pump with Viton tubing, 4,7. Swagelok 3-way ball valves for sampling, 6. Column placement, 9. Effluent waste flask. b) Photograph of lab setup showing the same component order and placement.

The effective porosity of the packed columns was estimated by McLaren (2023) using a one-dimensional mass transport model and data from a bromide tracer study. The estimated effective porosity was then used to calculate the pore volume of each column. The final specifications of the packed columns are summarized in Table 4.2.

Table 4.2: Specifications of the columns after packing

Parameter	KC-PAC	A	A-BA	A-PAC	A-PAC-BA
Column length (cm)	37.1	36.8	37.0	36.8	36.9
Inner diameter (cm)	3.75	3.75	3.75	3.75	3.75
Cross-sectional area (cm ²)	11	11	11	11	11
PAC zone length (cm)	6	--	--	6	6
Dry BS mass (g) ¹	716.98	754.18	743.22	768.00	752.23
Wet BS mass (g)	853.60	897.89	884.84	914.347	895.56
Effective porosity (-)	0.339	0.311	0.301	0.299	0.310
Pore Volume (mL)	138.91	126.43	123.06	121.63	126.30

Notes:

1. Estimated from known moisture content of BS measured from drying core samples and performing a mass balance.

4.1.3 Operation

The prepared AGW was stored in a mixing vessel before being injected into the Tedlar bag inside a glove bag filled with N₂ to ensure anerobic conditions. A peristaltic pump was used to feed the AGW through Teflon tubing into the SS inlet valve of the Tedlar bag. Once the AGW was inside the Tedlar bag, the necessary BTX additions were made through the valve using a sterile glass microliter syringe (Hamilton, Reno, NV, USA). Sodium sulfate was added using a disposable plastic syringe equipped with a Luer-lok fitting (Ait-Tite, Virginia Beach, VA, USA) attached to a 22-gauge needle (Exel Int., USA). The feed solution for the KC-PAC column had a 5% sodium azide solution added per L of AGW using a disposable plastic syringe equipped with a Luer-lok fitting attached to a 22-gauge needle. Once the necessary additions were made to the AGW, each Tedlar bag was covered in an opaque sheet to reduce light exposure and agitated on a platform shaker (Barnstead Thermolyne, Dubuque, IA, USA) for one day at 10 rotations per minute.

All column systems were operated for a nine-month acclimation period (denoted as Year-1) and to ensure breakthrough was achieved (i.e., appearance of BTX in the column effluent). During Year-1 operation the influent and effluent BTX concentrations, and flow rate were monitored. Target BTX influent concentrations during Year-1 were 3 mg/L per solute. The flow rate was estimated from the collected effluent volume and time interval. A target flow rate of ~ 0.024 mL/min was selected to provide a column residence time of about three days (i.e., one pore volume (PV) passing through the column every three days). Since the presence of a PAC zone would delay the time to breakthrough, a higher flow rate of 0.8 mL/min was used for applicable columns (KC-PAC, A-PAC, A-PAC-BA) for 1 to 2 months, after which time the flow rate was reduced to 0.024 mL/min. At the end of Year-1, the columns were taken offline to make improvements to the column system construction in response to observed limitations of the Tedlar bags used (e.g., leakage, corrosion, BTX sorption to liner material) and column tubing (switch from transparent Teflon tubing initially used to SS tubing, and the addition of SS filter holders as described above).

Following the Year-1 acclimation period, the columns were run for an additional 14 months at the nominal flow rate of 0.024 mL/min, denoted as Year-2. The KC column was run for an additional 10 months (24 total) to observe desorption behavior. To investigate increasing BTX column loading, the target influent BTX concentrations during Year-2 were sequentially increased from 5 mg/L per solute, to 10 mg/L per solute, and then to 20 mg/L per solute. Concomitantly the influent sulfate concentration was increased from a target concentration of 5 mg/L to 20 mg/L and then to 30 mg/L.

Once breakthrough was observed at the 5 mg/L BTX loading level, the DGGTM cultures were injected into the A-BA and A-PAC-BA columns. These columns were disconnected from the flow lines, the influent and effluent tubing clamped, and then the column was positioned horizontally inside an anaerobic glove bag. A 20 mL culture mixture comprised of 6.66 mL each of DGG-BTM, DGG-TTM, and DGG-XTM was injected using the centre port of the column (17 cm from influent) using two injections from a 10 mL glass syringe. The volume of culture mixture added was calculated based on the volume needed to fill the pore space of a ~ 6 cm long zone within the column. The column was positioned horizontally during the injection to favour a uniform distribution of the culture mixture within the BA zone. The influent and effluent end of the columns were unclamped during the injection to allow the injected mixture to displace the existing fluid in

the column. After culture addition, the columns were clamped and placed horizontally in an anaerobic environment for seven days to allow the culture to grow on the substrate and attach to the BS and/or PAC to retain culture mass in this zone once flow was re-started. Following this period, the columns were reconnected to the flow lines, positioned vertically and the flow rate was gradually increased to ~ 0.024 mL/min over a period of six hours.

4.1.4 Sampling and Analytical Methods

During Year-1 and Year-2, 25 mL samples were collected from the influent and effluent every 1 to 3 weeks and used to measure:

1. pH, ORP, DO, and sulfate (5 mL)
2. $\text{BTX}_{(\text{aq})}$ (2 mL)
3. $\text{CO}_{2(\text{aq})}$ and $\text{CH}_{4(\text{aq})}$ (4.5 mL)
4. DIC (3.5 mL)

Influent samples (from component 4 in Figure 4.3a) were collected over the course of one day under a temporarily increased flow rate of 0.4 mL/min. Following influent sampling, the column was returned to the nominal flow rate (~ 0.024 mL/min) for three days before an effluent sample was collected to sample the same PV of fluid in the influent and effluent. The effluent sample was then collected (from component 7 in Figure 4.3a) over three additional days to allow for an adequate sample volume to be gathered. This sampling process took one week to complete for each sampling episode. Samples were collected from the two three-way SS ball valve by modifying the flow direction of the sampling port and inserting a 17-gauge SS needle (Ace Gladd, Vineland, NJ, USA) through the rubber septa. The needle was attached to a 100 mL glass syringe (Cadence Science, Inc., Cranston, RI, USA) with 2-way nylon stopcocks (Masterflex, Gelsenkirchen, DE). The aqueous sample was then transferred to designated vials. The pH, ORP, DO, sulfate, BTX, DIC, and sulfide were analyzed following the same methodology as described in Section 3.1.4.

$\text{CO}_{2(\text{aq})}$ and $\text{CH}_{4(\text{aq})}$

The aqueous sample collected for CO_2 and CH_4 analysis was transferred from the 100 mL glass syringe to a 4.5 mL glass vial with a Teflon lined septa (Thermoscientific, Rockwood, TN, USA)

that was preloaded with 0.045 mL of a 5% sodium azide solution. Then 1 to 2 mL of the aqueous sample was withdrawn into a syringe and diluted with pure helium (10 to 15 mL), vigorously shaken, and placed upright for ~30 minutes to equilibrate. The gas phase from the syringe was then injected into a GC as described in Section 3.1.4. The aqueous CO₂ and CH₄ concentrations were then calculated using the gas concentration from the calibration, the volume of aqueous sample water, the headspace volume, and the Oswald coefficient for gas solubility in liquids (Markham and Kobe, 1941). The MDL for the aqueous CO₂ and CH₄ concentrations were 17.15 mg/L and 0.38 mg/L, respectively.

DNA

Before the cultures were injected into the columns, DNA samples were collected of each culture in an Eppendorf tube (20 mL of DGG™-B,T,X respectively). Samples were then centrifuged for 15 min at 14,000 rpm, decanted and stored at -80°C before being sent to the University of Toronto for qPCR analysis (see Section 3.1.4 for details).

Solid phase sampling

At the termination of the column experiments, the active columns (A, A-BA, A-PAC, A-PAC-BA) were subjected to a detailed solid phase sampling regime to determine the spatial distribution of BTX and microorganism populations (Figure 4.4). For the bioaugmented columns (A-BA, A-PAC, and A-PAC-BA), samples were collected from 17 locations with a higher resolution in the middle of the column (Figure 4.4a). For the A column, samples were collected from five locations distributed evenly along the column (Figure 4.4b). The distribution and location of the samples were determined based on the nominal column length of 36 cm. In each area sampled, the ~10 mg solid sample collected was split evenly for BTX concentration measurements (~5 mg) and DNA analysis (~5 mg). The same analytical methods as those described for the solid phase microcosm samples in Section 3.1.4 was used.

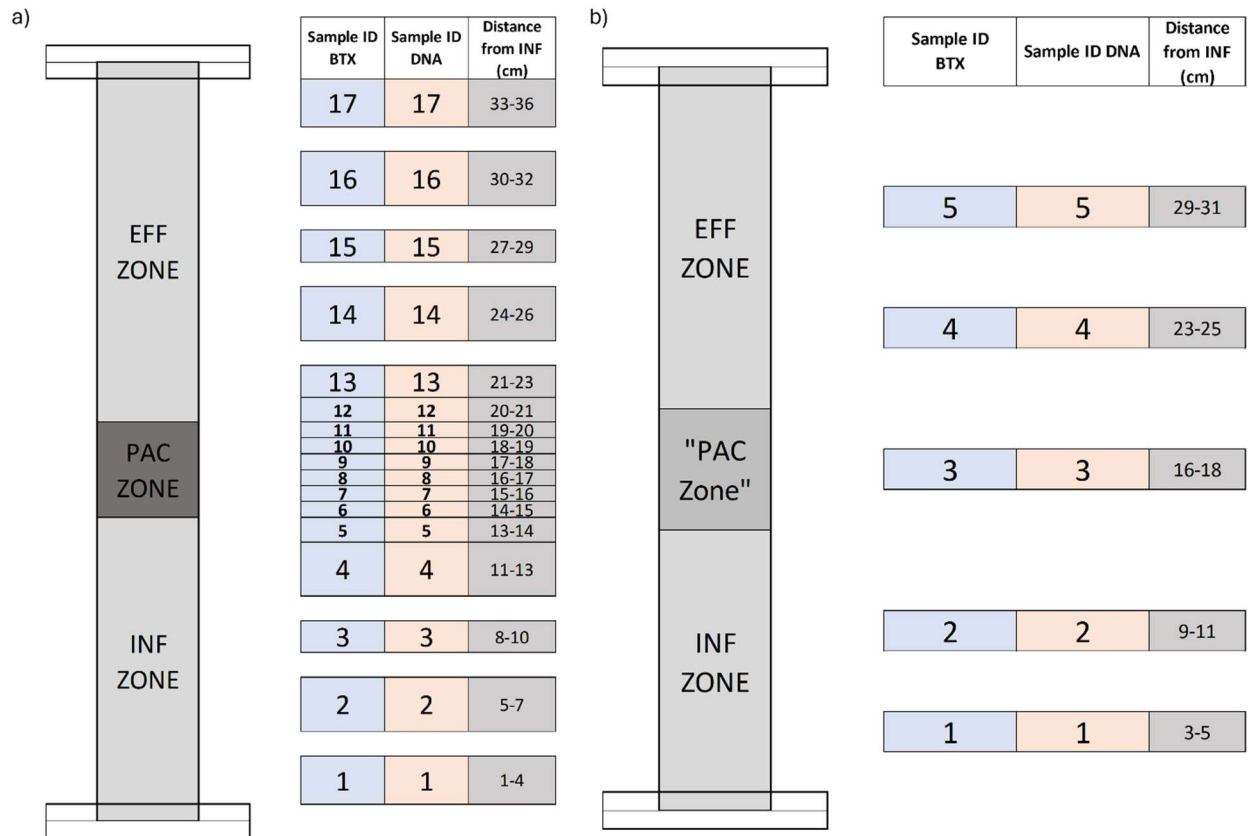


Figure 4.4: Two column layouts showing the influent (INF) zone, the effluent (EFF) zone, and PAC zone of the a) A-BA, A-PAC, and A-PAC-BA columns, and b) A column. The sampling locations for solid phase BTX and DNA samples are shown, along with their distance from the INF. “PAC ZONE” for the A and A-BA columns column indicate where PAC would be added if they were PAC columns.

4.1.5 Mass Balance Analysis

For Year-1 and Year-2, a BTX mass balance was performed for the active columns using the aqueous and solid phase mass to estimate the total mass of each solute, i , that includes what was biodegraded and all of the associated error:

$$m_{i,BD} = m_{i(aq),INF} - m_{i(aq),EFF} + \Delta m_{i(s)} \quad (4.1)$$

where $m_{i(aq),INF}$ = the cumulative influent aqueous mass, $m_{i(aq),EFF}$ = the cumulative aqueous mass that left the column, and $\Delta m_{i(s)}$ = the change in mass within the solid phase between the start to the end of Year-2 (beginning of Year-2 subtract end of Year-2).

The cumulative aqueous mass $m_{i(aq),INF}$ and $m_{i(aq),EFF}$ were calculated by adding the product of the average aqueous phase concentrations between two timepoints (t and $t-1$), the number of PVs that flowed through the column between the timepoints, and the column PV:

$$m_{i(aq),INF} = \sum_{t=2}^n (PV_{\#,t} - PV_{\#,t-1}) (PV) \frac{C_{i,t} + C_{i,t-1}}{2} \quad (4.2a)$$

$$m_{i(aq),EFF} = \sum_{t=2}^n (PV_{\#,t} - PV_{\#,t-1}) (PV) \frac{C_{i,t} + C_{i,t-1}}{2} \quad (4.2b)$$

where $PV_{\#}$ is the number of pore volumes at time t , PV = the column pore volume (mL) (Table 4.2), C_i = the aqueous concentration of solute i at time t , and n is the total number of timepoints. This method assumes a linear relationship between concentrations measured at each timepoint. While the uncertainty associated with these cumulative mass estimates is difficult to define, an assumed value of $\pm 10\%$ is considered reasonable.

The $\Delta m_{i(s)}$ was calculated as the change in the solid phase mass from the start of Year-2, $m_{i(s),S}$, to the end of Year-2, $m_{i(s),E}$ (i.e., $\Delta m_{i(s)} = m_{i(s),S} - m_{i(s),E}$). Given the f_{oc} of BS is very small (0.00024 [-], Xu and Thomson (2008)), the $\Delta m_{i(s)}$ was only calculated for the PAC Zone in the two columns with PAC (A-PAC, A-PAC-BA). The $m_{i(s),S}$ was assumed to be the solid phase mass present in the PAC Zone at the end of the Year-1 acclimation period. Since the column was intact (no solid phase data), the solid phase mass at the end of Year-1 was estimated based on the effluent aqueous concentrations at the end of Year-1:

$$m_{i(s),S} = m_{PAC} C_{i(s),PAC} \quad (4.3)$$

where m_{PAC} = the mass of PAC in the column, and $C_{i(s),PAC}$ = the sorbed concentration of solute i on the PAC at the end of Year-1 considering competitive sorption using ISIAS theory (Yonge and Keinath, 1986). The $C_{i(s),PAC}$ is calculated using the Freundlich sorption parameters for each solute i , $K_{f,i}$ ($\text{mg}^{1-n_f} \text{L}^{n_f} \text{g}^{-1}$) and $n_{f,i}$ (dimensionless):

$$C_{i(s),PAC} = K_{f,i}^{\left(\frac{n_{f,i}-1}{n_{f,i}}\right)} \left[\frac{K_{f,i}}{a_i} C_{i(aq)}^{n_{f,i}} \right]^{\frac{1}{n_{f,i}}} \left[\sum_i^N \left(\frac{K_{f,i}/a_i}{K_{f,i}} C_{i(aq)}^{n_{f,i}} \right)^{\frac{1}{n_{f,i}}} \right]^{(n_{f,i}-1)} \quad (4.4)$$

with

$$K_{f,i} = \frac{\sum_i^N (K_{f,i}/a_i)}{N} \quad (4.5)$$

$$n_{f,i} = \frac{\sum_i^N n_{f,i}}{N} \quad (4.6)$$

where $K_{f,i}$ = the weighted average of $K_{f,i}$ values ($\text{mg}^{1-\text{nf}}\text{L}^{\text{nf}}\text{g}^{-1}$), $n_{f,i}$ = the average of the $n_{f,i}$ values, a_i = the competition coefficient for each solute (dimensionless), and N is the total number of solutes. The values used for $K_{f,i}$, $n_{f,i}$, and a_i are listed in Table 4.3. The $C_{i(aq)}$ in Equation 4.4 was taken as effluent concentration at the end of Year-1, which was assumed to be representative of the aqueous BTX concentration throughout the column.

Table 4.3: Freundlich sorption parameters and multi-compound competition coefficients for BTX on Calgon WPC® powdered activated carbon ($\pm 95\%$ CI) adapted from Marrocco (2023).

