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FINAL REPORT

Application of Biofilm Covered Activated Carbon Particles as a Microbial Inoculum Delivery System in Weather PCB Contaminated Soils

Phase II

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14. ABSTRACT

Recent work with polychlorinated biphenyls (PCBs) contaminated sediments has used activated carbon (AC) to control in situ bioavailability of PCBs, with large reduction in the bioaccumulation of PCBs by aquatic organisms in field studies with sediment treated using 1-5% by weight granular activated carbon (GAC) (Ghosh et al., 2011). Sediment treated with GAC attained aqueous equilibrium PCB concentrations that were 85% and 92% lower than untreated sediment in one-month and six-month contact experiments, respectively (Zimmerman et al., 2004). It has been demonstrated that AC effectively outcompetes solid phases and benthic organisms for PCBs thus reducing PCB exposure to aquatic organisms.

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LIST OF ACRONYMS

AC	Activated carbon
BC	Bone biochar
DCE	Dichloroethylene
DNA	Deoxyribonucleic acid
DOD	Department of Defense
ECD	Electron capture detector
Eq.	Equation
GC	Gas chromatography
PCBs	Polychlorinated biphenyls
DHPLC	Denaturing high performance liquid chromatography
EPS	Extracellular Polymeric Substances
GAC	Granular Activated Carbon
MS	Mass spectrometry
PCE	Tetrachloroethylene
PE	Polyethylene
PNA-FISH	Peptide nucleic acid Fluorescence in situ hybridization
POM	Polyoxymethylene
SEM	Scanning electron microscopy
SERDP	Strategic Environmental Research and Development Program
TCE	Trichloroethylene
TCMX	tetrachloro-m-xylene
CLSM	Confocal Laser Scanning microscopy
DAPI	4',6-diamidino-2-phenylindole
Q-PCR	Quantitative polymerase chain reaction
VC	Vinyl chloride

EXECUTIVE SUMMARY

Objectives

Recent work with polychlorinated biphenyls (PCBs) contaminated sediments has used activated carbon (AC) to control in situ bioavailability of PCBs, with large reduction in the bioaccumulation of PCBs by aquatic organisms in field studies with sediment treated using 1-5% by weight granular activated carbon (GAC) (Ghosh et al., 2011). Sediment treated with GAC attained aqueous equilibrium PCB concentrations that were 85% and 92% lower than untreated sediment in one-month and six-month contact experiments, respectively (Zimmerman et al., 2004). It has been demonstrated that AC effectively outcompetes solid phases and benthic organisms for PCBs thus reducing PCB exposure to aquatic organisms.

Phase I of the SERDP project ER-2135 aimed at assessing how natural PCB dechlorination activity in sediments was affected by the addition of AC. Results showed that indigenous PCB dechlorinating bacteria were capable of PCB dechlorination even in the presence of a strong sorbent like AC. Although the results showed that there was a slight decrease in the overall concentration of parent congeners dechlorinated within the time frame studied, there was a significant increase in the extent of dechlorination in the presence of AC. The products of dechlorination were in the absence of AC tri-, tetra- and penta chlorinated congeners, whereas in the presence of AC the dechlorination products were mono- and di- chlorinated congeners. These dechlorination products generated in the presence of AC will likely make it possible for indigenous aerobic bacteria to complete the mineralization process in the presence of oxygen.

Bioaugmentation of PCBs in sediment and soils has been shown in several studies however, the dechlorination rates and the extent of PCB dechlorination obtained with bioaugmentation in weathered PCB contaminated sediments are often low (Krumins et al., 2009). Therefore, to further enhance the degradation of weathered and low concentration PCBs in sediment, a combination of adsorbent sequestration and bioaugmentation in the form of biofilm covered sorptive particles is proposed. This approach benefits from bacteria being able to grow to a high density at the surface of sequestering sorbent. In addition, PCB dechlorinating bacteria in biofilms are surrounded by a hydrophobic layer enabling the bacterial cells to align in close proximity to adsorbent particles and thereby interact with hydrophobic PCBs that are adsorbed to particles (May et al. 2008). This biofilm community of microbes has greater cell density and activity than would be possible to obtain in free floating systems, where direct interaction between PCBs and the bacteria would occur less frequently. This interaction is required for optimal electron transfer and subsequent PCB dechlorination.

This biofilm based approach is a significant improvement compared to previous bioaugmentation studies, where liquid cultures with free floating bacteria were injected or in other ways supplied to the sediment with reduced efficiency. An additional positive effect is

that the dechlorinating bacteria will be fixed to the sorptive particles, so they will not be washed away or consumed by other microorganisms in the environment thereby increasing the potential for successful long term bioaugmentation. This concept was tested in the Phase I of project ER-2135, where biofilm based inoculum on AC particles was applied as a delivery system in Aroclor 1248 contaminated sediment with high total PCB concentrations. The results showed that bioaugmentation with the biofilm inoculum enhanced the PCB dechlorination compared to liquid inoculum and that the biofilm bacteria survived in the sediment environment and remained active throughout the 200-day experiment.

The intent of the work is to expand on the results with high PCB concentrations used in Phase I of project ER- 2135 by testing the application of dechlorinating bacterial biofilms on sorptive materials for treating sediments with lower total levels of weathered PCBs typically found in the environment. The goal for this research project is to obtain full degradation of PCBs in weathered sediment by focusing on the first and rate- limiting step of the initial anaerobic dechlorination and subsequently enable aerobic degradation by applying biofilm inoculum associated with AC as a delivery system.

Many Department of Defense facilities face challenges from contaminated sediments, and existing remediation options are slow and expensive. This project assisted in the development of a novel approach to addressing PCB-contaminated sediments and management tools that will be applied in situ, resulting in more efficient and cost-effective reduction of risk at these sites.

Technical Approach

Bioaugmentation performed with PCB degrading biofilm inoculum will be applied for both anaerobic and aerobic biodegradation processes. It is expected that in the proposed study, application of biofilm inoculum will increase reductive dechlorination of contaminated sediment containing low concentrations of weathered PCBs compared to bioaugmentation using liquid inoculum. Furthermore, mineralization and removal based on combined aerobic/anaerobic biofilm inoculum is expected. The experiments will be performed in mesocosms containing contaminated sediment from a DOD site of interest.

The use of inoculum originating from other locations than the one being bioaugmented has been shown in previous studies (Kjellerup et al. 2008; Payne, et al., 2013). Here, bacterium DF1 (originating from Charleston Harbor, SC) not only survived in other environments than its native environment such as contaminated soil, but was also capable of reductively dechlorinating weathered PCBs at this location (Kjellerup et al. 2008; Payne, et al., 2013). This has also been shown in recent bioaugmentation studies, where liquid DF1 inoculum has been applied simultaneously with AC, but not as a biofilm (Payne, et al., 2011, Payne, et al., 2013).

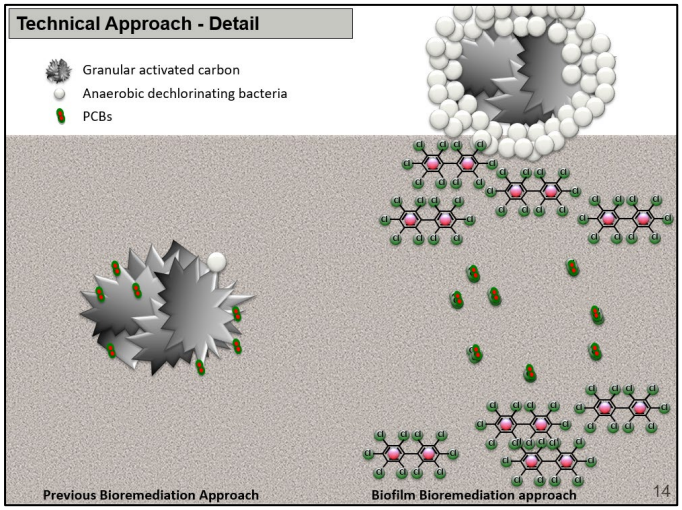
The effects of bioaugmentation including the formation of dechlorinating biofilms on AC particles and the role of these microbes in the degradation of PCBs were studied in this work. Active PCB dechlorinating cultures were cultured by the research team. The biologically based "tool box" was expanded during Phase II of project ER-2135 and these methods aid in the evaluation of dechlorinating biofilm samples: 1) PCR using selective primers to rapidly characterize the 16S rDNA sequences of bacteria associated with specific dechlorination patterns. Different PCB dechlorinating strains exhibit congener specificity depending on the relative positions of chlorines on the biphenyl ring; 2) Q-PCR assay quantifying PCB dechlorinating species in sediments in a rapid and more sensitive way.; 3) DHPLC (Denaturing High Pressure Liquid Chromatography) based assay for rapid monitoring of the diversity of PCB dechlorinating species. The separation of the individual bacterial species is performed with a chromatographic column according to their genetic sequence; 4) Microscopic techniques such as Fluorescence In Situ Hybridization, SybrGreen and DAPI staining for identification and visualization of bacterial populations. The bacteria are labeled with a specific probe targeting dechlorinating bacteria and they can subsequently be visualized in the fluorescence microscope allowing both qualitative and quantitative analyses; 5) Confocal Laser Scanning Microscopy of in situ biofilm samples using the SybrGreen stain was applied to evaluate the formation and structure of the biofilm on different AC and bone biochar (BC) surfaces; 6) Scanning Electron Microscopy (SEM) for high resolution images of the bacterial biofilms as well as elemental analysis of the surface and associated biofilm. Using this comprehensive approach, assessment of the changes that occur in the PCB-dechlorinating biofilm populations covering the sorptive particles can be rapidly assessed; and; 7) Metagenomic analysis of potential organohalide respiring bacteria in bioaugmented environmental samples also assisted in the evaluation of community shifts over time.