Compound	$K_{f,i}$ ($\text{mg}^{1-\text{nf}}\text{L}^{\text{nf}}\text{g}^{-1}$)	$n_{f,i}$ [-]	a_i [-]
Benzene	36.1 ± 3.77	0.484 ± 0.045	1.416 ± 0.383
Toluene	88.2 ± 7.70	0.421 ± 0.044	1.432 ± 0.158
<i>o</i> -Xylene	131 ± 20.0	0.371 ± 0.099	1.080 ± 0.078

The $m_{i(s),E}$ was estimated using the 5 mg sub-samples collected from the active columns at the end of Year-2 (Section 4.1.3). The total solid phase mass of each solute, i , was obtained by:

$$m_{i(s)} = \sum_{s=1}^z AL_s \rho_{BS} C_{s,i(s)} \quad (4.7)$$

where A = the cross-sectional area of the column (cm^2), L_s = the length of the sample segment S (cm), ρ_{BS} = the bulk density of BS (1.81 g/cm^3), and $C_{s,i(s)}$ = the solid phase concentration of solute i in segment S (mg/g), and z is the total number of sample segments. Finally, the $m_{i,BD}$ was calculated using Equation 4.1 for Year-2 only and over the entire experimental period (Year-1 + Year-2).

4.2 Results and Discussion

During the Year-1 acclimation period, the measured flow rate varied from 0.017 to 0.76 mL/min, and the BTX mass injected was variable as each system required different BTX loading rates to achieve breakthrough. The average flow rate, linear velocity, and residence time for the column and PAC zone during Year-2 are listed in Table 4.3 along with the average influent and effluent pH, ORP, and DO values. The influent and effluent pH remained near 7 for all columns, indicating near neutral pH conditions (Table 4.3). The influent ORP of all columns was ~180 mV, and the effluent ORP for the active columns was, in general, much lower indicative of biodegradation; however, the ORP was highly variable as suggested by the large standard deviation. The KC-PAC column did not have a decrease in the effluent ORP as no activity was expected in this system (Table 4.3). Finally, both the influent and effluent DO in the KC-PAC column were higher than the active columns due to its lack of biodegradation and inability to support aerobic activity. Conversely, the active columns support biodegradation with the aerobic pathway preferred, thus any oxygen present was utilized first. Sulfate decreased by > 99% in all active columns (Appendix B3, Table B.2), suggesting that a shift in the column took place from sulfate reducing conditions (as sulfate was utilized) to methanogenic conditions.

The stepwise increase in influent concentrations from 5 to 10 and then to 20 mg/L was achieved for benzene, toluene, and *o*-xylene in the KC-PAC, A-BA, A-PAC, and A-PAC-BA columns. The A column had influent concentrations of benzene and toluene decrease to 14 to 15 mg/L by PV ~35 and to 3 to 4 mg/L by PV 60, following the target step increase at PV ~35 to 20 mg/L. These influent concentrations were lower than expected, and are suspected to be caused by losses of benzene and toluene mass in the Tedlar bag despite the Year-1 efforts to optimize the Tedlar bag used.

Given Year-1 was an acclimation period, the results discussed here focus on data collected during Year-2 only. Time series of aqueous BTX, DIC and methane concentrations at the influent and the effluent are presented with respect to PVs. One effluent PV is equal to the time it takes one parcel of fluid contained in the pore space to travel through the column. To determine the PV associated with an influent sample, one PV was added to the effluent PV value at the time the influent sample

was collected. Bulk BTX concentrations, and qPCR targets from sub-samples collected from the columns at the end of the experiment are also discussed.

Table 4.3: Average flow rate, residence time, pH, ORP, and DO during Year-2 operation.

Parameter	KC-PAC	A	A-BA	A-PAC	A-PAC-BA
Flow rate (mL/min)	0.021	0.021	0.025	0.025	0.023
Average linear velocity (cm/min)	0.006	0.006	0.008	0.008	0.007
Residence time (days)	4.54	4.18	3.41	3.32	3.77
PAC zone (6 cm width) residence time (days)	0.735	--	--	0.541	0.615
Average Influent Conditions					
pH	7.15 ± 0.15	6.58 ± 1.35	7.00 ± 0.25	6.98 ± 0.21	6.88 ± 0.21
ORP (mV)	181 ± 28.8	175 ± 48.3	175 ± 29.5	188 ± 40.1	175 ± 54.9
DO (mg/L)	3.59 ± 1.67	0.48 ± 0.28	0.77 ± 0.39	0.67 ± 0.28	0.72 ± 0.71
Average Effluent Conditions					
pH	7.25 ± 0.14	6.95 ± 0.14	6.77 ± 1.39	7.09 ± 0.16	6.93 ± 0.16
ORP (mV)	195 ± 38.8	9.38 ± 94.7	45.0 ± 114	21.2 ± 118	8.10 ± 116
DO (mg/L)	2.52 ± 1.38	0.43 ± 0.18	0.53 ± 0.30	0.47 ± 0.19	0.43 ± 0.22

4.2.1 Biodegraded Mass Estimates

Table 4.4 summarizes the BTX mass attributed to biodegradation during Year-2 estimated from the mass balance calculations. A summary of aqueous phase cumulative influent and effluent masses, the estimated solid phase mass at the beginning of Year-2 (end of Year-1), and the solid phase mass in the PAC zone at the end of Year-2 are provided in Appendix B3, Table B.1.

The mass of benzene attributed to biodegradation was highest in the A-PAC-BA system (~ 25%), while the A, A-BA, and A-PAC columns had < 20% benzene mass reduction (Table 4.4). A minimum threshold of 20% mass reduction was assigned to account for experimental variability and estimation uncertainty. The larger apparent biodegraded benzene mass in the column with both

PAC and BA (as opposed to either PAC or BA alone) suggests that the combination of PAC + BA increased the biodegradation of benzene. The mass reduction of toluene attributed to biodegradation was ~ 95% in the A-PAC column and > 99% in the A-BA and A-PAC-BA systems, demonstrating that systems with either BA, PAC, or both were all highly effective at reducing toluene mass. The mass balance for the active column (A) indicated apparent toluene biodegradation but to a lesser degree (~ 70%) than the columns with BA and/or PAC. The mass reduction of *o*-xylene attributed to biodegradation was highest in the A-BA (~ 90%) and A-PAC-BA (~ 80%) columns compared to their non-BA counterparts. Furthermore, the A-PAC column had a slightly higher estimated loss of *o*-xylene mass attributed to biodegradation than the A column (~ 25% versus < 20%). The A-BA column had higher overall BTX mass reduction than the A-PAC column, as they performed similarly for benzene (< 20%) and toluene (> 99% and ~ 95%, respectively), but the A-BA column had higher *o*-xylene mass reduction (~90% versus ~25%). These mass balance estimates suggest that systems with BA are needed to significantly biodegrade *o*-xylene (> 80%) regardless of the presence of PAC.

Table 4.4 Percent reduction in BTX mass during Year-2 column operation. A minimum threshold of 20% mass reduction was assigned to account for experimental variability and estimation uncertainty.

Column	Benzene % reduction	Toluene % reduction	O-xylene % reduction
A	< 20 %	~ 70 %	< 20 %
A-PAC	< 20 %	~ 95 %	~ 25 %
A-BA	< 20 %	> 99 %	~ 90 %
A-PAC-BA	~ 25 %	> 99 %	~ 80 %

4.2.2 Killed Control System

The time series of aqueous BTX concentrations, TIC and methane concentrations, and cumulative aqueous BTX mass for the KC-PAC column are shown in Figure 4.5. This column serves as a baseline for the active columns, showing the expected behavior for a system with PAC but with no biodegradation taking place. The effluent response to increases in BTX influent concentration was delayed due to sorption onto PAC, but eventually reached near influent concentrations at PV 52 (influent concentrations of BTX were 20.14, 19.37, and 18.38 mg/L, respectively, and effluent concentrations were 19.47, 18.27, 14.68 mg/L, respectively). Benzene and toluene effluent

concentrations reached near the influent concentrations at PV 30, indicating that the system reached equilibrium. *o*-Xylene effluent concentrations also increased towards influent concentration values, but remained ~ 4 mg/L lower, indicating the system had not reached equilibrium by PV 52. The lack of biodegradation in the column is confirmed by the absence of methane production (Figure 4.5b), as well as stable influent and effluent sulfate concentrations at ~ 25 mg/L (Appendix B4, Figure B.1). Together, the KC-PAC column results demonstrate the ability of the PAC to sequester PHCs and thereby increase the time for toluene and *o*-xylene to reach the effluent concentration compared to the system without PAC.

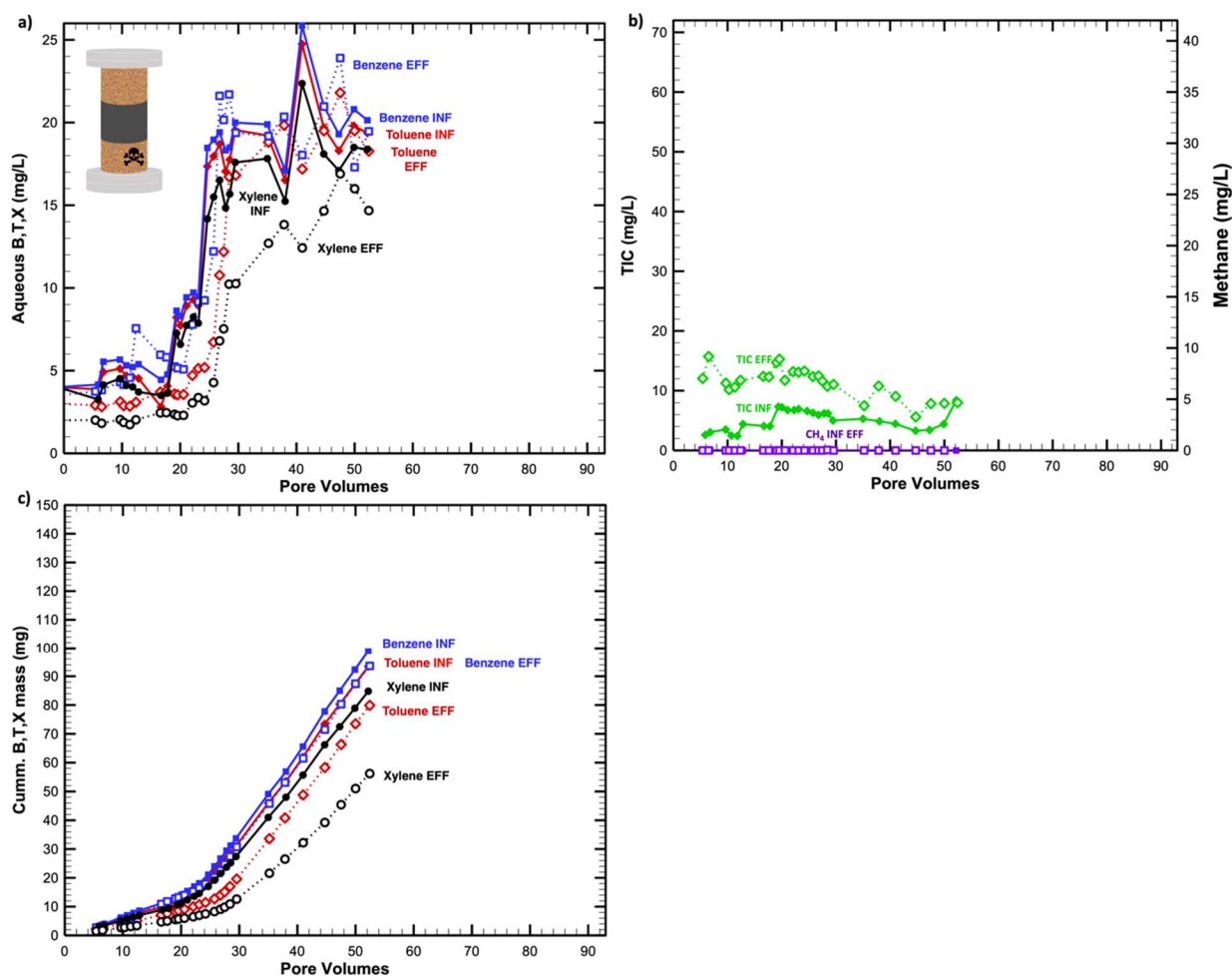


Figure 4.5: a) Aqueous phase BTX concentration (influent and effluent), b) TIC concentration and methane concentration c) Cumulative aqueous phase BTX mass for the KC-PAC column.

Figure 4.5c highlights the cumulative BTX mass over time in the influent and effluent. At PV 52.19, there was a small difference between the influent and effluent benzene cumulative mass of

5.3 mg. At the same PV, toluene had a larger difference between the influent and effluent cumulative mass (13.5 mg), and *o*-xylene had the largest difference (28.6 mg), suggesting these solutes were preferentially sorbed to the PAC under the assumption of minimal losses of these BTX compounds to other sources. This order of mass storage is a function of competitive sorption and the sorption affinity of each solute to PAC, following the order of *o*-xylene, then toluene, then benzene (high to low) (Daifullah and Girgis, 2003). This pattern of preferential sorption seen from the cumulative BTX mass is consistent with the aqueous concentration time series data (Figure 4.5a).

4.2.3 Active Systems Without PAC

The time series of aqueous BTX concentrations, TIC and methane concentrations, and cumulative aqueous BTX mass for the active (A) column is shown in Figure 4.6. The effluent concentration of benzene mirrored the influent behavior, which was expected given there was no BA in this column and the lack of a substantial native benzene degrading community present in the BS (Toth et al., 2021). The toluene cumulative mass was lower in the effluent than the influent and the deviation increased as the experiment progressed; the difference was 16.5 mg at PV 35.6 and 65.7 mg at PV 81.5. The increasing difference suggests that toluene mass was being removed from the system through biodegradation since sorption to BS was very low. The low sorption to BS was expected based on the low f_{oc} of BS (0.00024 [-], Xu and Thomson (2008)) and was confirmed by the very low (< MDL) solid phase toluene concentrations at the end of the experiment (Appendix B5, Figure B.6). The *o*-xylene effluent concentration was ~1-2 mg/L lower than the influent concentration until PV ~40, which may be due to minor *o*-xylene biodegradation. Beyond PV~40 the influent and effluent had the same concentration, which suggests that biodegradation halted despite the expectation that the biodegradation rate would remain consistent or increase. The TIC and methane concentrations (Figure 4.6b) further support that toluene and minimal *o*-xylene biodegradation took place; by PV 35 methane increased from <MDL to ~ 25 mg/L and TIC increased from ~ 15 to ~ 45 mg/L between the influent to the effluent. Significant sulfate reduction took place in the A column, as all the sulfate added ($229.1 \text{ mg} \pm 10\%$) (Appendix B4, Figure B.2).

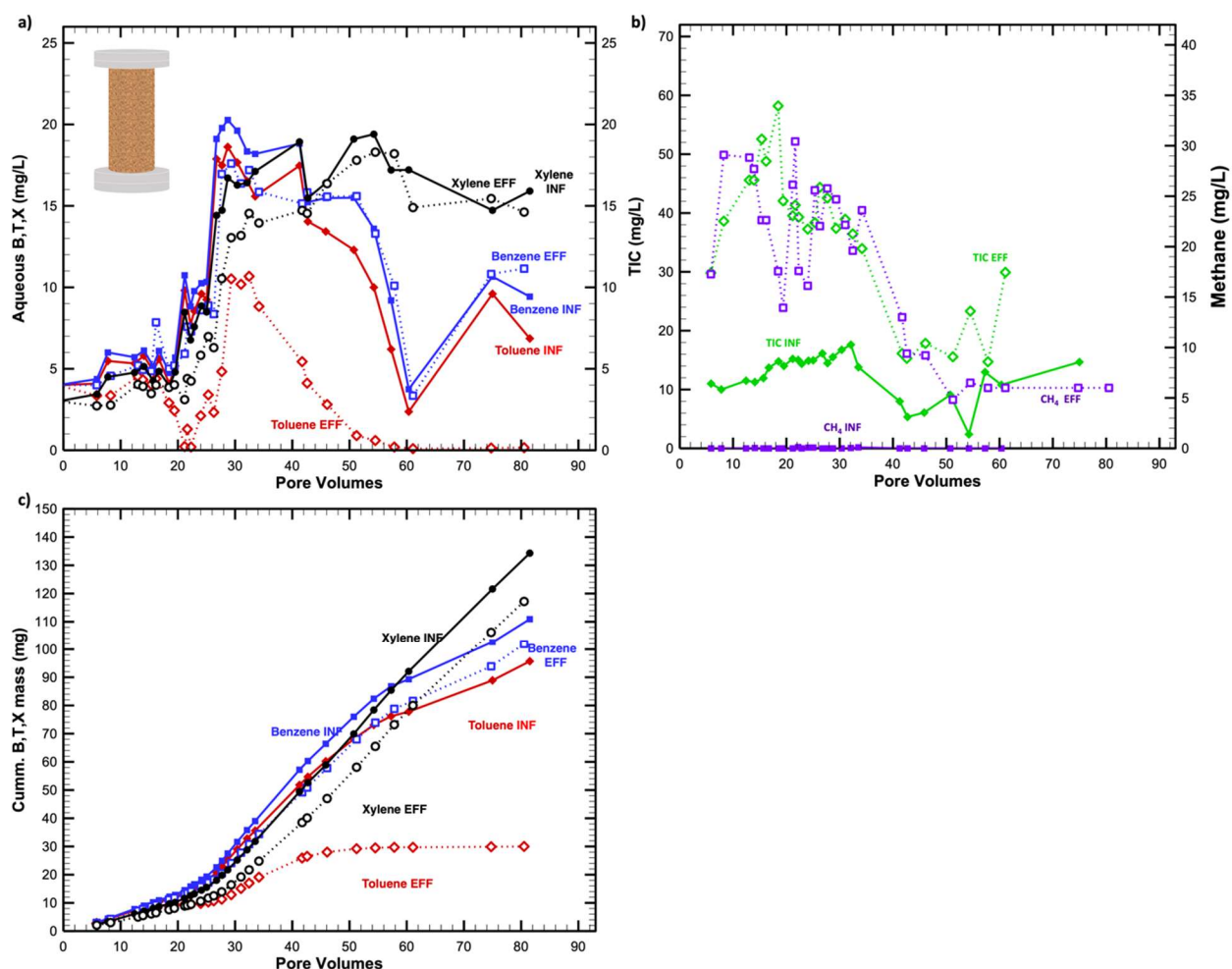


Figure 4.6: a) Aqueous phase BTX concentration (influent and effluent), b) TIC concentration and methane concentration c) Cumulative aqueous phase BTX mass for the A column.