Results and Discussion

Seven separate studies were conducted to address the project objectives:

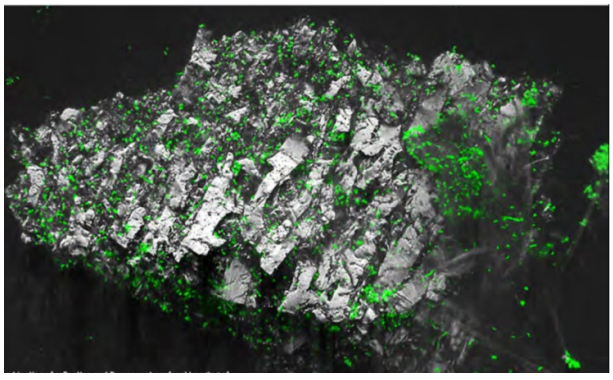
Chapter 1. History of the project and timeline.

In this chapter, we describe the project timeline including the initial SEED project that transitioned into the SERDP project ER-2135, which we are reporting on now.



The image shows the working hypothesis for the project that the dual approach of a) adsorption of PCBs from the sediment porewater onto activated carbon and b) biofilm formation on the activated carbon that will provide a robust delivery vehicle for bioaugmentation into PCB contaminated weathered sediment.

Chapter 2. Community analysis of biofilm formation on activated carbon particles.
In this chapter, we show that aerobic and anaerobic biofilms could successfully be grown on GAC surfaces (F400). Augmented mesocosms of Grasse River sediment with biofilm covered GAC showed increased Aroclor 1248 degradation. This is illustrated in the image below.

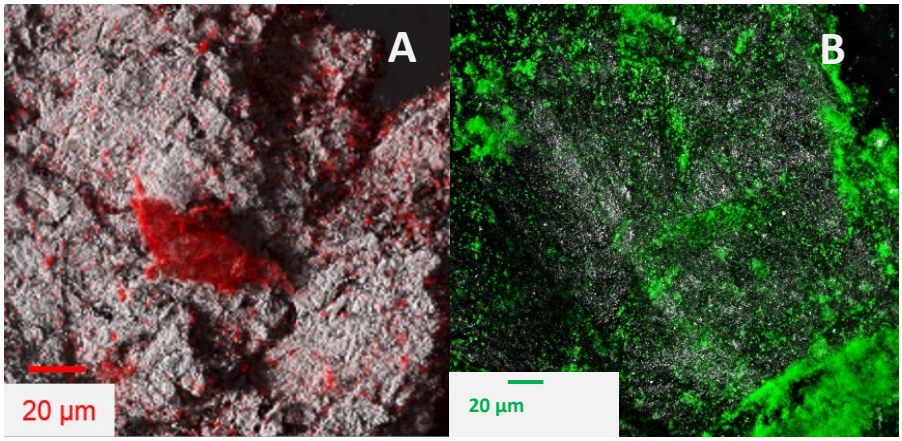


The image shows DF-1 biofilm formed on the surface of GAC particle. The bacteria were labeled with the DNA specific stain SybrGreen that only targets DNA (i.e. bacterial cell material) and not the background such as GAC and/or media components.

Chapter 3. Characterization of *Paraburkholderia xenovorans* LB400 biofilm development on various sorptive materials for potential aerobic biodegradation of organohalide contaminants.

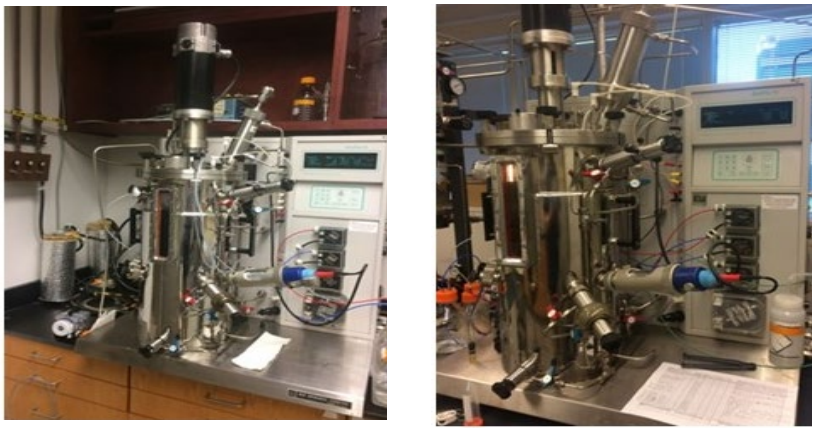
In this chapter, we show that biofilms made up by *Paraburkholderia xenovorans* strain LB400 developed on each of the tested sorptive materials including but not limited to

Coconut AC, Calgon BL (PAC), Calgon TOG LF, acai pit biochar, bone biochar, hardwood activated biochar, peanut hull biochar, sand, XAD and graphite foil and powder. The importance of these parameters were assessed: surface area, electrical conductivity, sorption capacity, porosity and pore volume. The biofilms did not display a preference for growth on any particular sorptive carrier material. Advanced microscopy tools were developed and evaluated the biofilm formation using various fluorescent dyes. An example of the combined microscopic approaches is shown below.



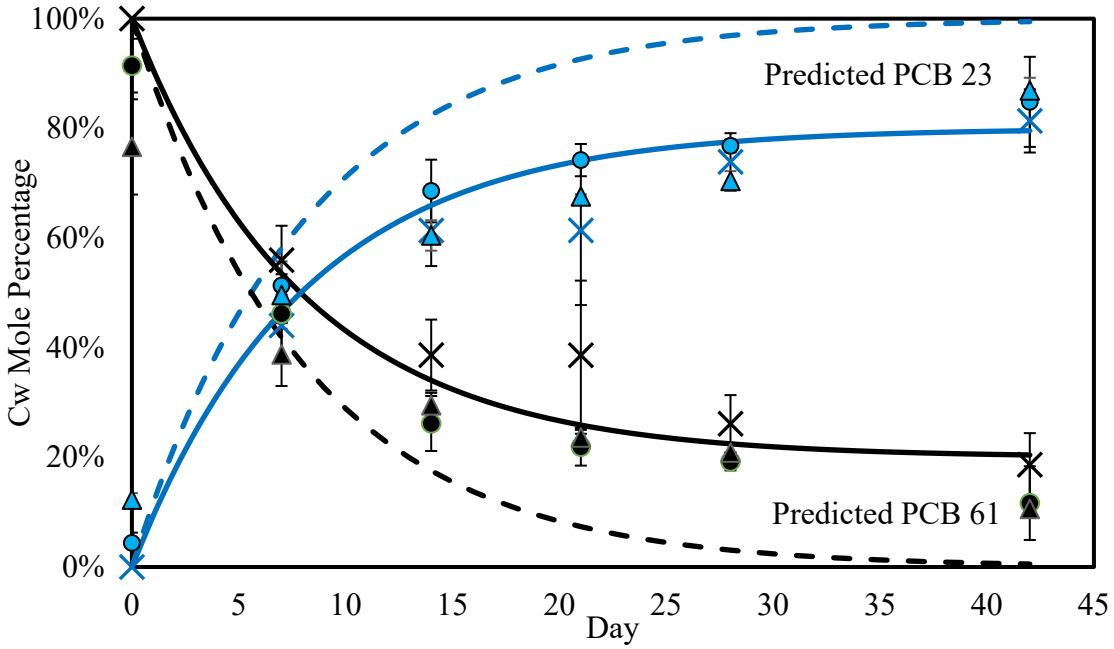
Biofilm of LB400 formed on coal GAC and stained with wheat germ agglutinin (WGA) [CLSM: Fluorescence/reflection].

Chapter 4. *Dehalobium chlorocoercia* DF1 maintenance, scale up and optimization.
In this chapter, we show that the anaerobic dechlorinating bacterium *Dehalobium chlorocoercia* DF1 (DF1) could be scaled up, grown and maintained using tandem 20 L bioreactors to numbers that were applicable and effective for full scale implementation. The reactors that were used for this are shown in the figures below.