After PV 38, the influent toluene and benzene concentrations lowered rapidly from ~ 15 mg/L to 2 – 3 mg/L over ~ 20 PVs, while the *o*-xylene concentration remained relatively constant. The decrease in toluene and benzene concentrations is accompanied by a similar decreasing trend in TIC and methane concentrations, suggesting an issue with the delivery of BTX at the influent (feed solution). Additionally, the effluent TIC and methane concentrations before PV 30 are stoichiometrically higher than expected given the low BTX concentrations in the column influent. This large amount of methane and TIC generation is not explainable through complete conversion of BTX mass in the system before PV 30.

Figure 4.7 shows the time series data of aqueous BTX concentrations, TIC and methane concentrations, and cumulative aqueous BTX mass for the active column containing the bioaugmentation cultures (A-BA). Similar to the A column, the solid phase concentrations of BTX at the end of the experiment were < MDL for benzene and toluene, and < 10 mg/kg bulk for *o*-xylene (Appendix B5, Figure B.7), indicative of very low storage of BTX mass by the BS.

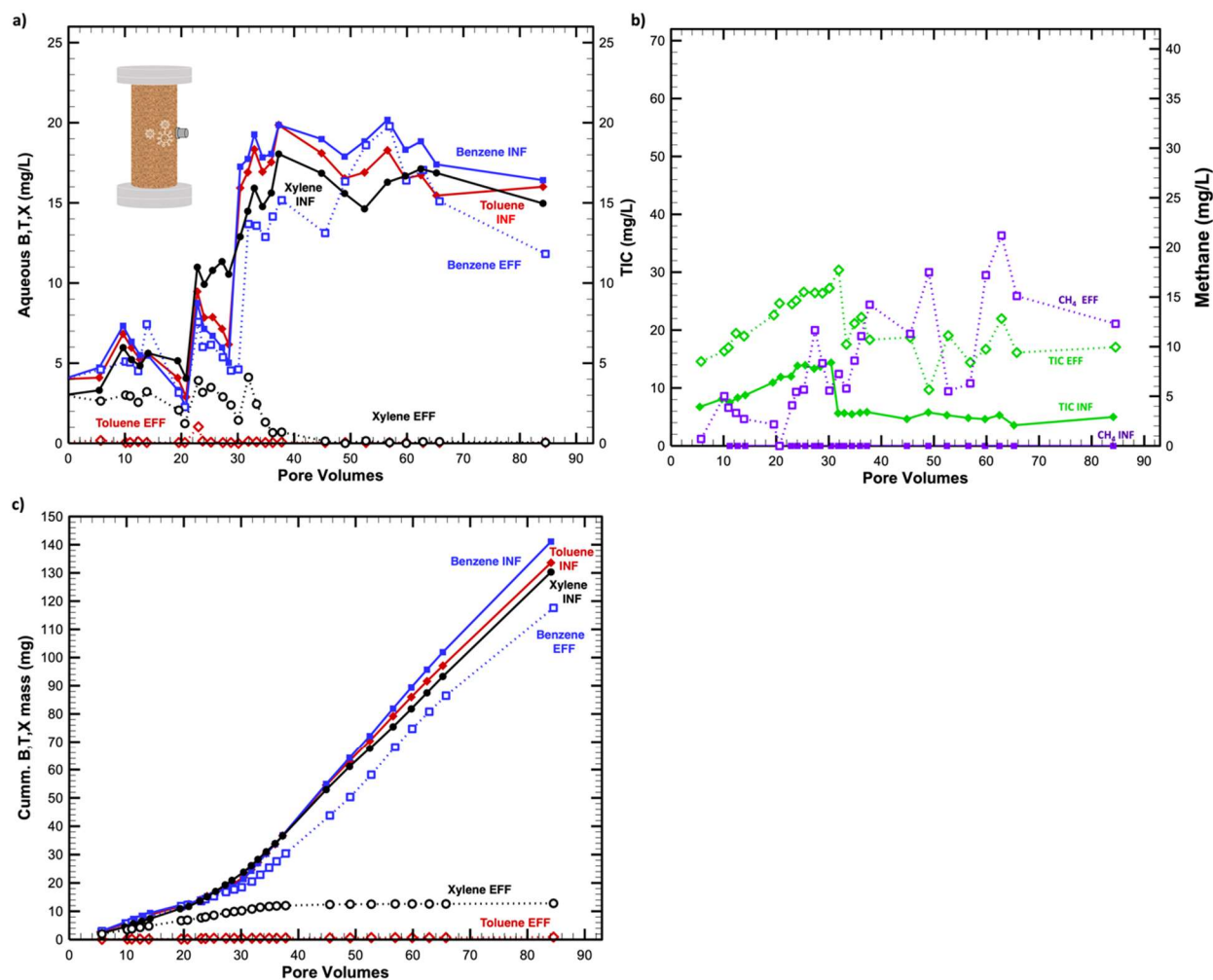


Figure 4.7: a) Aqueous phase BTX concentration (influent and effluent), b) TIC concentration and methane concentration c) Cumulative aqueous phase BTX mass for the A-BA column.

Toluene effluent concentrations were < or near MDL for the entire experiment (Figure 4.7a) which suggests biodegradation of toluene occurred. Effluent *o*-xylene concentration remained at ~3 mg/L until PV 30. Once the influent *o*-xylene concentration reached 20 mg/L (PV ~30), the *o*-xylene effluent concentration decreased from 3 mg/L to < MDL (PV 30 to 40), possibly due to increasing microbial populations in the presence of higher *o*-xylene concentrations. The benzene effluent

concentrations (Figure 4.7a) were consistently ~5 mg/L lower than the influent concentration from PV 35 to 45, and 3 to 5 mg/L lower than the influent concentration from PV 65 to 85, which suggests that benzene was biodegraded. Since both toluene and *o*-xylene were depleted during the 20 mg/L influent concentration step at > PV 50 (Figure 4.7a), they would not hinder benzene biodegradation, allowing for benzene biodegradation to take place. The elevated TIC and methane concentrations throughout the experiment (Figure 4.7b) supports that biodegradation occurred within the column, with notable increases in effluent methane corresponding to increases in BTX mass loading (after PV 40). All the sulfate added to the column ($231.9 \text{ mg} \pm 10\%$) was utilized, suggesting significant sulfate reduction took place (Appendix B1, Table B.2).

The cumulative BTX mass in the A-BA column (Figure 4.7c) further highlights the biodegradation behaviour within this column. The cumulative effluent mass of benzene closely followed the cumulative influent mass and was 13 mg lower the end of the experiment, suggesting biodegradation occurred. In contrast, *o*-xylene cumulative effluent mass increased to 12.8 mg by PV 45 and then remained steady. Toluene biodegradation occurred in both the A and A-BA columns, but *o*-xylene biodegradation only occurred in the A-BA column.

Figure 4.8 presents the qPCR targets versus distance from the column influent from the A and A-BA columns. The communities highlighted from the qPCR test were ORM2, Desulfosporosinus (DSP), and Peptococcaceae (PEP), which are key fermenters in benzene, toluene, and *o*-xylene biodegradation, respectively. ORM2 was not present in the A column (Figure 4.8a), which supports that no benzene biodegradation took place. DSP was present closer to the influent (10^5 copies/g at 5 cm from the influent) of the A column and declined towards the effluent end, demonstrating as toluene degraded, the population of degraders also declined. PEP was non-existent in this column, which supports the observation that no *o*-xylene biodegradation occurred.

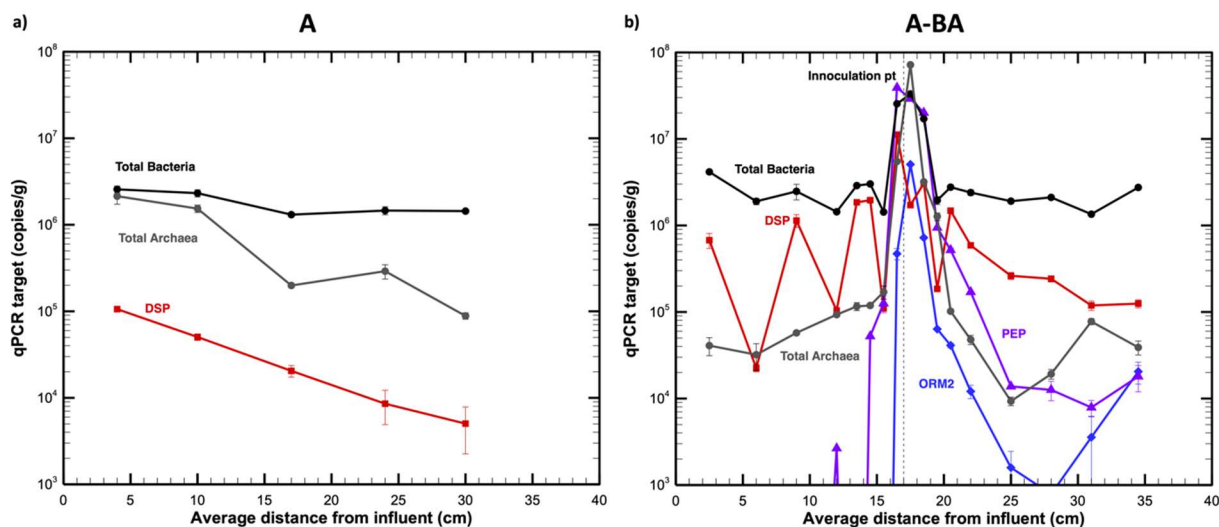


Figure 4.8: qPCR targets versus average distance from the influent (INF) for the a) A column, and b) A-BA column. The dotted line indicates the location of the culture addition at 17 cm from INF. The shaded area between 14 and 20 cm from influent represents the location of the PAC zone.

For the A-BA column (Figure 4.8b), ORM2 was present in the zone of culture addition ($> 10^6$ copies/g at 18 cm from the influent), which provides evidence that the necessary fermenters for benzene biodegradation were present. There is also evidence of an increase in population of ORM2 at the end of the column, but the population did not reach the $> 10^7$ copies/g needed to support benzene biodegradation (Toth et al, 2021). Increases to the length of the column may have provided additional residence time to allow a population to sufficiently grow downgradient of the zone of culture addition. DSP was present upgradient of the culture addition zone at $\sim 10^6$ copies/g, and peaked in the addition zone to 10^7 copies/g. The population of DSP slowly declined over the column length presumably due to decreasing toluene concentrations as a result of biodegradation. PEP followed a similar trend to DSP; upgradient of the addition zone it had a population of $\sim 10^5$ copies/g, then spiked at the start of the addition zone to $\sim 10^8$ copies/g, followed by a slow decline through the column as *o*-xylene concentration declined due to biodegradation.

4.2.4 Active Systems With PAC

The time series of aqueous BTX concentration, TIC and methane concentrations, and cumulative aqueous BTX mass for the active column containing PAC (A-PAC) is shown in Figure 4.9. Toluene concentrations remained $< \text{MDL}$ or slightly above ($< 1 \text{ mg/L}$) throughout the experiment. *o*-Xylene also remained within 1 mg/L of MDL until PV 40, after which it began to increase. As with the A

column, complete *o*-xylene biodegradation was not expected for this column, and thus the concentration increase was likely due to breakthrough

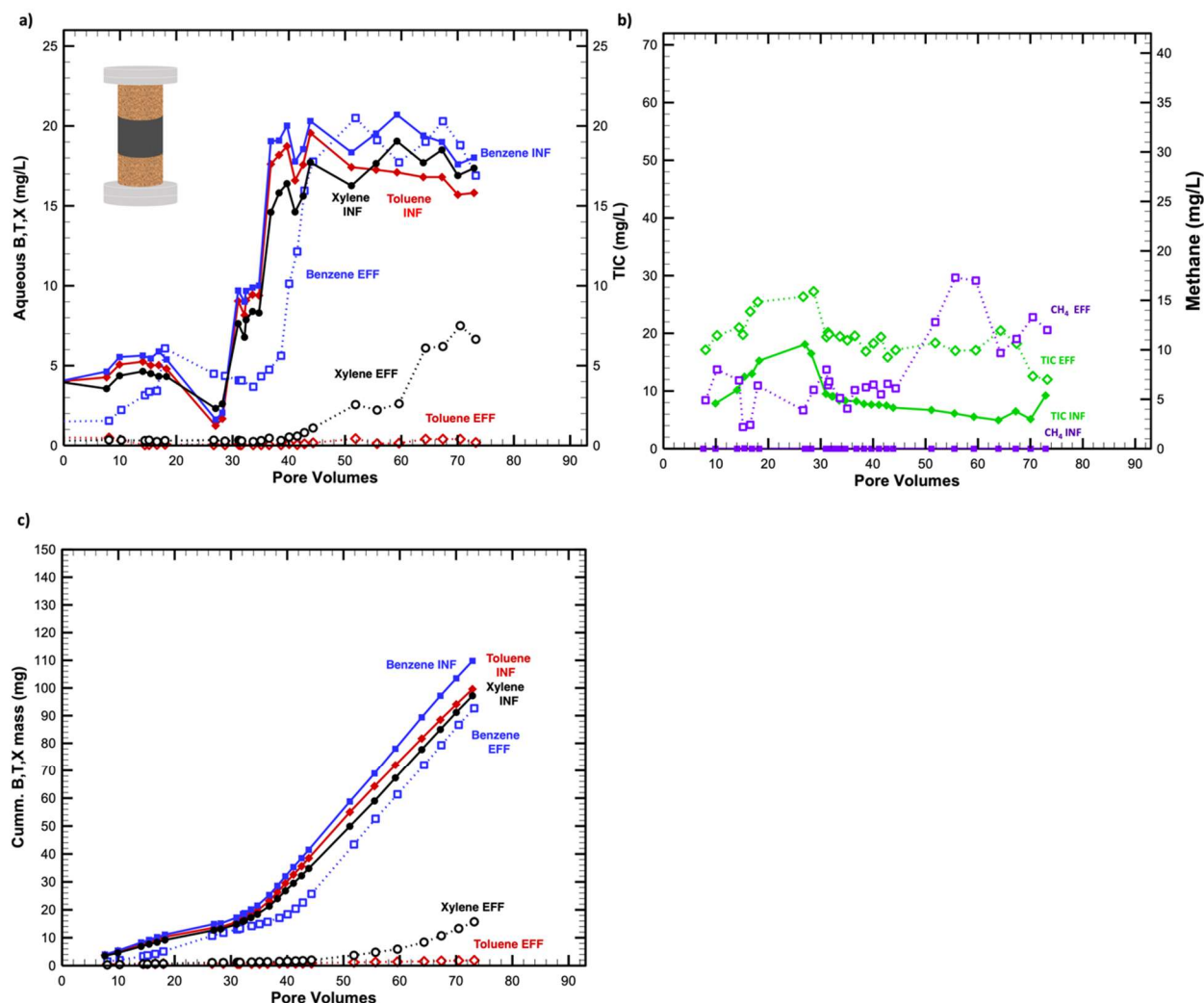


Figure 4.9: a) Aqueous phase BTX concentration (influent and effluent), b) TIC concentration and methane concentration c) Cumulative aqueous phase BTX mass for the A-PAC column.

The increased effluent TIC and methane concentrations (Figure 4.9b) suggest that biodegradation occurred. Additionally, the complete depletion of the injected sulfate over the experimental duration (209.8 mg) is indicative of significant sulfate reduction (Appendix B4, Figure B.4). These results suggests that toluene biodegradation, and possibly small amounts of *o*-xylene biodegradation, took place. The A-PAC column results further support the conclusion made from the KC-PAC column that PAC increases the time for toluene and *o*-xylene to reach the effluent compared to a system without PAC. Consistent with the mass balances estimates (Table 4.4),

minimal biodegradation of benzene occurred as the effluent and influent benzene concentrations were essentially the same by PV 50. This behavior mimics what was observed from the KC-PAC column.

The time series of aqueous BTX concentration, TIC and methane concentrations, and cumulative aqueous BTX concentrations for the active column with a PAC zone and bioaugmentation addition (A-PAC-BA) are shown in Figure 4.10. The toluene and *o*-xylene concentrations remained below or slightly above MDL throughout the experiment, indicating that biodegradation of these compounds occurred. The biodegradation of toluene and *o*-xylene is further supported by the elevated levels of methane and TIC generated throughout the experiment (Figure 4.10b) and by the depletion of sulfate (Appendix B4, Figure B.5). Similar to the A-PAC column, the benzene effluent concentration in the A-PAC-BA column followed the increasing influent concentrations with a lag, but was lower than the influent concentration throughout the experiment, due to sorption to the PAC and minimal biodegradation (~ 25% reduction, Table 4.4)

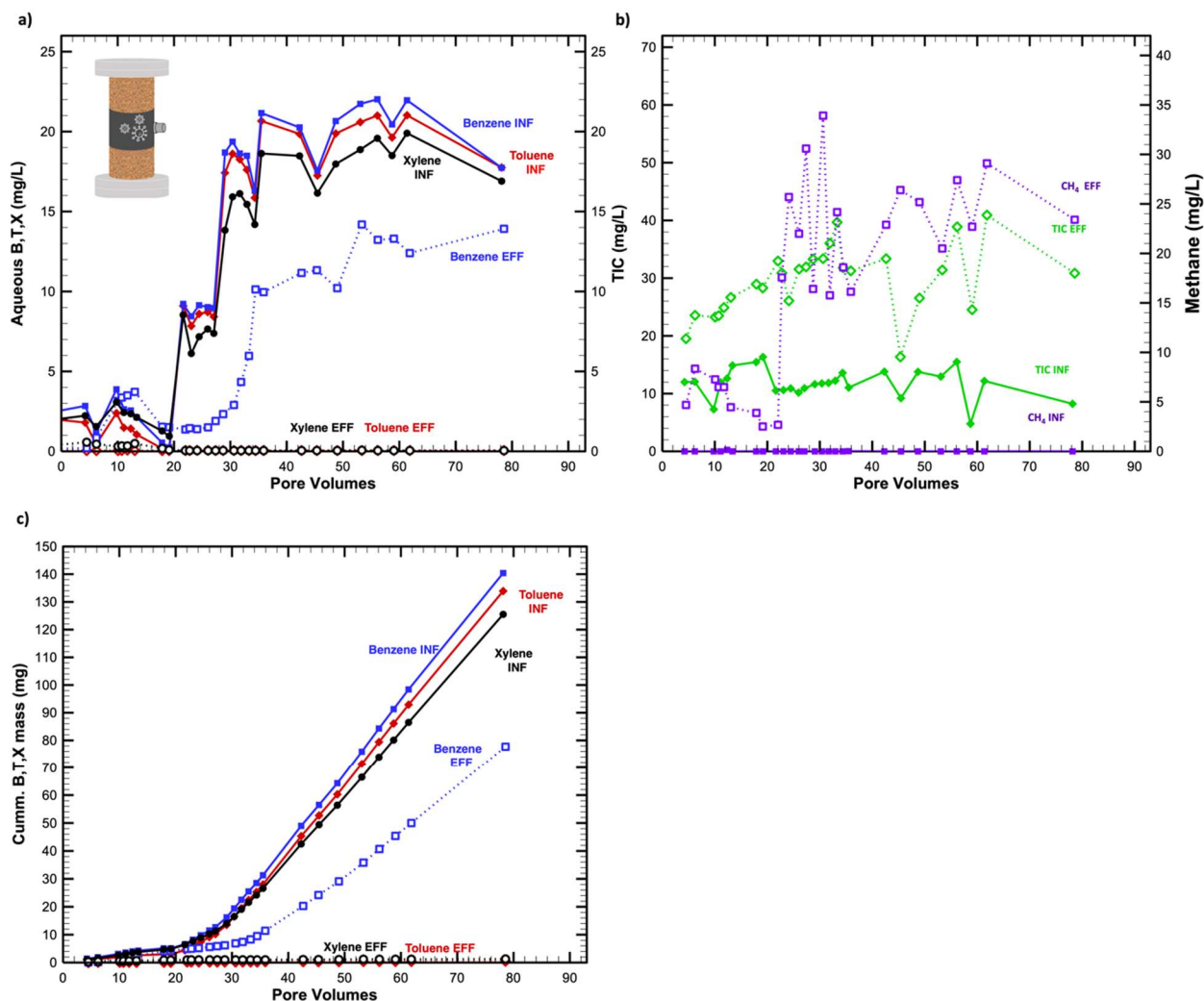


Figure 4.10: a) Aqueous phase BTX concentration (influent and effluent), b) TIC concentration and methane concentration c) Cumulative aqueous phase BTX mass for the A-PAC-BA column.

Figure 4.11 shows the bulk solid BTX concentration (in mg target PHC compound per kg of bulk material) for the A-PAC (Figure 4.11a) and A-PAC-BA (Figure 4.11b) columns. For clarity, Figure 4.11c and Figure 4.11d show the PAC zone only (14 to 20 cm from the influent end). The A-PAC column had higher solid phase toluene concentrations at the beginning of the PAC zone (e.g., 34.9 mg/kg at 14 cm from the influent) then declined towards the end of the zone (4.34 mg/kg at 19 cm from the influent) due to biodegradation (Figure 4.11a and Figure 4.11c). Conversely, *o*-xylene and benzene solid phase concentrations increased through the PAC zone from 487.8 mg/kg to 921.4 mg/kg and from 13.4 to 31.9 mg/kg, respectively. The increase in the solid phase concentrations of *o*-xylene and benzene was due to toluene biodegradation in the aqueous phase

leading to subsequent desorption from the PAC. The higher *o*-xylene solid phase concentrations compared to benzene is due to its higher sorption affinity to PAC.

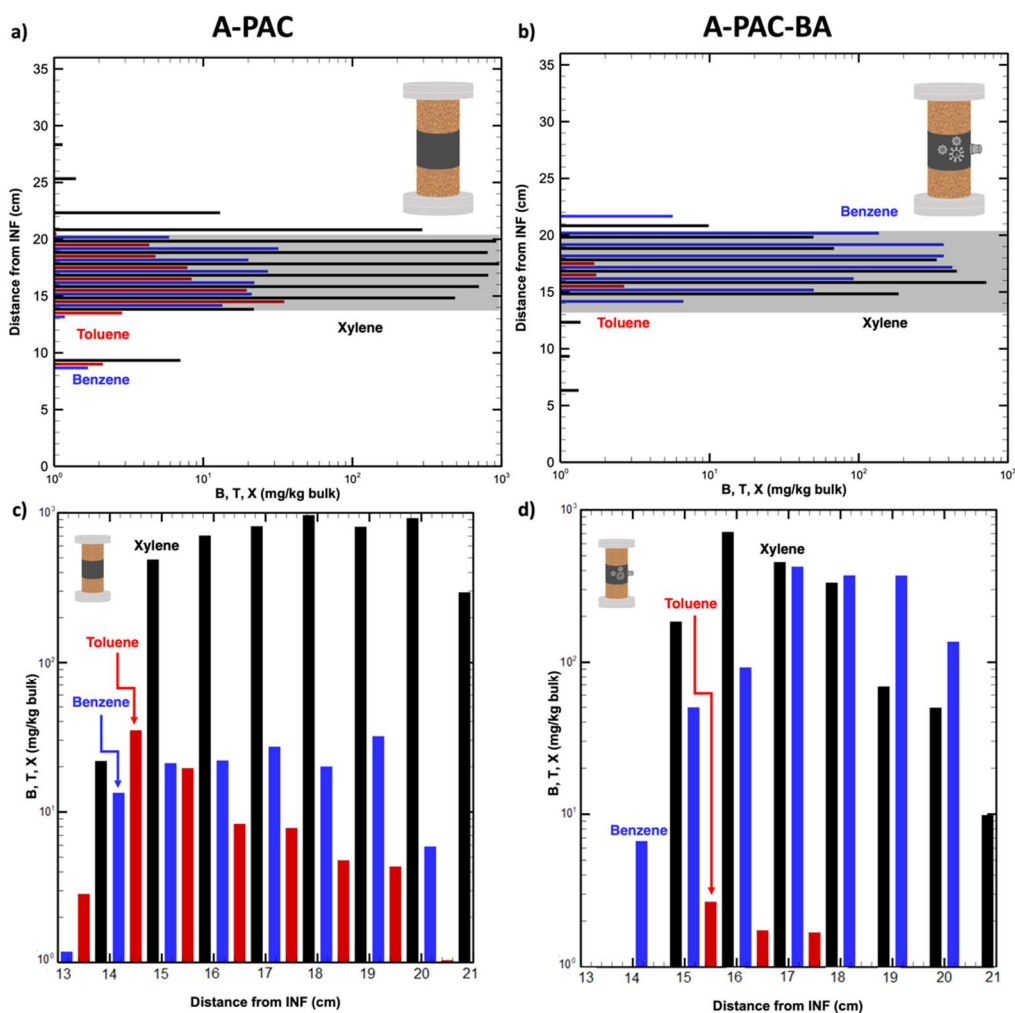


Figure 4.11: Solid phase BTX concentration distributions in a) A-PAC, and b) A-PAC-BA columns for the whole column length. Grey Zone highlights the PAC zone of c) A-PAC, d) A-PAC-BA columns.

Solid phase toluene in the A-PAC-BA column at the start of the PAC zone (2.67 mg/kg at 14 cm from the influent) declined through the zone ($< \text{MDL}$ by 19 cm from the influent) due to biodegradation and subsequent desorption (Figure 4.11b and Figure 4.11d). Concentrations of *o*-xylene also declined through the PAC zone from 717.0 mg/kg at 15 cm from the influent to 49.7 mg/kg at 19 cm from the influent. *o*-Xylene concentrations were higher at the start of the PAC zone compared to toluene due to its higher sorption affinity and the order of biodegradation ($T > X > B$). This decreasing trend for *o*-xylene supports the conclusions based on the aqueous

concentration data that *o*-xylene biodegradation occurred within the PAC zone. This decreasing trend of *o*-xylene was not evident in A-PAC column where *o*-xylene bulk phase concentrations increased through the PAC zone.

Benzene solid phase concentrations in the A-PAC-BA column increased over the length of the PAC zone (from 49.9 mg/kg at 15 cm from the influent to 424.16 mg/kg at 17 cm from the influent), as toluene and *o*-xylene biodegradation through that section of the PAC zone made sorption sites available for additional benzene sorption to PAC. As in the A-PAC column, the solid phase BTX concentrations through the PAC zone of the A-PAC-BA column followed the order of competitive sorption; when *o*-xylene concentrations started to decrease, more benzene mass sorbed. The decrease in the solid benzene mass concentration to 136.59 mg/kg at 20 cm from the influent from 424.16 mg/kg at 17 cm from the influent supports that some benzene biodegradation (~25% reduction in the total mass injected, Table 4.4) occurred.

Similar to the A column, ORM-2 was non-existent in the A-PAC column (Figure 4.12a) since it is not naturally present in the BS. Therefore, minimal benzene biodegradation was expected and was confirmed by the qPCR results. DSP was present near the influent (10^5 copies/g at 5 cm from the influent), indicating an area of heightened biodegradation of toluene. Between 5 and 10 cm from the influent, DSP populations decreased, but rebounded at the start of the PAC zone (3×10^5 copies/g at 16 cm from the influent) before steadily decreasing through the PAC zone. The trend in DSP population mimics the trend in the toluene solid phase concentration (Figure 4.11), which showed that toluene mass decreased through the PAC zone and suggests that heightened areas of toluene biodegradation occurred in an area near the beginning of the PAC zone. PEP population increased to 5×10^4 copies/g in the middle of the PAC zone where toluene mass concentrations had decreased. This population is able to support *o*-xylene biodegradation activity, and therefore the qPCR data indicates that *o*-xylene biodegradation occurred primarily in the central portion of the PAC zone.

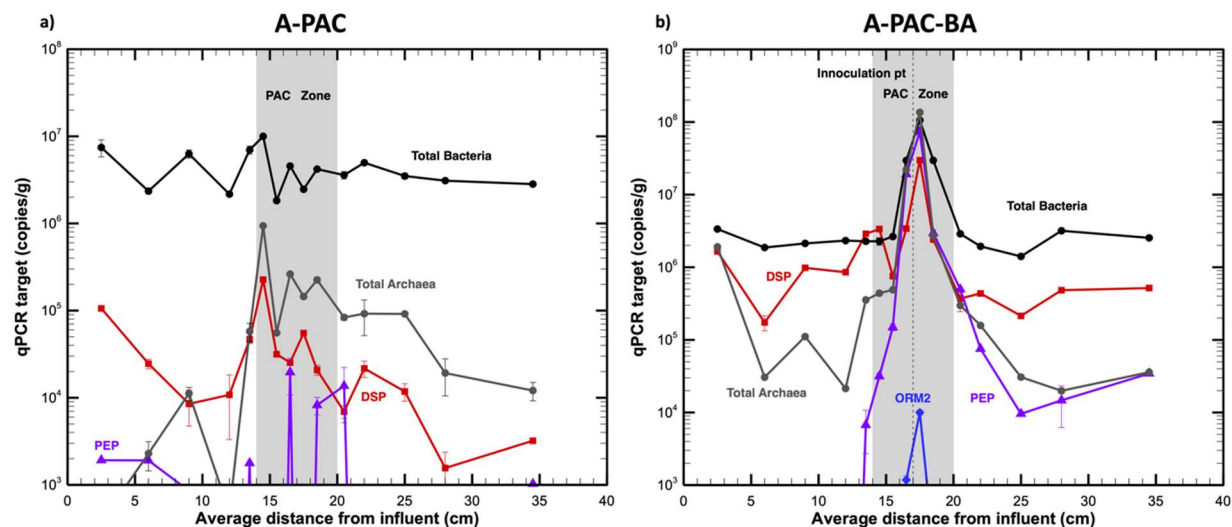


Figure 4.12: qPCR targets versus average distance from the influent (INF) for the a) A-PAC column, b) A-PAC-BA column. The dotted line indicates the location of the culture addition at 17 cm from INF. The shaded area between 14 and 20 cm average distance from influent represents the location of the PAC zone.

DSP was present in the A-PAC-BA column before the culture addition zone at $\sim 10^6$ copies/g and peaked at 5×10^7 copies/g within the BA zone (Figure 4.12b). The population of DSP slowly declined through the column as the toluene mass was depleted due to biodegradation. PEP followed a similar trend to DSP but was not present before the BA zone. At the start of the BA zone, the abundance of PEP spiked to $\sim 10^8$ copies/g, with a slow decline as the *o*-xylene mass was degraded.

Finally, the A-PAC-BA column (Figure 4.12b) had a small ORM2 presence at $\sim 10^4$ copies/g near the BA injection zone, which is low in relation to the $> 10^7$ copies/g suggested by Toth et al. (2022) to be the required population to support significant benzene biodegradation. Despite the ORM2 population being low, the $\sim 25\%$ mass reduction and complete biodegradation of toluene and *o*-xylene suggest minor benzene biodegradation took place. It is possible that the qPCR results from the A-PAC-BA column were unrepresentative of ORM2 populations. This explanation would also explain why a larger ORM2 population was found in the A-BA column versus the A-PAC-BA column ($> 10^6$ copies/g versus $\sim 10^4$ at 18 cm from the influent, respectively) despite the A-BA column having $< 20\%$ benzene mass reduction.

4.3 Summary

The objective of the column experiments was to assess how PAC coupled with bioaugmentation affects the biodegradation of BTX compounds in anaerobic environments. The hypothesis was that the presence of PAC will enhance the biodegradation of BTX in systems with and without bioaugmentation. In the active column without PAC or bioaugmentation (A), the presence of toluene biodegradation was supported by the toluene mass reduction and qPCR results, indicating that BS has the potential for toluene biodegradation. The addition of PAC resulted in increased biodegradation of toluene and *o*-xylene, indicated by the higher percent reduction of toluene and *o*-xylene mass in the A-PAC column compared to the A column, and further supported by the presence of toluene and *o*-xylene degrading microbial populations. The active column with bioaugmentation (A-BA) showed evidence of both toluene and *o*-xylene biodegradation through increased mass reduction compared to both the A and A-PAC systems, which was further supported by the qPCR data. Despite the high ORM2 populations in the A-BA column, the biodegradation of benzene was < 20%. The active column with both PAC and bioaugmentation (A-PAC-BA) had a reduction of toluene mass comparable to the A-BA system, but a lower reduction of *o*-xylene mass and a higher reduction of benzene mass. The qPCR data supports toluene and *o*-xylene biodegradation in the A-PAC-BA column, but there was a lack of definitive evidence for increased benzene biodegradation, as ORM2 populations were lower than in the A-BA column.

In summary, the column systems performed differently at reducing the mass of injected benzene, toluene and *o*-xylene than expected. PAC did not enhance the biodegradation of toluene in the presence of BA, as the A-PAC, A-BA, and A-PAC-BA columns all had mass reductions attributed to biodegradation of > 95%. The A-BA column had higher *o*-xylene mass reduction than both the A-PAC and A-PAC-BA columns, suggesting the presence of PAC did not improve *o*-xylene biodegradation. However, due to competing lines of evidence no conclusion could be reached regarding whether PAC improved the biodegradation of benzene.