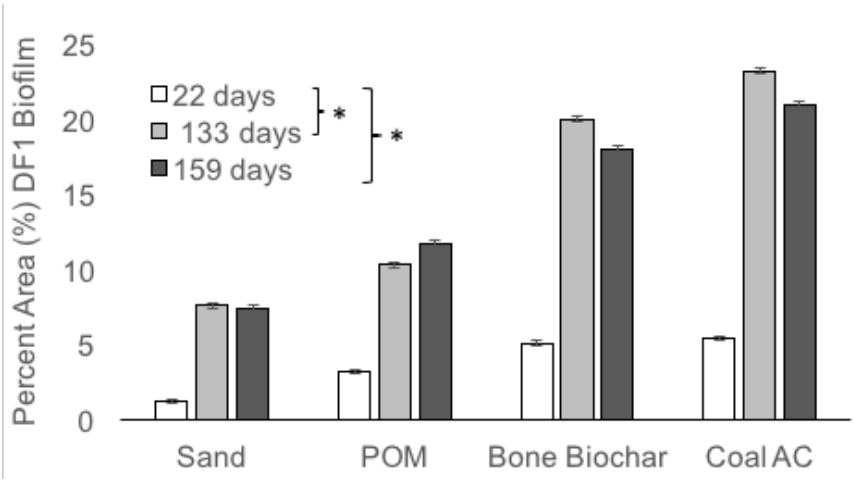
Photos of the tandem 20L bioreactors used to anaerobically grow and maintain DF1 cultures.

Chapter 5. Kinetics of PCB microbial dechlorination explained by freely dissolved concentration in sediment microcosms.
In this chapter, we show that the rate of the single congener PCB 61 (2,3,4,5-CB) was found to be linearly dependent on the freely dissolved concentration in both sediment and in sediment-free microcosms. PCB microbial dechlorination kinetics can be predicted in sediments based on these findings. This is illustrated in the figure below, where PCB 61 and the break down product PCB 23 are shown as the concentrated and predicted concentrations.



Accumulation of PCB 23 (blue) and depletion of PCB 61 (black) at three different aqueous concentrations in spiked sediment: 0.005 nM, (x), 0.015 nM (●), 0.134 nM (▲). Dashed lines are predictions based on the first order biological rate constant measured in a sediment-free system. Solid lines are predictions that include a slow desorption fraction of 20%.

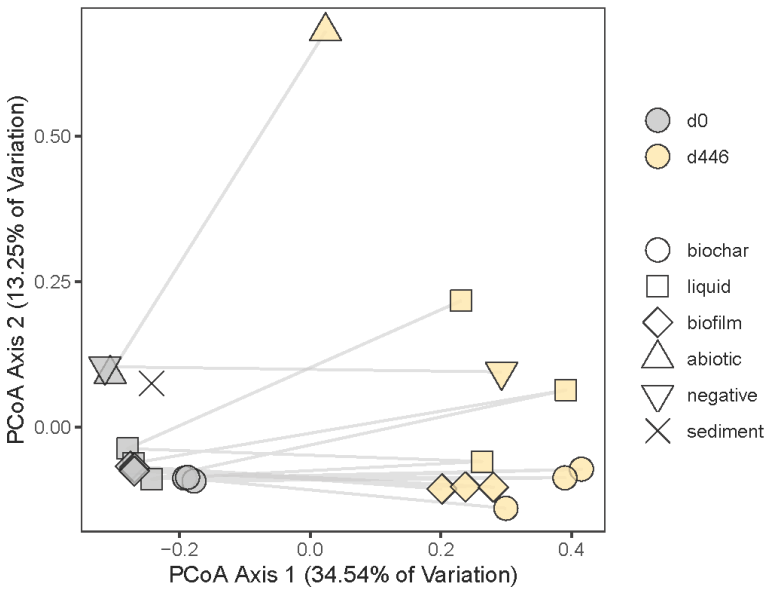
Chapter 6. Colonization and growth of *Dehalobium chlorocoercia* DF-1 anaerobic dehalorespiring biofilms on carbonaceous sorptive amendments.
In this chapter, we show that dehalorespiring biofilms formed on all carrier materials tested with a preference for sorptive carbonaceous materials, which subsequently was shown to be the most important mechanism for formation of biofilm. Biofilm-based inoculum can be an efficient approach for bioaugmentation of PCBs in sediment. The figure below shows that coal based activated carbon (here: F400) has the highest surface area coverages of the tested materials.



Biofilm area coverage of DF1 biofilm on evaluated materials. Error bars show standard deviation (n = 10 images). The asterisk indicates a statistically significant difference between time-points.

Chapter 7. Application of biofilm-based inoculum delivery system for organohalide respiration of native PCBs in sediments.

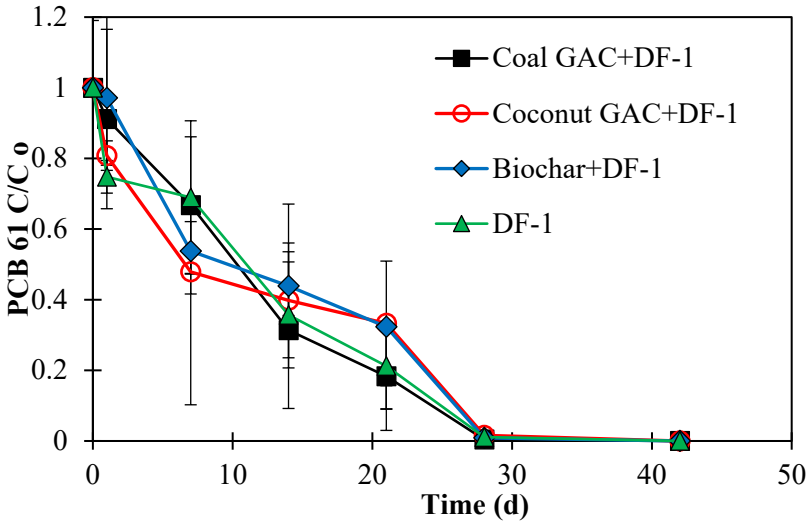
In this chapter, we showed that dechlorination of weathered PCBs in Grasse River sediment occurred in the presence of biochar, liquid bacterial inoculum and biofilm formed on biochar. All treatments improve the dechlorination efficiency, but the dechlorination rate was highest for the biofilm inoculum. Contaminated sediment that was bioaugmented with biofilms formed on biochar showed enhanced microbial community stability thus making this approach advantages for field applications of bioaugmentation. The stability of the microbial community indicates that dechlorinating bacteria remain active in the sediment over time thus re-augmentation might not be required. The stability of the biofilm communities is shown in the figure below, where the community compositions after 446 days remain similar (yellow diamonds) for the sediment inoculated with biofilm.



Principal coordinates analysis of microbial communities from different groups (Lines connect the same mesocosms at different time points) showing that the biofilm communities (yellow diamonds) cluster closely together thus remain similar and stabile.

Chapter 8. Influence of Black Carbon Material on Organohalide respiration by *Dehalobium chlorocoercia.*

In this chapter, we show that enhanced kinetic effects on organohalide respiration were not observed in the presence of black carbon materials when the black carbon was first equilibrated with sediments followed by the addition of DF1. The effects may be different when the dechlorinating bacteria are applied as a biofilm coating on black carbon and is able to be exposed to sediment-bound PCBs before PCBs in the liquid. In the figure below, we show that PCB 61 dechlorination is resulting in first order rates independent of the condition.



Dechlorination activity of DF1 in the presence of the black carbon materials. PCB 61 concentrations (nM) measured were normalized to the initial aqueous concentration of PCB 61$_o$ resulting in similar first order rates for each microcosm. Each data point is the mean of triplicate microcosms.

Implications for Future Research and Benefit

Many Department of Defense facilities face challenges from contaminated sediments, and existing remediation options are often limited to expensive or slow approaches. This project aimed at developing a novel approach to addressing PCB-contaminated sediments and management tools by performing laboratory-based studies that subsequently can be transferred and expanded for application in situ. The goal is to more efficiently and in a cost-effective manner to reduce the risk from PCB contamination in sediment at these sites.

The results from this project have expanded the knowledge about mechanisms and rates for bioremediation of weathered PCB contaminated sediment. In addition, we have developed several new analytical approaches that will be applicable for treatment of other environmental compartments that are contaminated with PCBs and other persistent organic pollutants. These approaches include a predictive tool for PCB microbial dechlorination kinetics, quantitative methods for determining biofilm formation and presence on materials as well as scale up of microbial inoculum.

The project assessed the advantages (and limitations) for applying bioremediation and in particular biofilm-based bioremediation using sorptive materials such as activated carbon and biochar from different feed stocks. It was shown that sorption was a key mechanism for the enhanced biofilm formation on activated carbon and that the microbial communities formed on sorptive materials increased the stability of the overall microbial community in PCB contaminated sediment.