From the data compiled, it is inconclusive whether the presence of PAC enhanced the overall biodegradation of BTX when combined with bioaugmentation under anaerobic conditions. PAC coupled with BA (A-PAC-BA) was able to remove benzene more efficiently but at the cost of lower *o*-xylene removal compared to BA alone (A-BA). The difference in overall biodegradation of BTX in systems with PAC and BA versus BA alone is insignificant and consistent with the

microcosm results where the biodegradation rates between the A-PAC-BA and A-BA systems were not statistically different.

Chapter 5

Conclusions and Recommendations

5.1 Conclusions

Two experiments were performed to assess the effectiveness of PAC coupled with bioaugmentation as a potential *in situ* technology to address benzene, toluene, and *o*-xylene (BTX) contamination in anaerobic groundwater environments. The objective of the research reported in this thesis was to test if PAC and bioaugmentation synergistically enhance degradation. The hypothesis examined was that the presence of PAC will increase the biodegradation of BTX in systems both with and without bioaugmentation under anaerobic conditions. A microcosm experiment was conducted to represent static conditions, and a column experiment was performed that mimicked *in situ* conditions. All experiments were conducted under anaerobic conditions and included systems with and without the presence of a coconut-based PAC and bioaugmentation DGG™ cultures for benzene, toluene and *o*-xylene. The experiments were conducted using Borden aquifer material (BS) and artificial groundwater.

Two microcosm experiments were performed concurrently: one with toluene only, and the other one with benzene, toluene, and *o*-xylene. Microcosm experiments included killed controls (KC; autoclaved BS with biocide), starved controls (SC; indigenous microbes and no PHCs), positive controls (PC; no BS), active treatment (A; BS indigenous microbes and no PAC), and active treatment with PAC (A-PAC; indigenous microbes and PAC). These microcosm types included a series with and without culture addition (BA). The microcosms were sampled over 270-360 days following a repetitive and sacrificial sampling strategy at designated timepoints. Aqueous PHC, and sulfate concentrations were monitored as well as pH, ORP, DO, and gas phase carbon dioxide and methane during repetitive sampling events. During sacrificial sampling events, aqueous dissolved inorganic carbon (DIC) and solid phase PHC concentrations were monitored. At the conclusion of the microcosm experiments, solid phase samples were collected to characterize the microbial populations.

The KC, PC, and SC systems provided valuable information on background toluene-only and BTX biodegradation activity. As expected, the KC microcosms (autoclaved and biocide addition) showed no evidence of biodegradation as there were no viable microbial population present, while the PC microcosms (BA culture without BS) confirmed that the added BA culture provided viable biodegradation activity. The SC systems (no BTX addition) captured the background

biodegradation present in the BS with no added BTX. Data from the bioaugmented systems without PAC (PC-BA, and A-BA) confirmed that methanogenesis occurred based on TIC and methane generation, decreased aqueous BTX concentrations, and the presence of BTX fermenters. No statistically significant difference (at the 95% confidence level) in biodegradation rate was observed between the active systems with and without PAC that did not receive bioaugmentation (A versus A-PAC) suggesting that the presence of PAC did not have an effect. However, this conclusion may be due to insufficient time (< 1 year) to allow for methanogenic populations to proliferate. Finally, the active systems with BA (A-BA versus A-PAC-BA) degraded similar masses of BTX, and biodegradation rates were not statistically different (at the 95% confidence level). Therefore, the results from the microcosm experiments do not support that PAC increases the total mass of BTX that is biodegraded or the biodegradation rate either by itself or in combination with bioaugmentation in static anaerobic systems. The presence of PAC reduced the aqueous concentrations of BTX in the microcosm bottles and evidence of the bioregeneration of sorption sites was captured through the compartmental mass distribution analysis.

The column experiments used benzene, toluene, and *o*-xylene and included the following treatment types: killed control (KC; autoclaved BS with biocide and a PAC zone), active (A; BS and no PAC zone), and active with PAC (A-PAC; indigenous microbes and PAC zone). The active types included columns both with and without BA injection. The columns were subjected to an acclimation period of nine months followed by a 14-month experimental period where pH, ORP, DO, sulfate, aqueous BTX concentrations, aqueous carbon dioxide and methane, and DIC were monitored. At the end of the experiment, the columns were sub-sampled and bulk BTX concentrations were measured and DNA analyses were conducted. A mass balance was completed for each column (excluding KC-PAC) to estimate overall biodegraded mass.

The biodegradation of toluene and *o*-xylene were supported by observed mass reductions across the column (influent vs effluent) and changes in microbial populations in the active columns. Although the presence of a PAC zone improved the total toluene mass reduction for systems without bioaugmentation, toluene biodegradation was near complete ($\geq 95\%$ mass reduction) in the A-PAC, A-BA, and A-PAC-BA systems. Therefore, the results do not show evidence that PAC enhanced the biodegradation capacity of toluene when combined with bioaugmentation. *o*-Xylene had the highest total mass reduction in the A-BA system, suggesting that PAC coupled

with bioaugmentation did not improve *o*-xylene biodegradation compared to bioaugmentation alone. The column experiments were inconclusive regarding whether PAC enhanced the biodegradation of benzene; columns with PAC improved the total mass reduction of benzene, but qPCR results suggested a higher potential for benzene biodegradation in the column without PAC (A-BA). The column experiments, therefore, did not provide conclusive evidence to determine whether PAC enhances BTX biodegradation when combined with bioaugmentation.

Taken together, results from the microcosm and column experiments did not provide evidence that PAC and bioaugmentation work synergistically to enhance the biodegradation of BTX compounds in anaerobic groundwater environments. Although PAC did not improve the biodegradation of BTX in these experiments, bioaugmented systems both with and without PAC showed BTX biodegradation, confirming that the presence of PAC did not hinder biodegradation. For field-scale applications, the use of PAC in combination with bioaugmentation may still provide benefits by containing the BTX mass in a more spatially confined area for longer durations (> 20 years), which may aid in providing more time for biodegradation to occur. This effect would be more prominent in the field where sufficient PAC would be used to prevent breakthrough in 20 to 50 years as opposed to the ~2-month breakthrough period used in the column experiments.

5.2 Recommendations

Overall, the experimental design of the microcosm experiments was deemed successful with minimal variation in the microcosm systems indicating consistency across the bottle sets and the respective systems. Despite this observation, certain limitations were apparent during the experiment, which could be addressed with the following amendments to the experimental design:

- Microcosms were not sampled to determine aqueous BTX concentrations after each re-addition, so that the exact mass added was not known but was assumed to be a nominal amount (20 mg/L). Sampling the microcosms immediately after re-addition would allow for a more accurate calculation of theoretical TIC and methane generation over the experimental duration to be performed and provide a more reliable confirmation of the measured values.
- A complete mass balance based on measured values was not possible for the monthly samples as solid phase data was not collected for the repetitive sampling period. The

collection of solid phase data throughout the experimental period would allow for a more complete mass balance of BTX to be completed. This analysis would allow for the tracking of the mass of BTX in the different phases throughout the experiment, which can confirm whether the BTX mass is biodegraded or sorbed at each timepoint. This analysis would help provide a more mechanistic understanding of the interaction between PAC and BA in the systems.

- The microcosm bottles were not sampled for DNA at the beginning of the experiment (time 0) and it was unknown what the microbial populations were following the culture addition. Sampling the microcosms for DNA immediately following the culture addition would confirm the culture addition led to viable microbial populations within the bottles at the start of the treatment in each system.
- The temporary decline in the biodegradation rate that was observed in the toluene-only PC-BA microcosms were not consistent with the BTX PC-BA microcosms and may be a result of faulty inoculation; if the bioaugmentation did not provide high enough populations, the resulting biodegradation capacity would be hindered. Future experiments with larger culture doses would increase the chance of a successful addition of a viable BA population.

The column experiments successfully captured BTX biodegradation activity under flow through conditions. However, several limitations hindered the ability to make definitive conclusions about the effectiveness of PAC in improving the biodegradation of BTX when coupled with bioaugmentation. Additional column experiments that address the following limitations may be able to assist in testing the hypothesis that the presence of PAC will increase the biodegradation of BTX in systems both with and without bioaugmentation under anaerobic conditions:

- Only one column was run with each treatment type so that the variation in system behavior could not be captured. Experiments with additional columns (duplicate) would amend this limitation and allow for quantification of variability.
- Benzene biodegradation could not be definitively assessed because the experimental duration was not long enough to allow for microbial populations with low counts to proliferate. A longer experimental duration is needed to make conclusions about the effectiveness of PAC and bioaugmentation on benzene biodegradation.

- Further claims about the effectiveness of PAC coupled with bioaugmentation can be made through biodegradation rate calculations as well as mass balances on the methane and TIC generation. Such an analysis would generate additional lines of evidence to support conclusions.
- The size of the PAC zone was only 6cm in length. Flow rates through the column were selected to mimic in situ conditions, but the size of the PAC zone was selected based on a timeframe these experiments needed to be completed by. If the size of the PAC zone was increased to what would be utilized in field conditions (meters in width), the results may have been more conclusive/representative.

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Appendices

Appendix A

Supplemental Materials for Chapter 3

Appendix A1: Measured Sulfate Concentrations in Microcosms

Toluene-only Microcosm Systems

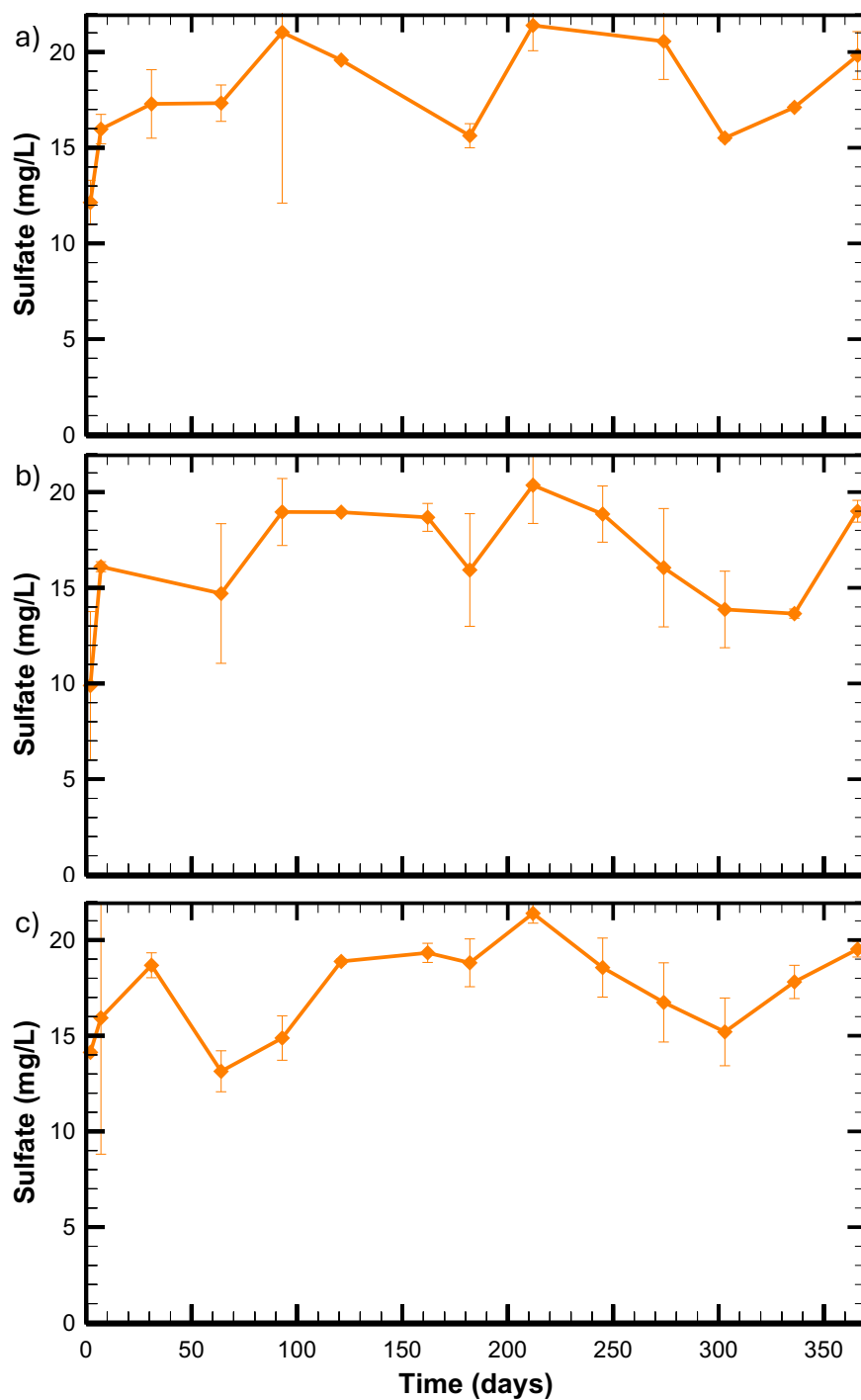


Figure A.1: Aqueous sulfate concentrations in the a) KC, b) KC-BA, and c) KC-PAC toluene-only microcosm systems. Error bars represent $\pm$ one standard deviation.

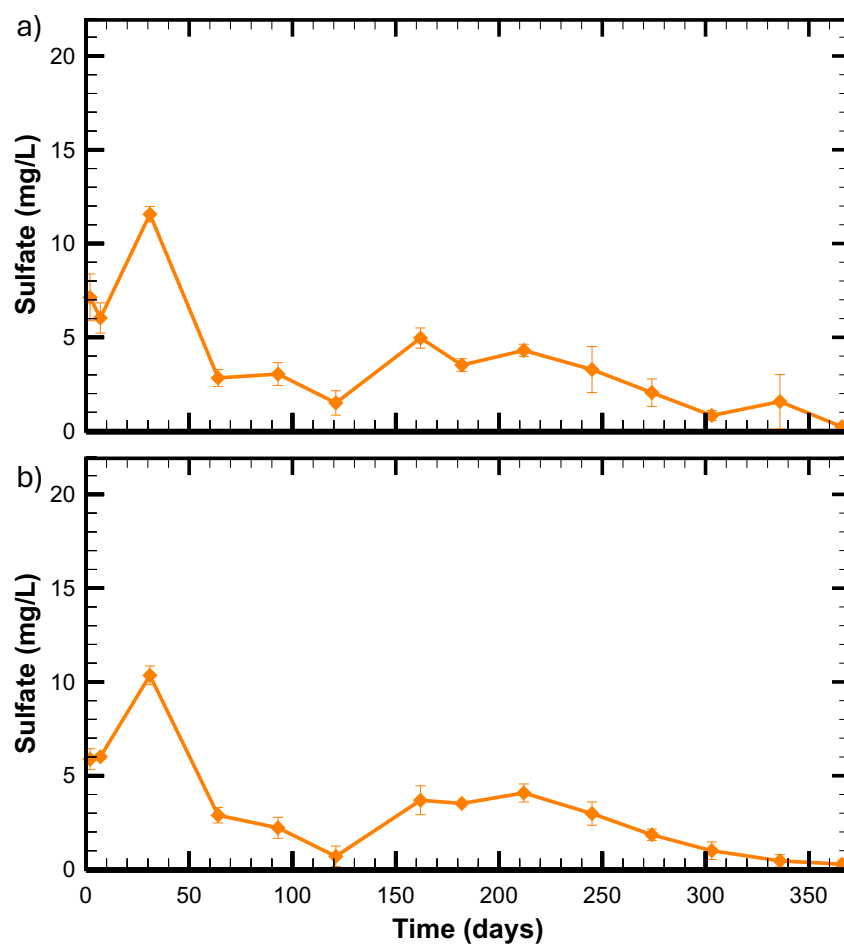


Figure A.2: Aqueous sulfate concentrations in the a) SC and b) SC-BA toluene-only microcosm systems. Error bars represent $\pm$ one standard deviation.

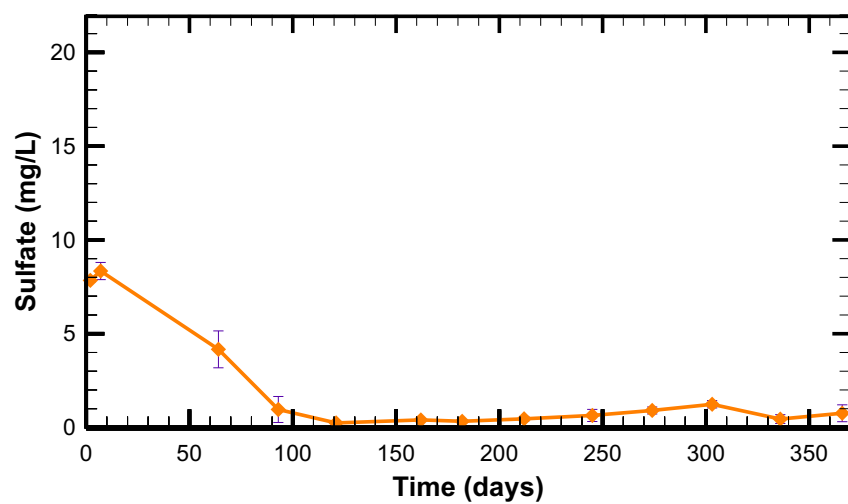


Figure A.3: Aqueous sulfate concentrations in the PC-BA toluene-only microcosm system. Error bars represent $\pm$ one standard deviation.

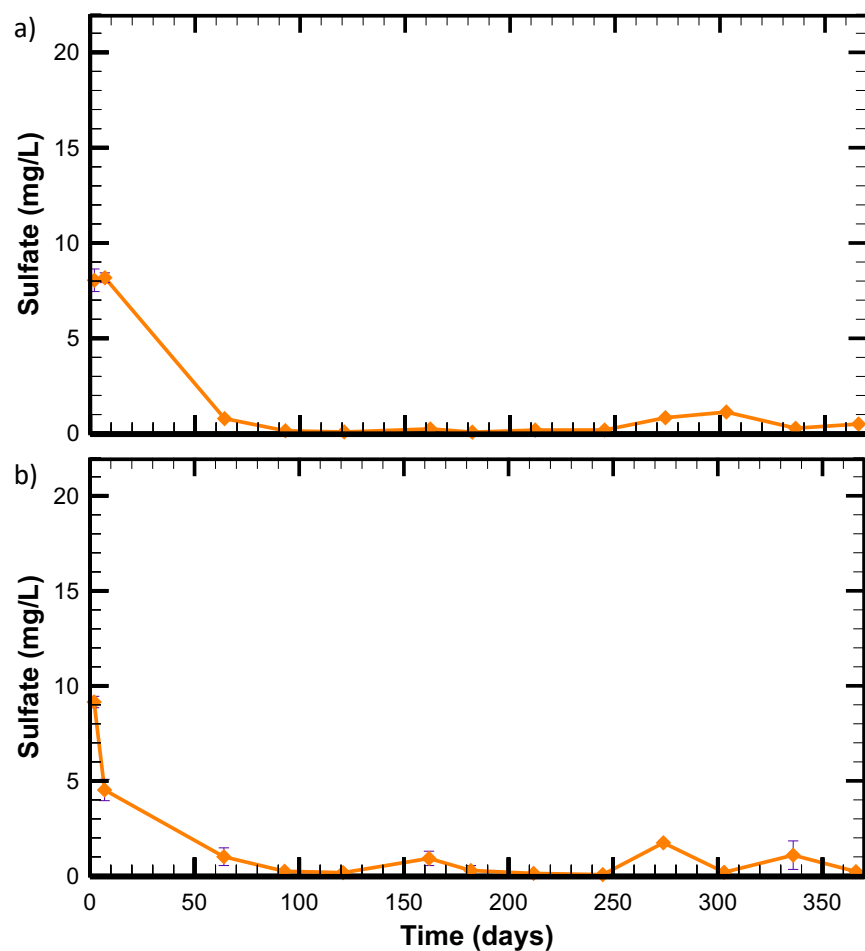


Figure A.4: Aqueous sulfate concentrations in the a) A and b) A-PAC toluene-only microcosm systems. Error bars represent $\pm$ one standard deviation.

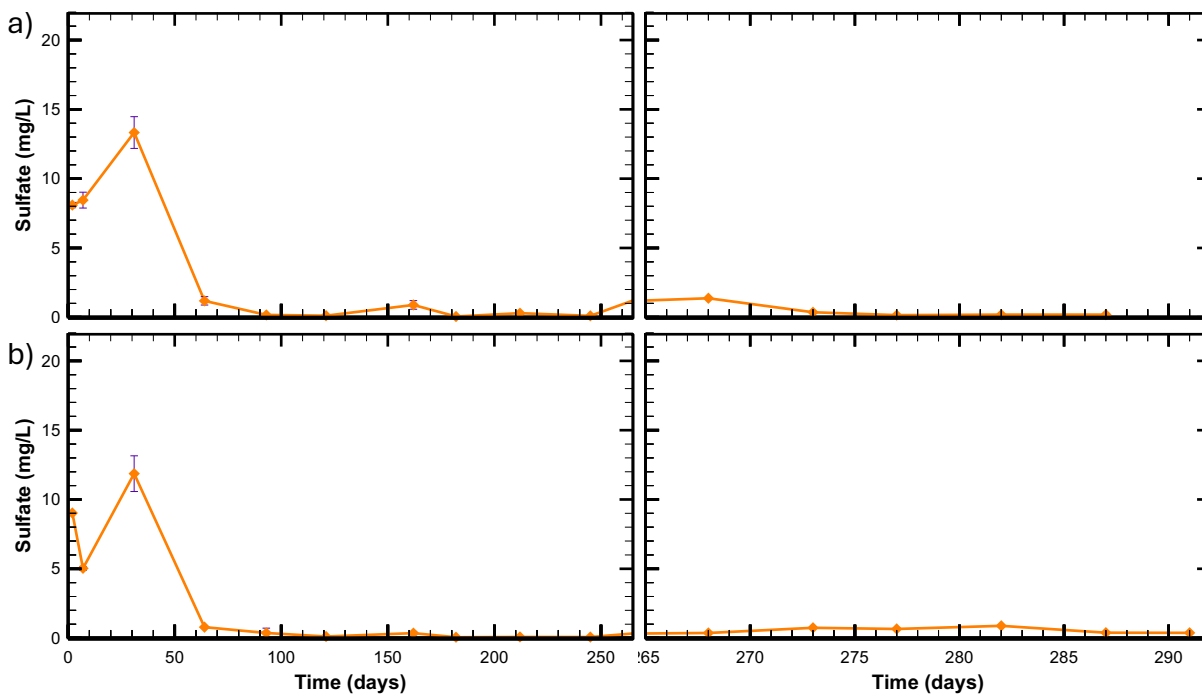


Figure A.5: Aqueous sulfate concentrations in the a) A-BA and b) A-PAC-BA toluene-only microcosm systems. Error bars represent $\pm$ one standard deviation.

BTX Microcosm Systems

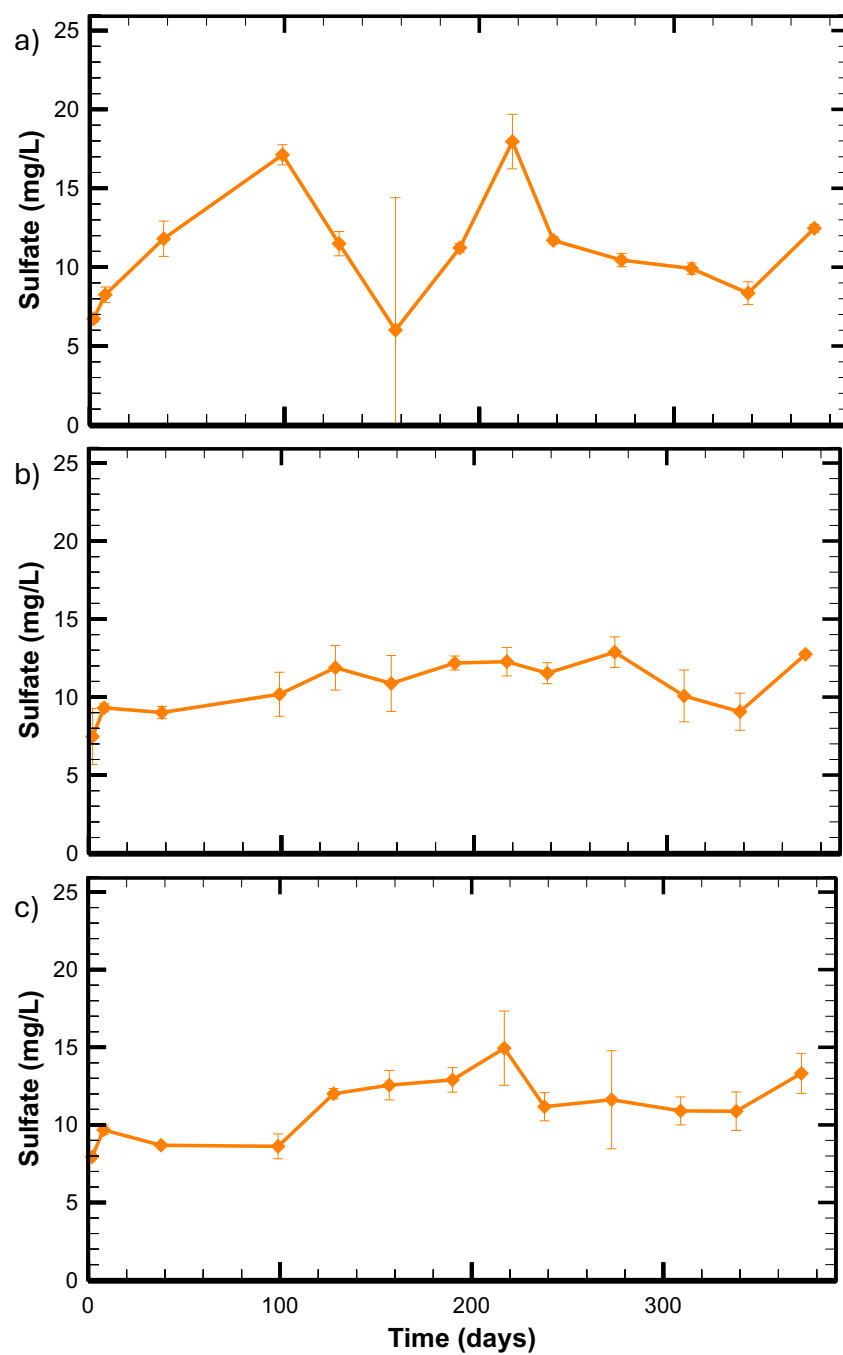


Figure A.6: Aqueous sulfate concentrations in the a) KC, b) KC-BA, and c) KC-PAC BTX microcosm systems. Error bars represent $\pm$ one standard deviation.

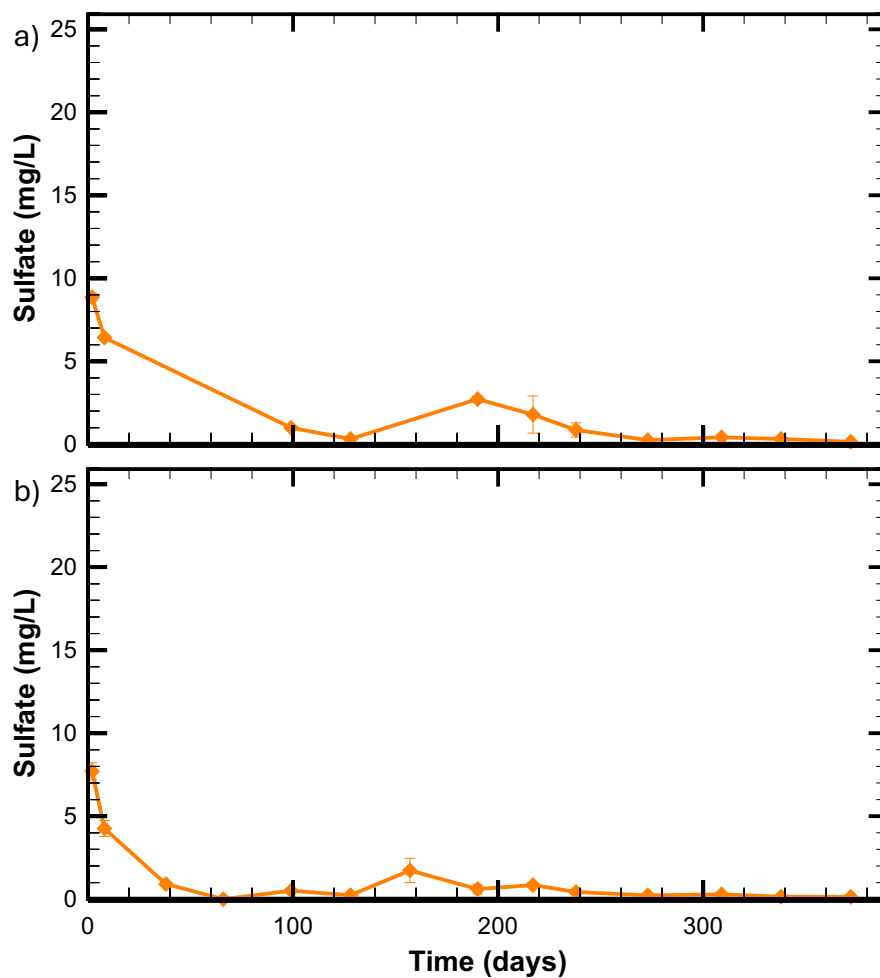


Figure A.7: Aqueous sulfate concentrations in the a) SC and b) SC-BA BTX microcosm systems. Error bars represent $\pm$ one standard deviation.

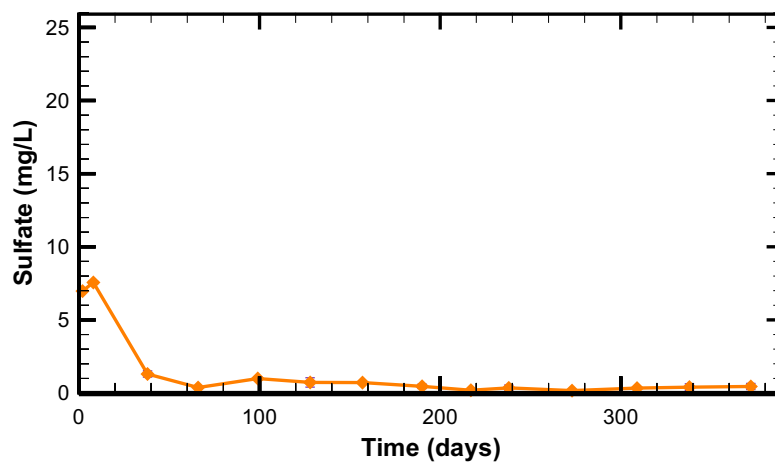


Figure A.8: Aqueous sulfate concentrations in the PC-BA BTX microcosm system. Error bars represent $\pm$ one standard deviation.

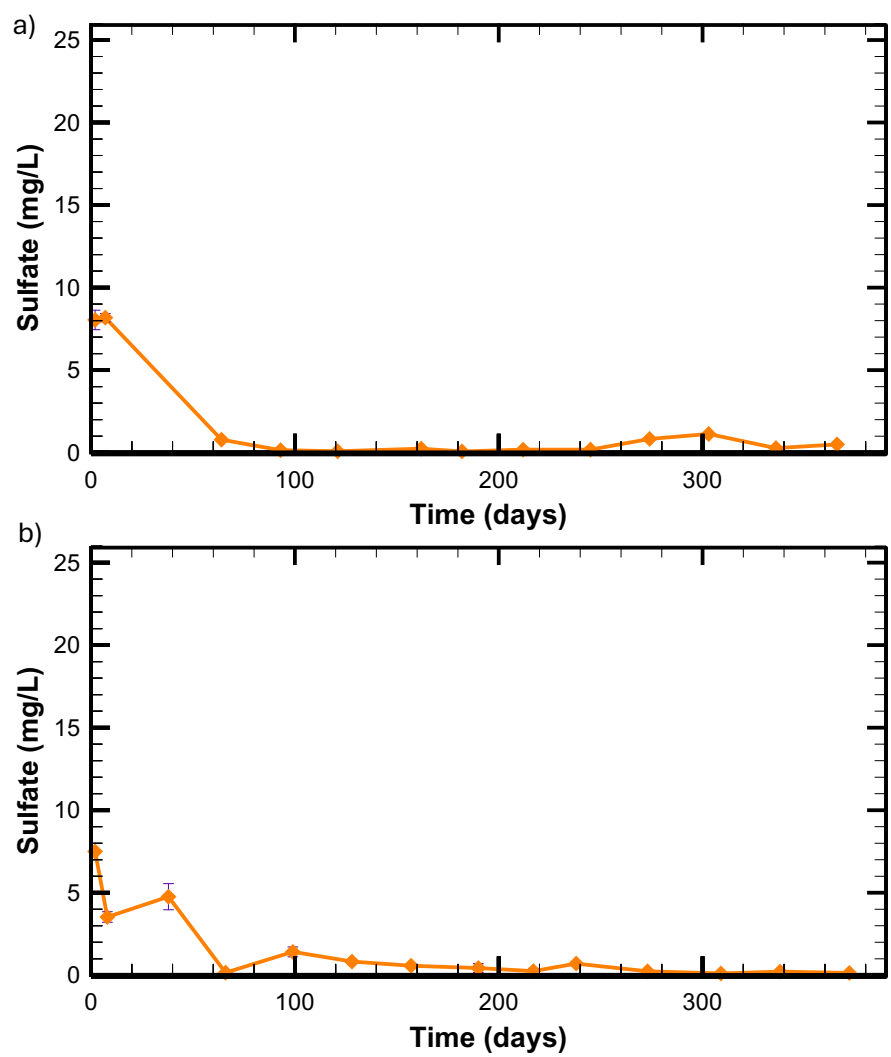


Figure A.9. Aqueous sulfate concentrations in the a) A and b) A-PAC BTX microcosm systems. Error bars represent $\pm$ one standard deviation.