The developed approaches can be further applied for bioremediation of PCBs and other persistent organic pollutants beyond contaminated sediment including stormwater, ‘mixed waters’ such as rising groundwater mixed with stormwater or sediment and/or other surface related waters. These environmental compartments are becoming increasingly important due to the impacts of climate change such as rising water tables and more unpredictable storms. Therefore, the presence of biofilms (stabile microbial communities) together with sorptive materials that can adsorp persistent organic pollutants might become even more beneficial in an increasingly dynamic environment.

Chapter 1 – Project overview and timeline

This chapter outlines the history of the project and the many events that have occurred during the project that altogether lead to an almost decade long project. Some of these events were related to 1) change of academic institution from Goucher College to University of Maryland for the PI Dr. Birthe Kjellerup, 2) issues with growth of microbial cultures for collaborators at University of Maryland Baltimore County (Ghosh and Sowers) and 3) latest the Global Pandemic COVID19. The timeline is explained in Table 1 below.

Table 1. Overview of project timeline for ER-2135.

Calendar Year	Institution	Project Phase
2011	Goucher College	SEED project start
2012	Goucher College	SEED project
2013	Goucher College	SEED project report; Follow-on proposal submitted and awarded → ER-2135
2014	Goucher College	Paused due to change of institution
2015	Univ. of Maryland	Project start ER-2135
2016	Univ. of Maryland	Ongoing research
2017	Univ. of Maryland	DF1 growth issues → Delays
2018	Univ. of Maryland	IPR Feb 2018: ER-2135 project stopped
2019	Univ. of Maryland	Tasks OBE (overcome by events)
2020	Univ. of Maryland	Project paused due to COVID19 → Delays
2021	Univ. of Maryland	Project paused due to COVID19 → Delays; Aug 2021: Project debrief

During the project, the following tasks from the original proposal have been completed (Table 2).

Table 2. Completed tasks for project ER-2135 as they are present in SEMS2. *OBE: Overcome By Events.

Task #	Task	Completed
3	Fundamental characteristics (Initial Lab Study)	Yes. White Paper 2 submitted January 2018
4	Isotherm studies	Yes. See this White Paper (#3).
5	Inoculum decision	Yes. White Paper 1. Submitted November 2017.
6	DOD site visits, sediment collection and characterization	Yes. See this White Paper (#3). Collected sediment from DOD sites will be used.
7	Setup of new analytical methods – Microbiology	Yes. See this White Paper (#3).
8	Setup of new analytical methods - Chemical and other	Yes. See this White Paper (#3).
9	Culture setup	Yes. See this White Paper (#3).
10	Not present in SEMS	
11	Biofilm inoculum (Anaerobic vs material)	Yes. Outlined in White Paper #3
12	Biofilm inoculum (Aerobic vs material)	Yes. See this White Paper (#3).
13	Removal methods for PCE on sorbents	OBE
14	Scale-up method develop: FBR approach	OBE
15	Scale-up method develop: MBBR approach	OBE
16	Standard inoculum assessment methods	OBE
17	Cost estimate	OBE
18	Decision on inoculum scale up method	OBE
19	Scale-up mesocosm experiment	OBE
20	Mesocosm studies - Set up	√ - Final report
21	Mesocosm studies - microbiological analysis	√ - Final report
22	Mesocosm studies - chemical and other analysis	√ - Final report
23	Reporting	Final report.

Chapter 2.
Community analysis of biofilms on granular activated carbon particles

Authors
Devrim Kaya, Sarah J. Edwards, Kevin R. Sowers, Staci L. Capozzi, Birthe V. Kjellerup

This chapter is a follow-up of the studies that were performed in the SEED Project (Phase 1, 2011-2013). These results were obtained after the final report was submitted in 2013, but provide important insights into the effect of bioremediation with both aerobic PCB degrading bacteria *Paraburkholderia xenovorans* strain LB400 (LB400) (the name was recently changed from *Burkholderia xenovorans* LB400) and an anaerobic PCB dechlorinating bacterium *Dehalobium chlorocoercia* DF-1 (DF1).

Highlights
- Aerobic and anaerobic biofilms were established on GAC surfaces.
- Augmented mesocosms of Grasse River sediment with biofilm covered GAC showed increased Aroclor 1248 degradation.

Objective
The objective of this study was to evaluate the effect of bioaugmentation of PCB contaminated sediment with microbial communities consisting of anaerobic and aerobic PCB biodegrading microorganisms. The specific objective of this study was to 1) Evaluate PCB transformation due to bioaugmentation with pre-formed biofilm on an activated carbon particle (results were shown in the Phase 1 Report) and 2) Assess the impact of the biofilm bioaugmentation on existing microbial communities in the PCB contaminated sediment.

Background
Polychlorinated biphenyls (PCBs) are toxic and persistent organic pollutants that adsorb to surfaces in the aquatic environment, where they are ingested by animals and accumulate in the food chain. Granular activated carbon (GAC) can minimize the aquatic exposure, but microbial transformation of adsorbed PCBs must also occur. Here, biofilm covered GAC particles were applied to enhance degradation PCBs in sediments due to simultaneous sequestration of PCBs together with increased biofilm activity and stability of the inoculum.

Biofilms were established on GAC surfaces by culturing anaerobic *Dehalobium chlorocoercia* DF-1 (DF-1) and aerobic *Paraburkholderia xenovorans* strain LB400 (LB400). Mature biofilms formed on the particles and were inoculated into mesocosms of PCB contaminated sediment from Grasse River, NY. Augmented mesocosms showed increased Aroclor 1248 (A1248) degradation. The biofilm inoculum was detected throughout the experiment. Evaluation with fluorescent stains showed biofilm covered GAC surfaces and thick layers of bacteria.

Biofilm covered GAC particles enhanced the PCB degradation in the mesocosms due to PCB adsorption onto the GAC ensuring direct contact between the PCB degrading biofilms and the adsorbed PCBs. Biofilms have previously been shown to enable simultaneous dechlorination by anaerobes coupled with biphenyl degradation by aerobic PCB degrading bacteria. Therefore, development of biofilms on GAC can enable complete onsite bioremediation of PCB contaminated sites.

Introduction

Despite their ban in the 1970es, weathered remains of mixtures of the 209 possible polychlorinated-biphenyl (PCB) congeners known as Aroclors still pollute harbor and river sediments (Lake et al., 1992; Fagervold et al., 2007; Martinez et al., 2010). PCBs are known endocrine disruptors posing risk to development via the fetal-placental environment and breast milk, culprits of neuropsychological degradation in the elderly, and likely carcinogens (Fitzgerald et al., 2008; Grandjean et al., 2008; Škrbića et al., 2010). Therefore, remediation of the globally ubiquitous class of PCBs is crucial due to their potential to accumulate to toxic levels in higher organisms (Versar, 1976).

Dredging and capping are the most popular remediation efforts for PCB contaminated sediments (EPA, 2005). Though dredging does remove PCBs with masses of sediment, besides being costly, it can also result in re-suspension of sediment, hence, result in higher water phase concentrations for bio-uptake and accumulation (Davis and Wade, 2003; Martins et al., 2012). Capping effectively controls PCB equilibrium, keeping them out of the water phase by sequestration with activated carbon (AC) and various other substrates (Zimmerman et al., 2004). However, PCBs still remain in the sediment and contaminated sediments can become recalcitrant to microbial degradation because of strong sequestering capacities of amendments and soil particles (Hatzinger and Alexander, 1995).

Anaerobic bacteria within the bacterial group *Chloroflexi* have been confirmed to have PCB dechlorinating activity (Fennell et al., 2004; May et al., 2006; Adrian et al., 2009). Several *Chloroflexi* species, *Dehalobium chlorocoercia* DF-1 (DF-1) and bacterium *ortho*-17 (*o*-17) specifically, have been isolated and characterized from native PCB-impacted sediments and are known dechlorinators (Cutter et al., 2001; Wu et al., 2002). Bacterium *ortho*-17 (o-17), an acetate-utilizing species, was discovered in the Baltimore Harbor estuarine sediment, which is mainly responsible for removal of *ortho* chlorines (Cutter et al., 2001). On the other hand, DF-1 and *Dehalococcoides mccartyi* (*Dhc*) strain 195 has been shown to attack double-flanked and/or flanked *meta*/*para* chlorines (Wu et al., 2002; Fennell et al., 2004). Lately, four new *Dhc* strains were identified as PCB dechlorinators such as *Dhc* CG-1, CG-4, CG-5, and JNA (Wang and He, 2013; Laroe et al., 2014; Wang et al., 2014). Especially, strain CG-1 is responsible of double-flanked *meta*-chlorine removals; strain CG-4 attacks flanked *para*-chlorines followed by flanked *meta*-chlorines; strain CG-5 primarily removes flanked *meta*-/*para*-chlorines (Wang and He, 2013; Wang et al., 2014). *Dhc* strain JNA is the first pure

culture carrying out a complex environmental dechlorination process and capable of chlorine removal from flanked *meta* positions of Aroclor 1260 (Wang and He, 2013; Laroe et al., 2014).

Complete mineralization of PCBs is possible through two subsequent steps: first anaerobic microbial dechlorination to a biphenyl structure with four or less chlorines, followed by an aerobic step where the biphenyl rings are broken, thereby enabling mineralization (Evans et al., 1996; Master et al., 2002; Payne et al., 2017). However, in sediment and soil *in situ* microbial degradation of PCBs under anaerobic conditions is a slow process (Bedard and Quensen III, 1995) due to their chemical and biological stabilities, low bioavailable concentrations and, in many cases, low number, diversity and activity of naturally occurring PCB degrading microorganisms (Abramowicz, 1995; Fagervold et al., 2005). On the other hand, bacteria capable of performing the aerobic step are ubiquitous. For example, *Paraburkholderia xenovorans* LB400 (LB400), aerobic organisms, can mineralize the biphenyl ring structure (Payne et al., 2017). Hence, *in situ* biological transformation of PCBs in sediments could not reduce their concentrations sufficiently within a reasonable period.

Bioaugmentation of PCBs in sediment and soils has been examined in several studies (Fagervold et al., 2011; Payne et al., 2011; Payne et al., 2017). It was found that the addition of cultured organisms stimulates indigenous communities to become more active in dechlorination, the mechanism for which is not yet understood. However, the growth of those organisms in the environment with very specific growth conditions are slow, making the application of planktonic cultures for bioremediation efforts a challenge. As a result, the dechlorination rates and the extent of PCB dechlorination obtained with bioaugmentation were low (May et al., 2008; Krumins et al., 2009). For these reasons, developing effective and efficient remediation tools is of utmost importance.

A combination of sequestration with dry AC together with bioaugmentation with liquid cultures of anaerobically dechlorinating bacteria has been examined and dechlorination was reported (Payne et al., 2011). Sediment treated with AC attains pore water PCB concentrations 85% and 92% lower than untreated sediment in one- and six-month contact experiments, respectively (Zimmerman et al., 2004). More recently, Payne et al. (Payne et al., 2017) has attained 94-97% reduction in pore water PCB concentrations in laboratory sediment mesocosms (2 L) spiked with 1.5% AC and bioaugmented with 5×10^5 DF-1 and LB400 cells per g of sediment. Such observations indicate that AC effectively outcompetes solid phases and benthic organisms for PCBs, resulting in lower PCB levels in the aqueous phase and reduced PCB exposure to aquatic organisms, including microbes.

In nature, metabolically active microbial communities typically live in polymeric encased matrixes called biofilms; capable of withstanding shear stress forces in the environment (Hall-Stoodley et al., 2004). The study of engineered biofilms used for bioremediation purposes is still in its infancy; however, the biofilm-like structure is inherent to the bacterial-

floc communities ubiquitous in wastewater treatment sludge, where PCBs often accumulate. In this study, our overall hypothesis is that the rate and extent of anaerobic dechlorination of PCBs in aquatic sediments can be enhanced by the presence of active microbial biofilms associated with surfaces of AC particles. This enhancement is due to simultaneous sequestration of PCBs and increased bacterial density at fixed surfaces leading to enhanced microbial PCB degradation. To show this, we explored the potential of coupling the PCB sequestering ability of AC and its capacity to donate electrons with the reductive dechlorination pathways of anaerobic biofilm-forming bacteria. By cultivating pure DF-1 biofilm cultures as dechlorinating consortia and using that as inoculum in PCB sediment mesocosms, we sought to analyze the effects of the biofilms on PCB bioremediation compared to various controls. Additionally, an aerobic biodegrader, *Paraburkholderia xenovorans* strain LB400 (LB400) was included in several mesocosms to elucidate the effect of both aerobic and anaerobic bioaugmentation on PCB dechlorination and mineralization.

Materials and Methods

Growth of anaerobic cultures for mesocosm inoculum.

***Dehalobium chlorocoercia* DF-1:** DF-1 (in co-culture with *Desulfovibrio*) was grown anaerobically in low-sulfate (<0.3 mM) estuarine medium (Berkaw et al., 1996) with 10 mM formate (Thermo Fisher Scientific) as electron donor, autoclaved *Desulfovibrio sp.* cell extract (1%, v/v) as a growth factor (May et al., 2008) and 0.5 mM titanium (III) nitrilotriacetate (Tinta) as a reductant (May et al., 2008) and 50 ppm PCB-61 (2,3,4,5-tetrachlorobiphenyl, purity >99%, Accustandard) dissolved in acetone (acetone/PCB-61: 0.1%, v/v) and used as an electron acceptor. Cultures were kept anaerobic in serum vials with Teflon caps at 30°C and sampled under nitrogen flow. Growth was monitored by GC analysis of PCB 61 dechlorination to PCB 23 (2,3,5-PCB) and by quantitative -PCR (q-PCR) of 16S rRNA gene copies (described below). DF-1 cultures were added as inoculum to mesocosms containing Grass River (GR), Massena, NY, USA, sediment (Table 1) when 40-50% PCB-61 dechlorinated to PCB-23 (Figure 1) either as liquid DF-1 cultures (DF-1 liq.) or as biofilm cultures formed on the GAC surfaces (details are below).

Aerobic cultures of LB400: The aerobic PCB degrading bacterium LB400 was grown aerobically in M9 media by using 10 mM biphenyl as the carbon source and electron donor (Payne et al., 2017) with and without 3% GAC. The growth was followed over several weeks by counting the total number of bacteria by fluorescent microscopy using 4', 6-diamidino-2-phenylindole (DAPI) fluorescent stain. Before addition to mesocosms, dechlorinating bacteria were enumerated via qPCR as described below.

Formation and Confirmation of Biofilm on AC particles.

Anaerobic Biofilm Development: Anaerobic based biofilm inoculum was developed on autoclaved GAC particles (Calgon Carbon Corporation; type46; mesh 50x200; Lot No. P-21553) by DF-1 in co-culture with *Desulfovibrio* and PCB-61 at 30°C in serum bottles sealed with Teflon-coated butyl stoppers (West Co., Lionville, PA) secured with aluminum crimp

seals under anaerobic conditions (Berkaw et al., 1996). DF-1 cultures were added as inoculum to mesocosms containing Grass River (GR), Massena, NY, USA, sediment on day 57 when 40-50% PCB-61 dechlorinated to PCB-23 (Figure 1-A1&A2) either as liquid DF-1 (DF-1 liq.) cultures or as biofilm cultures formed on the GAC surfaces.

GAC (2.6 g) was autoclaved and added to the related cultures (Table 1) on day 57, when approximately 40-50% dechlorination (PCB-61 to PCB-23) had been achieved (Figure 1-B1&B2), to facilitate biofilm formation. At this stage the bacterial cells would attach to the added GAC particles, colonize and develop a biofilm on the GAC particle surface, which was monitored by q-PCR (Figure 2) and scanning electron microscopy (SEM) (Figure 3).

Aerobic Biofilm Development: Biofilms consisting of LB400 on GAC particles were established by growing 15 plates of LB400 on M9 media containing 50 ppm biphenyl. These cultures grew for approximately 14 days on the plates in room temperature until visible biofilms could be observed on the plates. Next, LB400 cells were picked up from the 15 plates using sterile inoculation loops and placed into liquid M9 media without biphenyl to rinse the cells of the plates. Cells harvested from the M9 plates were inoculated in fresh sterile M9 medium with or without GAC (Table 1).

To verify the presence of biofilms formed on GAC particles prior to the setup of sediment mesocosms, anaerobic cultures of DF-1 or aerobic cultures of LB400 and GAC were mixed thoroughly and sampled for imaging at Montana State University's Center for Biofilm Engineering (CBE, Betsy Pitts) and Imaging and Chemical Analysis Laboratory (ICAL, Laura Kellerman). Cultures were shipped over night in anaerobic vials (DF-1) or aerobically (LB400) and were imaged the next day. Drops of sample were air dried onto silicon wafers and coated according to standard protocols for SEM analysis (Figure 4). Images obtained via CBE were acquired directly from the wet GAC samples, where the bacteria had formed biofilms. The samples were directly labeled with the fluorescent stain SybrGreen (Thermo Fisher Scientific) that target all DNA, but does not stain the background such as the GAC particles or components in the growth media (Figure 5). The fixation, hybridization and imaging for peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) were performed according to the manufacturer's instructions (Figure 6; AdvanDx, Woburn, MA, USA).

Mesocosm setup and sampling

GR sediment was collected in 2010 and homogenized using a coring device to collect the top 5 cm of the sediment (Dr. Upal Ghosh Laboratory, UMBC). The sediment was stored in an amber gallon glass jar, filled to the edge to avoid oxygen interference and was kept at 4ºC until the mesocosms were set up. Sediment were filled into glass tanks (mesocosms) ensuring that anaerobic layers of sediment would be present over layered about an inch of water from the sample as the sediment settled out after mixing with inoculum or appropriate controls. All

anaerobic mesocosm preparations were performed in an anaerobic glove box (5% H_2, 15% CO_2, 80% N_2) (Coy Laboratory, Grass Lake, MI, USA).