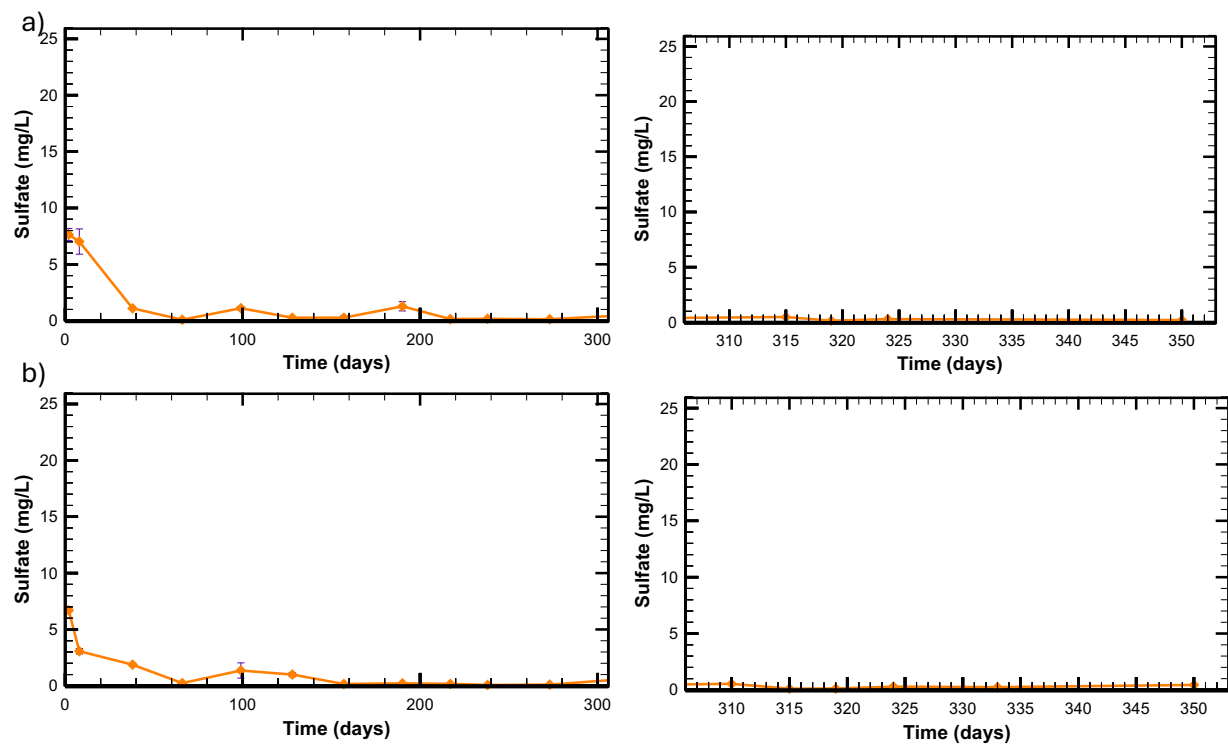


Figure A.10: Aqueous sulfate concentrations in the a) A-BA and b) A-PAC-BA BTX microcosm systems. Error bars represent $\pm$ one standard deviation.

Appendix A2: Statistical comparison of biodegradation rates

a) Method

Biodegradation rates were compared using a t-test and a 95% confidence interval. This method is used to test whether the slopes of two independent populations, β_1 and β_2 (i.e., the biodegradation rates), are equal. Both unpooled and pooled values of the error variances were used.

The null and alternative hypotheses are:

$H_0: \beta_1 = \beta_2$ i.e. $\beta_1 - \beta_2 = 0$ (the two slopes are the same)

$H_1: \beta_1 \neq \beta_2$ i.e. $\beta_1 - \beta_2 \neq 0$ (the two slopes are not the same)

The test statistic is:

$$t = \frac{\beta_1 - \beta_2}{\sqrt{s_{b1}^2 + s_{b2}^2}} \quad (\text{A.1})$$

where:

$$s_{b1}^2 = \frac{s_{Res1}^2}{\sqrt{n_1 - 1}} \quad s_{b2}^2 = \frac{s_{Res2}^2}{\sqrt{n_2 - 1}} \quad (\text{A.2; A.3})$$

and s_{Res1}^2 and s_{Res2}^2 are the error variances of the regression.

If the null hypothesis is true then:

$$\beta_1 - \beta_2 \sim N(0, s_{b1-b2})$$

Unpooled

In the unpooled case:

$$s_{b1-b2} = \sqrt{s_{b1}^2 + s_{b2}^2} \quad (\text{A.4})$$

Pooled

If the two error variances are equal, then they can be pooled, weighing each by their degree of freedom, and:

$$s_{b1-b2} = s_{Res} \sqrt{\frac{1}{s_{x1}^2(n_1-1)} + \frac{1}{s_{x2}^2(n_2-1)}} \quad (\text{A.5})$$

Table A.1: Excel equations used in the statistical analysis.

n	=COUNT(x)		
β	=SLOPE(y,x)		
s_{Res}	=STEYX(y,x)		
s_x	=STDEV(x)		
s_b	= $s_{Res}/(s_x * \text{SQRT}(n-1))$		

Pooled error variance method		Pooled error variance method	
		s_{Res}^2	$=((n_1-2)s_{Res1}^2 + (n_2-2)s_{Res2}^2)/(n_1+n_2-4)$
$s_{b_1-b_2}$	=SQRT($s_{b_1}^2 + s_{b_2}^2$)	$s_{b_1-b_2}$	$=s_{Res} * \text{SQRT}(1/s_{x1}^2(n_1-1) + 1/s_{x2}^2(n_2-1))$
t	$=(\beta_1-\beta_2)/(s_{b_1-b_2})$	t	$=(\beta_1-\beta_2)/(s_{b_1-b_2})$
df	$=n_1+n_2-4$	df	$=n_1+n_2-4$
α	0.05	α	0.05
p-value	=T.DIST.2T(t , df)	p-value	=T.DIST.2T(t , df)
t_{crit}	=T.INV.2T(α , df)	t_{crit}	=T.INV.2T(α , df)
Reject H_0 ?	=IF(p-value<a, YES, NO)	Reject H_0 ?	=IF(p-value<a, YES, NO)

b) Results**Table A.2:** Results of the t-test comparing the biodegradation rates of toluene between the A-BA and A-PAC-BA systems for the T-only microcosms.

	A-BA	A-PAC-BA
n	6	6
β	-0.26465	-0.30289
s_{Res}	0.465248	0.201997
s_x	2.738613	2.738613
s_b	0.075975	0.032986

Pooled error variance method		Pooled error variance method	
		s_{Res}^2	0.128629
$s_{b_1-b_2}$	0.082826	$s_{b_1-b_2}$	0.085234
t	0.461684	t	0.448644
df	8	df	8
α	0.05	α	0.05
p-value	0.656603	p-value	0.665592
t_{crit}	2.306004	t_{crit}	2.306004
Reject H_0 ?	NO	Reject H_0 ?	NO

Table A.3: Results of the t-test comparing the biodegradation rates of toluene between the A-BA and A-PAC-BA systems for the BTX microcosms.

	A-BA	A-PAC-BA
n	12	12
β	-0.21778296	-0.21168876
s_{Res}	0.536583469	0.404370752
s_x	5.376717485	5.376717485
s_b	0.030090107	0.022675986

Pooled error variance method		Pooled error variance method	
		s_{Res}^2	0.225718762
$s_{b_1-b_2}$	0.037677777	$s_{b_1-b_2}$	0.029786865
t	-0.16174535	t	-0.20459371
df	20	df	20
α	0.05	α	0.05
p-value	0.873129242	p-value	0.839957298
t_{crit}	2.085963447	t_{crit}	2.085963447
Reject H_0 ?	NO	Reject H_0 ?	NO

Table A.4: Results of the t-test comparing the biodegradation rates of *o*-xylene between the A-BA and A-PAC-BA systems for the BTX microcosms. Note: the first data point in the A-BA system was omitted because it was an outlier.

	A-BA	A-PAC-BA
n	12	12
β	-0.09007942	-0.098710298
s_{Res}	0.202897612	0.3013257
s_x	12.3251406	14.26374935
s_b	0.004963508	0.006369512

Pooled error variance method		Pooled error variance method	
		s_{Res}^2	0.065982309
$s_{b_1-b_2}$	0.008075091	$s_{b_1-b_2}$	0.006730673
t	1.068827399	t	1.282320125
df	20	df	20
α	0.05	α	0.05
p-value	0.297878659	p-value	0.214394917
t_{crit}	2.085963447	t_{crit}	2.085963447
Reject H_0 ?	NO	Reject H_0 ?	NO

Appendix B

Supplemental Materials for Chapter 4

Appendix B1: Ideal Breakthrough Time Estimation

Ideal breakthrough times were estimated for the columns that contained PAC zones following the method outlined by Wood (2002) and Lodewychx et al. (2004). The ideal breakthrough time for solute i (i.e., when 50% PAC saturation is reached) was calculated as:

$$t_{bi} = \frac{q_{ei} \times f_{PAC} \times \rho_{BS} \times V_{PAC}}{C_{0i} \times Q} \quad (B.1)$$

where:

q_{ei} = the equilibrium solid phase sorption capacity of the PAC for solute i (mg/g)

f_{PAC} = the fraction of PAC (-) = 0.005

ρ_{BS} = the bulk density of BS = 1.81 g/cm³ (Mackay et al., 1986)

V_{PAC} = the volume of the PAC zone = 66 cm³

C_{0i} = the initial aqueous phase concentration of solute i = 5 mg/L

Q = the column flow rate = 1.152 L/day

The q_{ei} was estimated using the single-solute Freundlich model parameters (K_{fi} and n_{fi}) or multi-solute improved simplified idea adsorption solution (ISIAS) competition factors calculated by Marrocco (2023).

Appendix B2: Bromide Tracer Test Details

The porosity, dispersity, and pore volume of the packed columns were estimated using a conservative bromide tracer test (McLaren, 2023). A continuous injection of a 754 mg Br/L sodium bromide (NaBr) solution was added to the columns, and the effluent bromide concentrations were monitored. An Ostrich Optimization model was fit to the time series of the measured effluent concentrations with the following model parameters: the length of the column (L), the Darcy Velocity ($q = Q/A$ where Q = flow rate (m^3/s), and A = cross-sectional area of the column (m^2)), and the initial concentration (C_o).

The nominal transport rate through the column was then extracted from the model as:

$$v = L / t^{\frac{1}{2}} \quad (\text{B.1})$$

where L is the length of the column (m), and $t^{\frac{1}{2}}$ is the modelled time it took for tracer concentration at leading edge of the plume to reach half the initial concentration of tracer solution (i.e., $C/C_o = 0.5$).

The column porosity, ϕ (-), was then calculated using:

$$\phi = q/v \quad (\text{B.2})$$

The column porosities were then used to calculate the pore volumes as:

$$PV = \phi \times L \times A \quad (\text{B.3})$$

where L and A are expressed in cm and cm^2 , respectively.

Appendix B3: Aqueous masses, solid phase masses, and mass reduction of BTX

Table B.1: Summary of aqueous cumulative influent and effluent masses, the estimated solid phase mass at the beginning of Year-2 (end of Year-1), and the sampled solid phases masses at the end of Year-2.

	A	A-PAC	A-BA	A-PAC-BA
Aqueous Mass (mg)				
Benzene IN	110.9	109.8	141.1	140.4
Benzene OUT	101.9	92.7	117.6	77.7
Toluene IN	95.7	99.6	133.6	133.9
Toluene OUT	30.0	1.9	0.9	0.5
<i>O</i> -xylene IN	134.3	97.2	130.3	125.5
<i>O</i> -xylene OUT	117.2	15.7	12.8	1.3
Solid Mass (mg)¹				
Benzene Year-2 Start	--	3.00	--	3.97
Benzene Year-2 End	--	2.85	--	29.00
Toluene Year-2 Start	--	9.75	--	11.06
Toluene Year-2 End	--	1.67	--	0.18
<i>O</i> -xylene Year-2 Start	--	59.21	--	61.27
<i>O</i> -xylene Year-2 End	--	100.15	--	36.30

¹Solid mass not estimated through ISIAS theory for columns without PAC present.

Table B.2: Year-2 cumulative sulfate utilization.

Column	Sulfate IN (mg)	Sulfate OUT (mg)
KC-PAC	157.3	152.9
A	230.6	1.6
A-PAC	211.9	2.1
A-BA	233.4	1.5
A-PAC-BA	217.6	1.5

* all values have inherent 10% estimated error associated with them.

Appendix B4: Measured Sulfate Concentrations in Columns

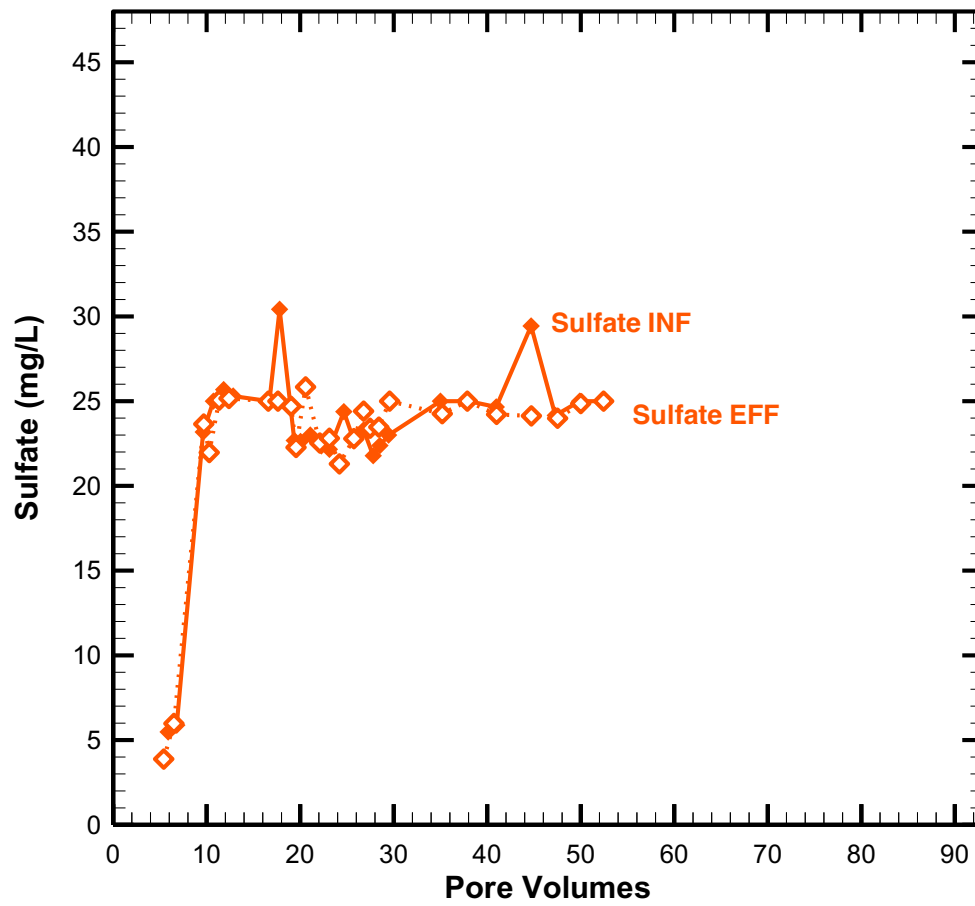


Figure B.1: Influent (INF) and effluent (EFF) aqueous sulfate concentrations in the KC-PAC column system.

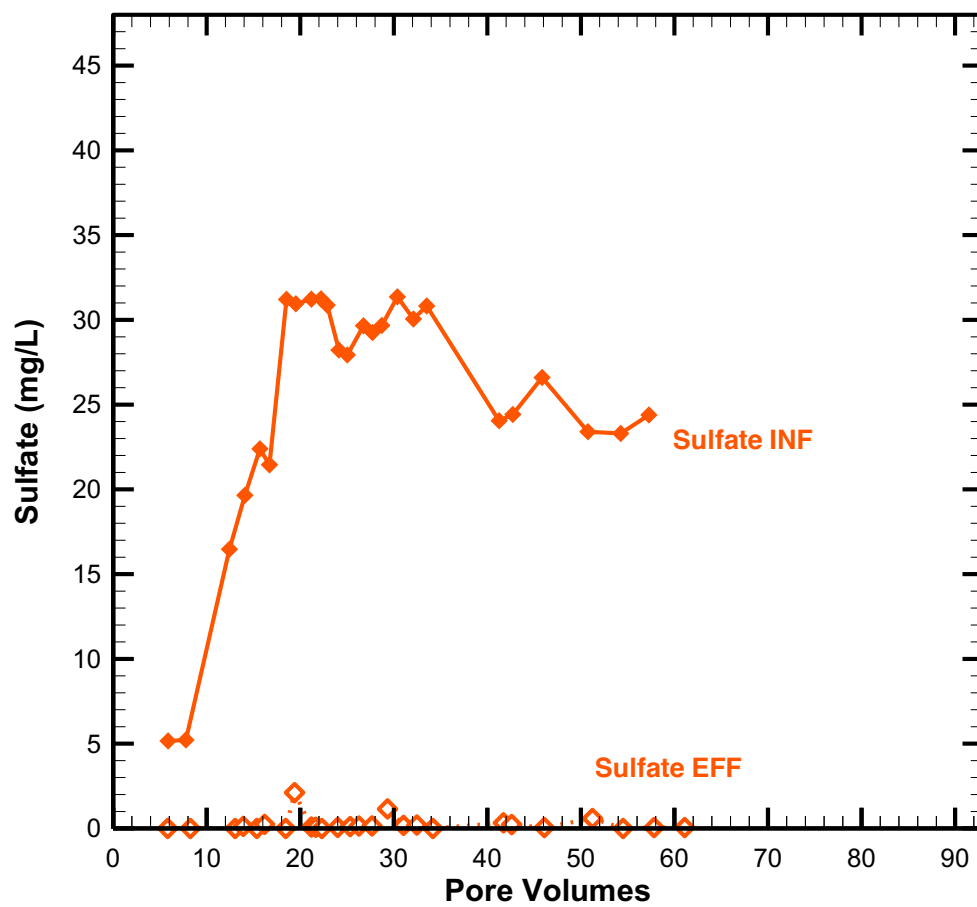


Figure B.2: Influent (INF) and effluent (EFF) aqueous sulfate concentrations in the A column system.