Anaerobic cultures of DF-1 with and without biofilm covered GAC were centrifuged at 12,000 rpm for 10 minutes to obtain an aggregate pellet which was then resuspended in 10 mL supernatant per mesocosm and added to respective mesocosms at approximately $2x10^6$ cells/g of GR sediment. Aroclor 1248 (A1248) as well as PCB-116 (2,3,4,5,6-PCB) were applied to respective experimental and control mesocosms at a final concentration of 50 ppm (Table 1). About 3% (by weight, 2.6 g) GAC was added to a control and mixed into the sediment (85 g). A control for abiotic transformation was prepared by autoclaving sediment for 45 minutes at 121°C and subsequently adding A1248.

Sampling and Analysis
Samples were collected from all cultures on day 0 (D0), D57, and D200 to evaluate the dechlorination rate and the number of dechlorinating bacteria.

PCB analysis
Extraction and analysis of PCBs followed a previously developed method (Fagervold et al., 2005; Payne et al., 2011; Kaya et al., 2017). Briefly, for every sampling date, triplicate samples (i.e., one mL aliquots of culture or 1 g of mesocosm sediment) were taken with sterile Pasteur pipets into clean glass culture tubes. To extract PCBs from any given sample, 5 mL of hexane (Fisher Scientific, PA) was added to the sample and on a wrist action shaker (Burrell Corp., PA) shaken for 3 hours for pure culture and 12 hours for mesocosm sediment. Just before extraction, samples with hexane were vortexed for 10 seconds. Cleanup of the samples followed the procedures previously applied for spiked samples (Kjellerup et al., 2012) using a glass column packed with copper/Florisil (Sigma Aldrich, St. Louis, MO) (1:4, by weight) and sodium sulfate (Fisher Scientific, PA) (Kaya et al., 2017). Surrogates (PCB-14, 65 and 166) were added to samples at the beginning of the extraction to ensure reproducible recoveries. Sediment sampling followed a grid pattern to ensure that the sampling would take place in independent areas at the different time points to increase the reproducibility of the results. Internal standards (i.e., PCB-30 and PCB-204) were added to the extracts before PCB analysis with an Agilent 6890 GC/ECD equipped with a DB-1 capillary column (60 m by 0.25 mm by 0.25 μm; JW Scientific) following a modified method of EPA method 8082 (Fagervold et al., 2005).

Calibration curves (10-point) were prepared as described previously (Payne et al., 2011). Calibration table consisted of 132 congener groups with co-elution, and in total, 173 congeners were identified in the GC method and the majority of these eluted off as single congeners. In cases where two or more congeners co-eluted, the amount was assumed to be equal for the congeners and split accordingly. Surrogate recoveries were around 83±5%. No surrogate recovery correction is performed on the presented data. PCB concentrations were measured as μg PCB per mL sample and converted to mol% by using molecular weight of

each PCB congener present in the samples (Kaya et al., 2017). The chlorines per biphenyl were obtained by calculating the relative number of chlorines in a homolog group by adding the sum of the measured masses of all congeners in a homolog group and multiplying by the number of chlorines and subsequently divide by the sum of all relative chlorine numbers. The data obtained from each of the three replicates were averaged and the standard deviation was calculated based on this. The dechlorination rate was calculated through the linear slope of the dechlorination curve by dividing total chlorine removed per biphenyl with the time elapsed in days and reported as Cl^-/bp.day (Kaya et al., 2017). All congeners were included in the analysis no matter how low the detected concentration was in the source data from the GC analysis. The detection level was defined as the lowest concentration of the individual PCB congeners used to establish the calibration curve used in the GC method, which was 4 µg/l. If a specific congener was detected at day 0, but not at day 200, the value for day 200 would be defined as 0 µg/l and this concentration would be used in the subsequent calculations.

DNA Extraction, Quantitative PCR, and Microbial Community Analysis.
Triplicate DNA samples from each sediment mesocosm (Table 1) were extracted from 0.25 mL of samples by using PowerSoil® DNA Isolation Kit (MoBio Laboratories). To monitor growth of pure cultures, DNA was extracted by using Bio-Rad InstaGene™ Matrix (Bio-Rad Laboratories). Extracted DNA samples had an A260 to A280 ratio was around 1.6 with A260 to A230 ratio around 2.0. The number of putative PCB dechlorinating microorganisms was quantified by using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories) and primers (348F/884R) specific to the 16S rRNA genes of putative dechlorinating clade within Chloroflexi (Fagervold et al., 2005) as described previously (Payne et al., 2011). Amplifications efficiencies of standards were 86(± 7) % with r^2= 0.99.

The diversity of the PCB dechlorinating organisms in the mesocosm samples was examined by using the DHPLC based assay by using a WAVE 3500HT system (Transgenomic, Inc.) as described and applied in previous studies (Kjellerup et al., 2008). An initial run was used to identify individual PCR fragments and determine their retention times. Individual peaks were eluted for sequencing from a subsequent run and collected with a fraction collector and sequenced by the BioAnalytical Services Lab (IMET, Baltimore, USA) using the BigDye® Terminator v3.1 (Applied Biosystems) as described previously (Kaya et al., 2017). Sequences were analyzed using the automatic nucleic acid aligner in the ARB software package.

Microbial community analysis of mesocosms

16S rRNA amplicon sequencing (Miseq paired-end) were performed on the samples collected at the beginning (day 0) and at the end of the treatment (day 200) by using universal primers covering all bacteria (using V3-V4 primers). In addition to that, day 200 samples were sequenced by using putative PCB dechlorinating Chloroflexi-specific primers (using 384F/884R). Preprocessing of the raw sequence data included read merging, quality trimming, artifact and contaminant removal. Next, high-quality passing sequences were clustered into OTUs de novo with the curated SILVA database. Additional analysis was performed using alpha- and beta-diversity characterization and PICRUSt for functional inference. The latter approach has the potential for hypothesis development based on predictive functions.

Results and Discussion

Cultivation of anaerobic bacterial inoculum using DF-1.

The bacterium *Dehalobium chlorocoercia* DF-1 (DF-1) was used as a model organism for reductive PCB dechlorination by anaerobic biofilms on activated carbon particles. Several cultures were prepared during the study for observation of growth as well as preparing inoculum for the mesocosm experiment. The culturing of pure DF-1, both planktonic and in biofilms, is a slow and fastidious process. Though DF-1 has great potential, its characterization and growth optimization are still in its infancy. To observe the dechlorination activity of the DF-1 cultures the relative mol% dechlorination of PCB-61 to PCB-23 (*para*-dechlorination product of PCB-61) was calculated based on the amount of each congener obtained through extraction and analysis by gas chromatography. Cultures 1, 2 and 3 showed a decrease in mol% PCB-61 (Figure 1.A1) with subsequent increases in %PCB-23 (Figure 1.A2) until day 56 with the fastest rates of turnover occurring between day 0 and day 35. Between day 56 and 70, %PCB-61 increased in cultures 1 and 3 (Figure 1.A1), which likely was due to loss of PCB-23 during analysis, since the cultures were grown in a closed system and the calculations are based on relative mol%. In the same time period the dechlorination seemed to continue in culture 2 seen as increased mol% of PCB-23 (Figure 1.A2).

The cultures 4, 5 and 6 (Figure 1.B1-B2) were treated the same was as cultures 1, 2 and 3 until day 56, when activated carbon was added at 3% to each of the cultures. At day 0, the results showed that the cultures 5 and 6 contained approximately 35% and 25% PCB-23, respectively, whereas culture 4 contained about 10% PCB-23 (Figure 1.B2). Before day 57, culture 4 demonstrated decreases in %PCB-61 (Figure 1.B1) with the fastest rate of turnover occurring between day 35 and 56. Cultures 5 and 6 showed limited activity until day 35, when both cultures showed dechlorination with increases in %PCB-23 until day 56 (Figure 1.B1, B2). Cultures 4-6 all seem to display the highest dechlorination rates of PCB-61 to PCB-23 between days 30 and 56. Unexpectedly, all cultures amended with GAC (cultures 4-6) presented an increase in %PCB-61 and a decrease in %PCB-23 after day 57 (Figure 1.B2). However, the reason for this is very likely that equilibrium between the added GAC and the

PCBs had not been reached after only two weeks following the addition and not that the cultures became inactive. This is supported by the increased cell numbers by qPCR (Figure 2) and by the advanced microscopy (Figure 7 and 8) that the bacteria had formed biofilm that were present at the surface and were active. On day 0 (immediately after transfer), the qPCR data showed that the cell numbers in the cultures ranged between 1.3-7.3x10^5 cells/ml (Figure 2). By day 35, all cell numbers had decreased, likely due to a lag phase where the bacteria adapt to the new conditions. Between day 35 and day 70 cell numbers increased in all cultures (except in culture 4) by an average of 3.6x10^5 cell/ml. Overall, cultures 1, 2, 4, and 6 demonstrated net increases in numbers of dechlorinating bacteria averaging 2.12x10^5 cells/ml. Cultures 3 (no GAC) and 5 (GAC) showed a net decrease in bacterial numbers of 5.45x10^5 and 3.83x10^5, respectively (Figure 2).