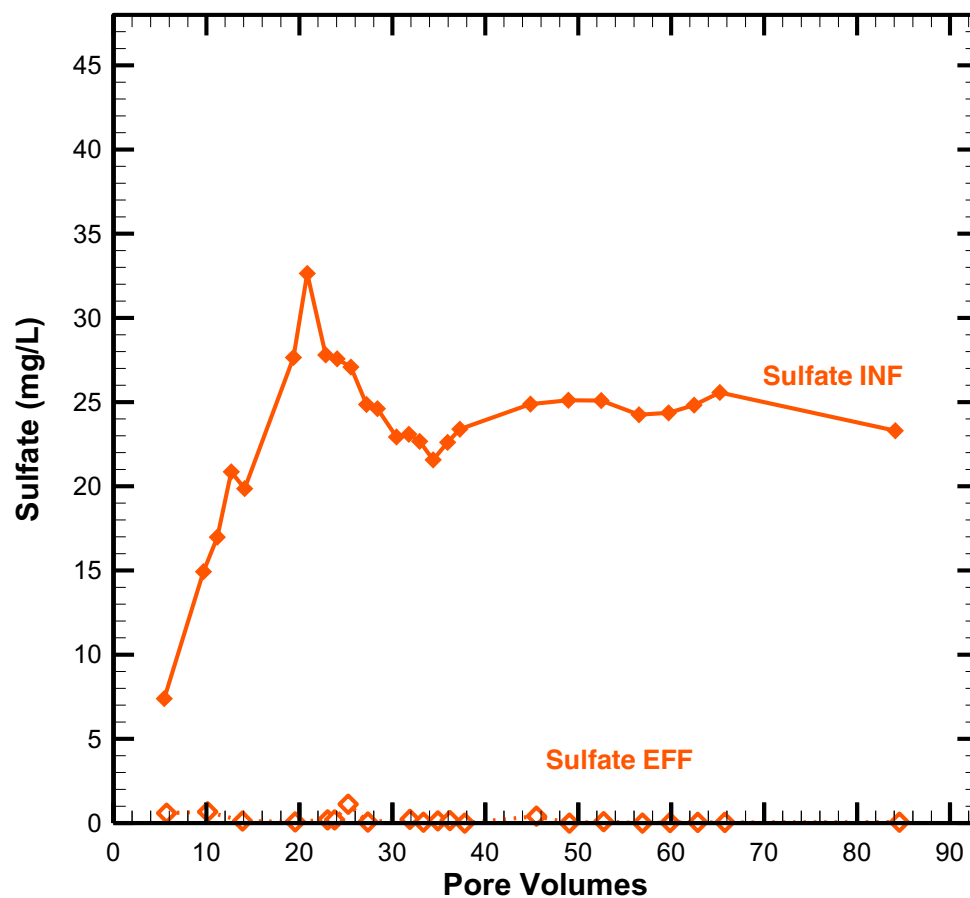


Figure B.3: Influent (INF) and effluent (EFF) aqueous sulfate concentrations in the A-BA column system.

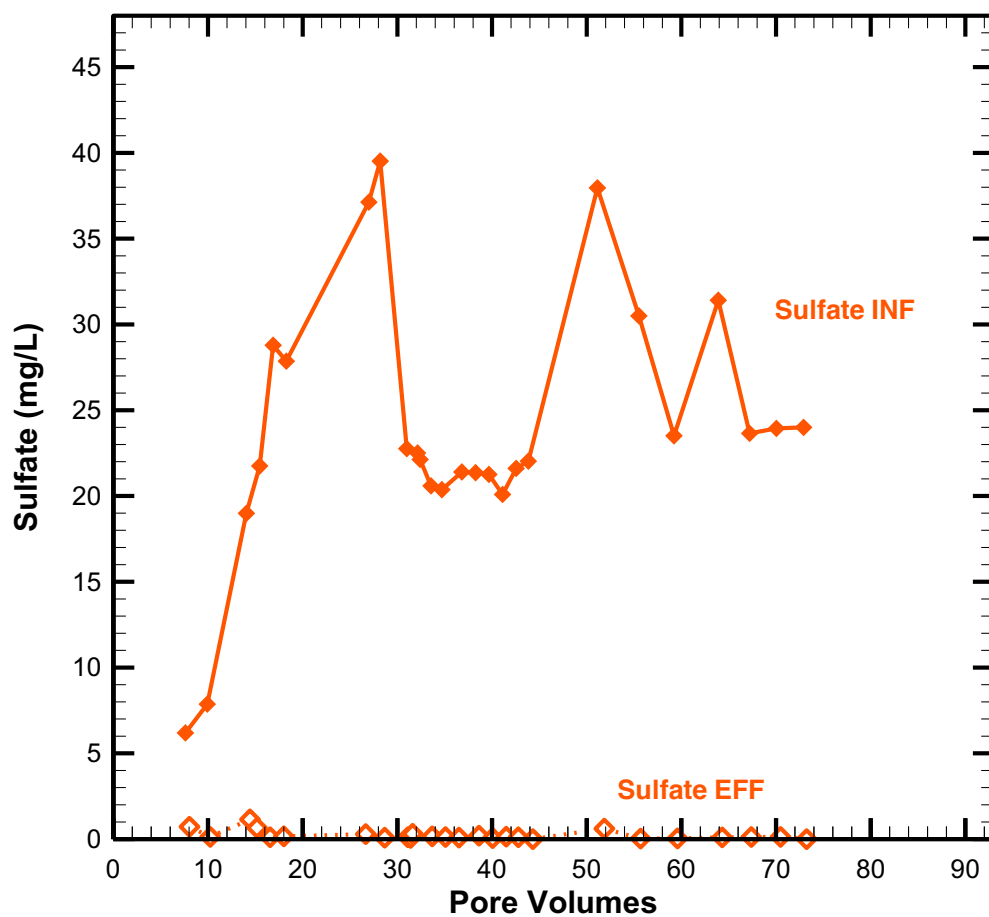


Figure B.4: Influent (INF) and effluent (EFF) aqueous sulfate concentrations in the A-PAC column system.

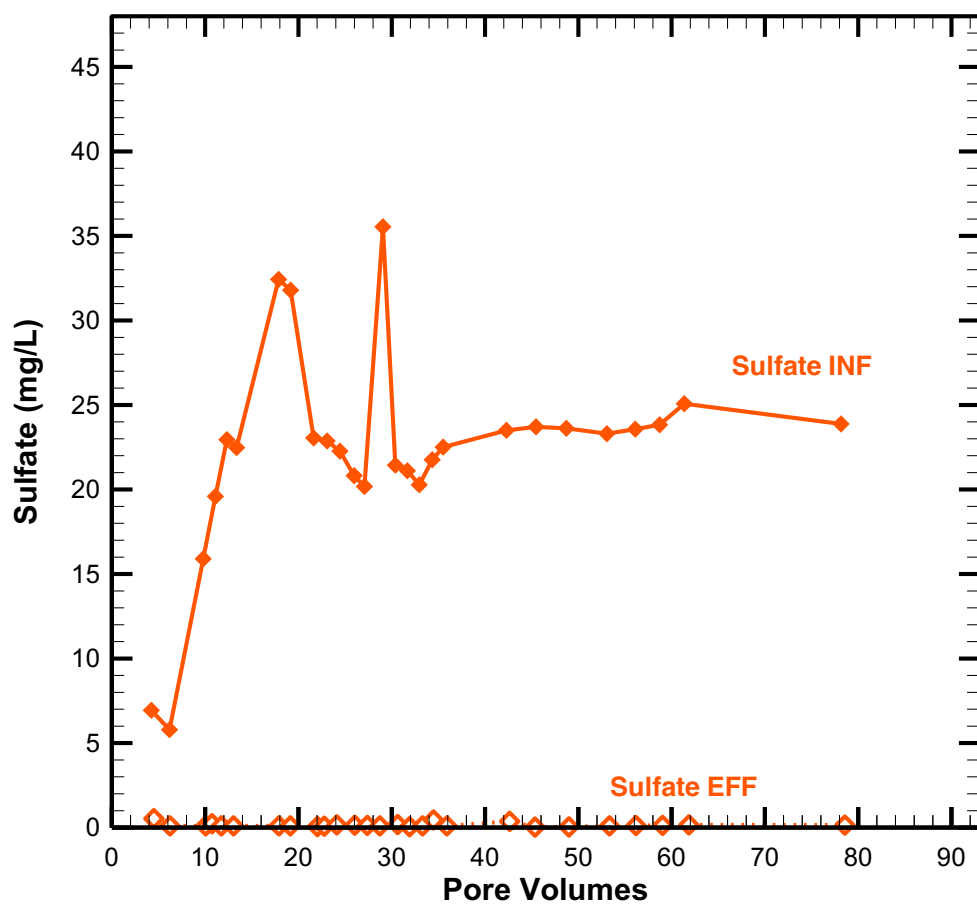


Figure B.5: Influent (INF) and effluent (EFF) aqueous sulfate concentrations in the A-PAC-BA column system.

Appendix B5: Solid phase concentrations in the A and A-BA columns

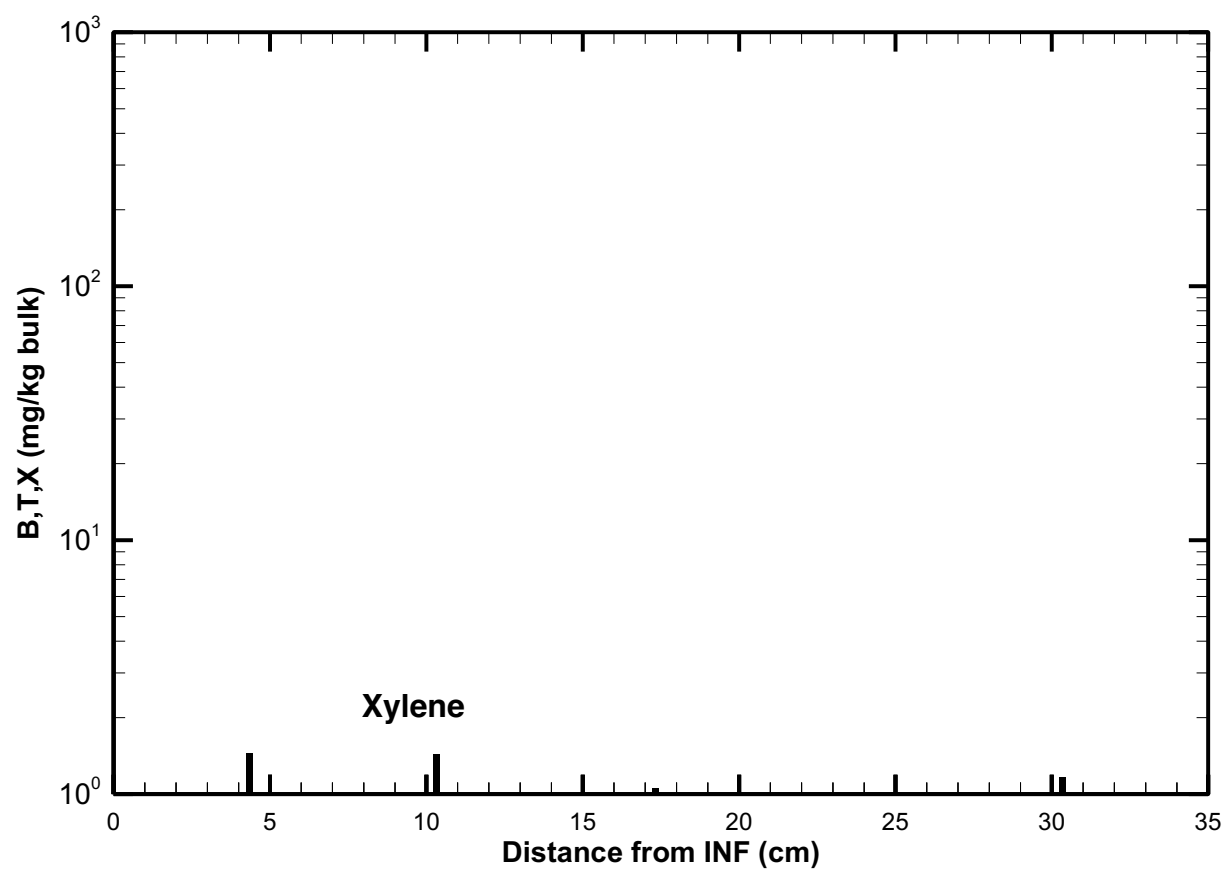


Figure B.6: Solid phase BTX concentrations in the A column system.

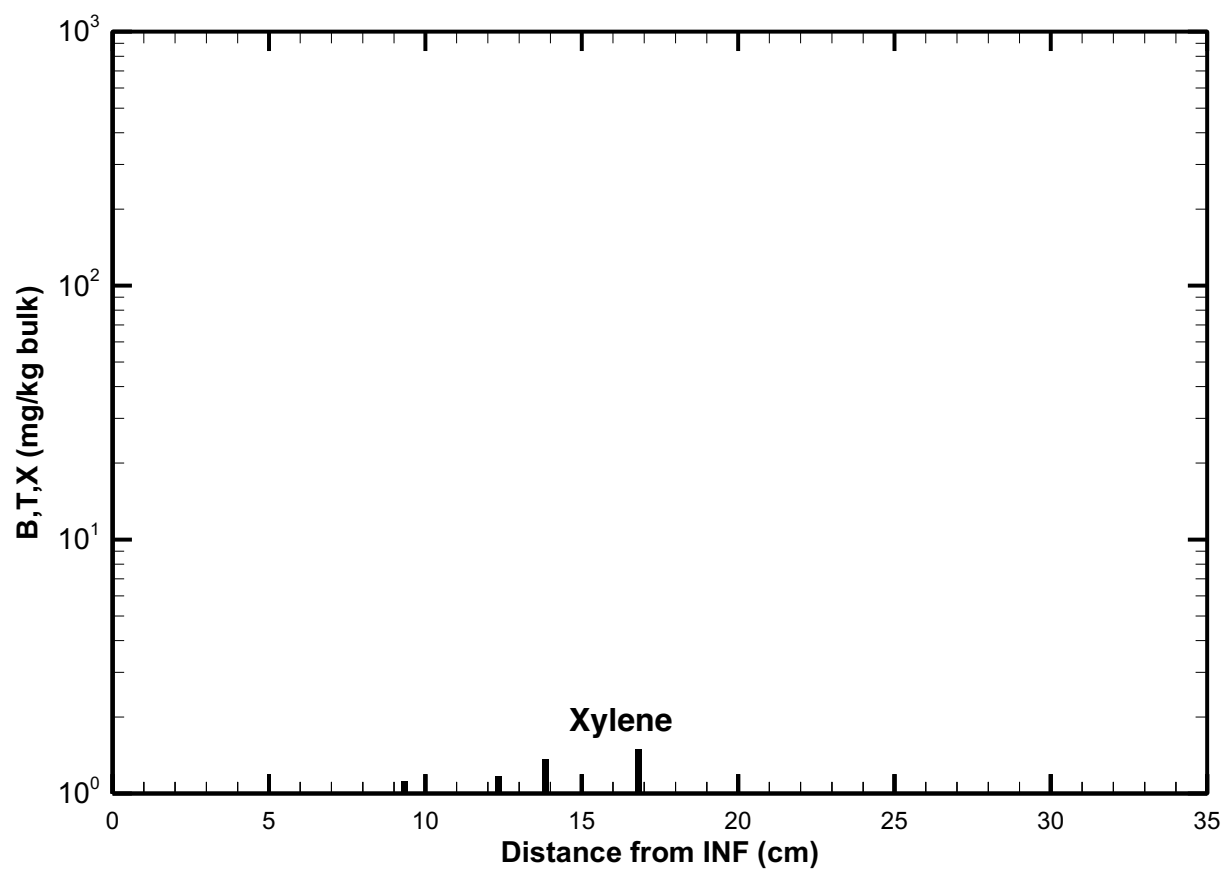


Figure B.7: Solid phase BTX concentrations in the A-BA column system.