Before the addition to the sediment mesocosms, DF-1 cultures 1-6 were sampled for imaging on day 70 by scanning electron microscopy. Images confirmed that biofilm had formed on the GAC particles (Figure 3.A-C) to a greater extent (estimated, not measured with image analysis) than in the cultures without GAC (Figure 3.D-F). Clumps of DF-1 had formed and are characterized as biofilms in cultures 1-3, but these biofilms were not as expansive as the GAC biofilms, cultures 4-6. Biofilm formation on GAC did not discriminate between seemingly smooth or rough surfaces.

Cultivation of aerobic bacterial inoculum using LB400.
The aerobic PCB degrading bacterium, LB400, was examined for its capability to form biofilms on activated carbon surfaces. It was grown in minimal media with 50 ppm biphenyl as substrate to cultures with and without 3% GAC. The growth was followed over several weeks by counting the total number of bacteria by direct cell count using an epifluorescent microscope. The initial results show that LB400 is an excellent and very fast biofilm former. Within only two weeks a biofilm had formed on GAC particles and there were significantly more cells attached to the surface of GAC particles than were present in the liquid culture media. SEM images were obtained every 1-2 weeks in order to follow the biofilm formation on the activated carbon surfaces and the bacterial cells adhered to the surface and formed robust biofilms (Figure 9).

Effects of treatments on PCBs dechlorination.
Grasse River (GR) sediment has been historically impacted with Aroclor 1248 former aluminum production at an industrial site during the1930s (EPA, 2005). GR sediments had an average of 2.99 ±0.3 chlorines per biphenyl. PCB analysis indicated that di-, tri-, tetra-, and penta-chlorobiphenyl homologs were dominant, accounting for of 41.2±0.10, 31.5±0.11, 18.0±0.01, and 6.9±0.07 in mol%.

The efficacy of GAC addition and biougmentaton on the dechlorination of A1248 was tested by bioaugmenting mesocosms containing weathered A1248 contaminated GR sediments with anaerobic PCB halorespiring (DF-1) and aerobic PCB degrading bacteria (LB400) delivered

both alone as a liquid culture or a biofilm formed on GAC and concurrently as biofilm formed on GAC (Table 1). Total chlorines per biphenyl (Cl^-/bp) in sediment mesocosms spiked with A1248 was 3.78 ± 0.3. PCB dechlorination was observed in all mesocosm except for autoclaved sediment mesocosm. Figure 10A shows PCB dechlorination rates observed during 200 days of incubation in sediment mesocosms amended both GAC alone or with simultaneous addition of dechlorination bacteria (i.e., DF-1, LB400, or both) either as in liquid culture or as in biofilm form on grown on GAC. The dechlorination rate in sediment control (no augmentation/GAC amendment), spiked with A1248 so all the others, was 3.73 ±0.5 x 10^{-3} Cl^-/bp.day. This could be due to biostimulation of indigenous dechlorinating organisms with receipt of A1248. When sediment was amended with GAC, A1248 dechlorination rate, 5.41 ±0.1 x 10^{-3} Cl^-/bp.day, was increased by about 45%, while in mesocosms augmented with DF-1 in liquid form (no GAC), the rate (4.76 ±0.4 x 10^{-3} Cl^-/bp.day) increased by about 28%, compared to sediment control. But, with simultaneous DF-1 liquid culture augmentation and GAC amendment, the A1248 dechlorination rate was increased by about 60% to 5.98±0.3 x 10^{-3} Cl^-/bp.day, compared to sediment control. Highest A1248 dechlorination rate, 7.92 ±0.5 x 10^{-3} Cl^-/bp.day, was achieved in sediment mesocosms that were bioaugmented with DF-1 biofilm grown on GAC, which is about two-fold higher than that of sediment control. A similar trend was observed with LB400 culture amendments. For example, A1248 dechlorination rate (Figure 10A) was higher in sediment mesocosms augmented with LB400 biofilm grown on GAC (5.99±0.5 x 10^{-3} Cl^-/bp.day) compared to the mesocosms augmented with LB400 liquid culture and amended with or without GAC amendment (4.60±0.3 x 10^{-3} and 3.78±0.2 x 10^{-3} Cl^-/bp.day, respectively).

On the other hand, the dechlorination rate at 4.46 ±0.1 x 10^{-3} Cl^-/bp.day observed in sediment mesocosms treated with both DF-1 (anaerobic) and LB400 (aerobic) biofilms grown on GAC (Figure 10A) was surprisingly lower than those treated with either DF-1 or LB400 biofilms on GAC by about 44% and 26%, respectively. The reason for this observation is not clear, but could be related to the how this set of mesocosms were prepared; LB400 biofilms on GAC were developed under aerobic condition and added to sediment mesocosm together with anaerobically grown DF-1 biofilms on GAC and incubated under anaerobic conditions throughout the experiment. Due to the set-up of the experiment, some oxygen might have been carried to the anaerobic system with LB400 biofilm on GAC resulting in inactivation or death of anaerobic bacteria as well as the aerobic bacteria considering the anaerobic conditions. This argument is supported with the decrease (about 17%) observed in the number of the dechlorination bacteria at day 60 (2.1 ±0.9 x 10^5 copies/ml) compared to day 0 (2.5 ±0.6 x 10^5 copies/ml) (Figure 10).

The dechlorination in abiotic control (autoclaved sediment mesocosm) with rate at 0.39 ±0.5 x 10^{-3} Cl^-/bp.day) was insignificant as opposed to that of the biotic ones. Similarly, no increase in the number of dechlorinating bacteria was observed in abiotic controls. On the other hand, in all biotic experiments the number of dechlorinating bacteria increased by about 7- to 36-fold ranging from 1.9 ±0.1x 10^6 to 4.19 ±0.3 x 10^6 copies/ml (Figure 11), indicating

the inoculum not only remained in the sediment but also increased significantly throughout the experiment (200 days). The inoculum added as DF-1 biofilm was shown to endure the environmental conditions and multiply about 10 times (Figure 11). This anaerobic biofilm inoculum was also able to survive and establish an active dechlorinating population when added to the sediment mesocosm. These observations are helpful to draw two main conclusions: 1) GAC amendment accelerated dechlorination rate significantly ($p: 0.018<0.05$); 2) cultures in biofilm forms performed better. In line with the dechlorination rate, GAC amendment regardless of bioaugmentation resulted in higher extension of dechlorination compared to those without GAC amendment. The overall A1248 dechlorination was between 21% and 42%, the highest being achieved by DF-1 biofilm on GAC (Figure 10B). Dechlorination was achieved mostly through meta and para chlorine removals (Figure 10B). However, rare ortho dechlorination (by about 8-19 mol% (relative to initial) decrease in total number of ortho chlorine) was also observed at the end of the experiment. The highest ortho dechlorination was observed in sediment amended only with GAC (19.4 %) followed by DF-1 biofilm culture grown on GAC, (19%), as opposed its liquid form with GAC (7.5%). On the other hand, about 4% increase was observed with DF-1 liquid culture (no GAC). But, this observation seemed to be due to the presence of GAC rather than biofilm itself, as similar trend was observed in mesocosm augmented with LB400 culture (Figure 10B). Indeed, statistical analysis (student-t test) indicated GAC amendment favored dechlorination in ortho positions significantly ($p: 0.042<0.05$).

A detailed look at the dechlorination taking place in the sediment that was augmented with the anaerobic DF-1 biofilm (Figure 12) showed the removal of more extensively chlorinated congeners (below the x-axis) and the dechlorination products (above the x-axis). With a close look on the distribution of PCB homologs, it can be seen that most of dechlorination was achieved through dechlorination of tetra- (decreased from 53% to 28%) and tri-chlorinated (decreased from 36% to 4%) PCB congeners. The inoculation with this biofilm delivery vehicle showed that mono- (58% increase), and di-chlorinated (4% increase) congeners were formed as a result of dechlorination from a wide range of extensively chlorinated congeners. On the other hand, in mesocosms with LB400 biofilm (Figure 13), about 7%, 32%, and 11% decrease was observed in mol% of penta-, tetra, and tri-chlorinated congeners resulting in an increase in di- (41%) and mono-chlorinated (13%) congeners.

Effects of treatments on Microbial Community Composition.

Microbial community analysis was conducted to reveal temporal differences in taxonomic composition based on treatment regimen within and among mesocosms. Results gleaned from the universal bacterial analysis showed stability amongst the microbial communities over time (Figure 14). However, more sensitive information was obtained with regard to halorespiring communities using the Chloroflexi-specific primer set. Substantial enrichment was observed in Chloroflexi members in mesocosms over 200 days in Figure 15.

Analysis of the microbial populations using the universal primer set targeting all bacterial groups (V3-V4) showed limited difference between the mesocosms independent of the specific augmentation. The reason for this might be the level of analysis, where differences between specific groups of organohalide respiring bacteria cannot be distinguished. Organohalide respiring bacteria commonly make up less that 1-2% of all bacteria in sediment this cannot easily be detected using the universal approach. Instead analysis using specific primers such as 348F-884R can be applied.

The analysis of the sequencing data using the primer set detecting putative PCB dechlorinating Chloroflexi showed that similar groups are present in all mesocosms but in different abundances. The mesocosm, where liquid DF1 culture was added showed higher detection of *Dehalococcoides* and *Dehalogenimonas*. These groups of bacteria are not closely related to DF1 that was added thus the results might be a result of the semi-quantitative nature of sequencing. DF1 belongs to the group of *Dehalobium* that was solely detected in the mesocosm, where DF1 biofilm formed on GAC was added. This showed that augmentation with biofilm formed on GAC was the only approach, where DF1 was detectable after 200 days of incubation in the mesocosms.

Environmental Implications

To compare the efficacy of a bioaugmentation inoculum, we applied both biofilm cultures as well as planktonic DF-1 and LB400 both alone or with simultaneous addition of GAC to PCB impacted sediment from Grass River, NY. The mesocosm inoculated with DF-1 biofilm did not demonstrate discrimination of specific congeners and exhibited formation of less extensively chlorinated byproducts, which likely promoted enhanced dechlorination activity by the indigenous dechlorinating population. As cell numbers for sediment (14 fold), DF-1 liquid culture without GAC (21 fold) and with GAC (12 fold) and DF-1 biofilm culture on GAC (10 fold) mesocosms increased throughout the experiment, it can be concluded that cell abundance is in correlation with dechlorination rates and extends. The results implicate that the biofilm structure, while not increasing overall cell abundance to levels observed in planktonic (liquid) form, does increase dechlorination rates as the highest dechlorination was observed with biofilm. When sediment samples from the mesocosms were analyzed for the presence of the augmented biofilm, these particles could be observed in the mixed sediment samples thus supporting the qPCR results that showed that the biofilm inoculum remained in the sediment throughout the experiment (data not shown). This supports the fact that biofilm inoculum is a robust delivery system for bioaugmentation.

In addition to anaerobic processes, experiments investigating the biofilm formation by the aerobic degrader *Paraburkolderia xenovorans* LB400 and the effect in sediment were performed. The results showed that LB400 is an efficient and fast biofilm former that readily adheres to activated carbon surfaces. In the mesocosm, since the sediment remained anaerobic, the indigenous dechlorinating bacteria continued the anaerobic processes, while LB400 performed aerobic degradation, which has been observed in other projects as well

(Tillmann et al., 2005). As LB400 is a PCB degrader, LB400 associated enzymes are capable of opening the biphenyl ring structure and transform the molecule into a linear structure; this changed structure was not measured as a product in this experiment. However, the results showed decrease in congeners with 6 or less Cl^-/bp with increase in the congeners with 2 or less Cl^-/bp, which indicate that breakdown was taking place by LB400. Similar results have been seen in other sediment environments since it is known that LB400 can oxidize PCBs with up to 4-6 chlorine substitutions on the biphenyl rings (Bedard et al., 1986). This is particularly relevant for sediments contaminated with "lighter" Aroclor mixtures such as Aroclor 1248 and 1242.

In summary, the potential to culture anaerobic PCB degrading bacterial biofilms for bioremediation is validated by these experiments. Furthermore, we show that cultures with higher diversity are capable of dechlorinating highly chlorinated mixtures to mono- and di-chlorinated congeners which are then available for aerobic mineralization. This study also demonstrates that the prospect of exploiting both aerobic and anaerobic mechanisms to dechlorinate and mineralize the biphenyl ring structure to subsequently get rid of PCBs altogether is plausible but more thorough investigation is needed.

Tables and Figures

Table 2.1 Experimental setup of sediment mesocosm treatments

Sediment Mesocosm Treatment	No	Sediment	GAC	A1248	External Inoculum (~10^5 cells/ml)
Sediment (no augmentation)	1	+	-	50 ppm	-
GAC	6	+	3%	50 ppm	-
DF-1 liq.	5	+	-	50 ppm	DF-1 liquid
DF-1 liq. & GAC	7	+	3%	50 ppm	DF-1 liquid
DF-1 biofilm on GAC	8	+	3%	50 ppm	DF-1 biofilm
LB400 & DF-1 biofilm on GAC	16	+	3%	50 ppm	LB400 & DF-1 biofilm
LB400 liq	9	+	-	50 ppm	LB400 liquid
LB400 liq. & GAC	10	+	3%	50 ppm	LB400 liquid
LB400 biofilm on GAC	11	+	3%	50 ppm	LB400 biofilm
Autoclaved sediment	14	+	-	50 ppm	-

Table 2.2. Species more than 2 fold upregulated based on DNA sequencing (16S).

Taxon	Fold increase	Example organism/family	Function
Acidobacteria	2-35	*Holophagaceae*	Anaerobic cellulose degrader
Actinobacteria	3	*Micromonosporaceae*	Sediment bacterium
Alphaproteobacteria:	2-10	*Rhodospirillaceae*	Metal (Se, Zn) reduction in soil
Betaproteobacteria:	16	*Zoogloea*	Remediation of pharmaceutical and personal care products
Deltaproteobacteria	2-5	*Desulfobulbaceae*	Toluene-Degrading
Gammaproteobacteria	4	*Methylomonas*	Anaerobic methane oxidation
Armatimonadetes	3-5	-----	Anaerobic ammonium oxidation
Bacteroidetes	2-3	*Niabella*	Soil/Rhizosphere bacterium
Chloroflexi	2-8	*Anaerolineae*	Anaerobic degradation of oil-related compounds
Cyanobacteria	2-4	*Nostocaceae*	Nitrogen transformation
*Elusimicrobia**	3-4	*Elusimicrobiales*	Degradation of aromatic compounds
Firmicutes	4	*Ruminococcaceae*	Anaerobic cellulose degradation
*Lentisphaerae**	3	*Lentisphaeria*	Petroleum-hydrocarbon degradation
Nitrospirae	2	*Nitrospirales*	Acid tolerant fermentation
Planctomycetes	3	-----	Aromatic hydrocarbon degradation & denitrification
TM7	2	-----	Acidic mine drainage
Unknown Bacteria	2-6	-----	Related to soil/ sediment biofilms
Archaea	2	*Methanoregula*	Degradation of C7-8 iso-alkanes (methanogenic conditions)

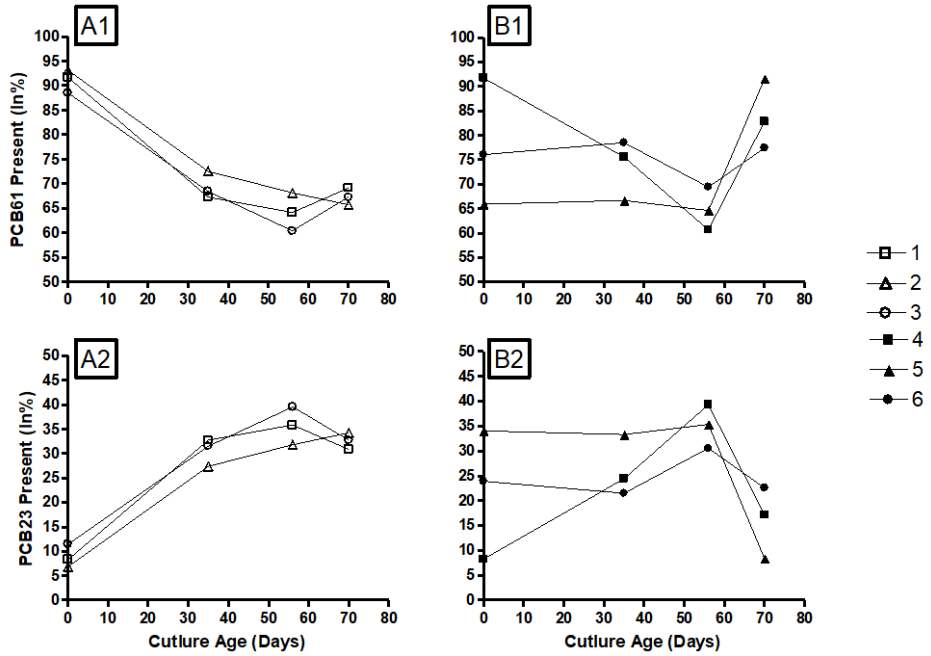


Figure 2.1 Comparison of the growth of DF-1 cultures in the absence (A1, A2) and presence (B1, B2) of activated carbon (same cultures as in Fig. S1). All cultures were transferred at Day 0 and grown in minimal media with 50 ppm PCB-61 (2,3,4,5-PCB). PCB-23 (2,3,5-PCB) is the product of double-flanked *para*-dechlorination of PCB-61 by DF-1. Activated carbon was added to cultures 4, 5 and 6 on day 57.

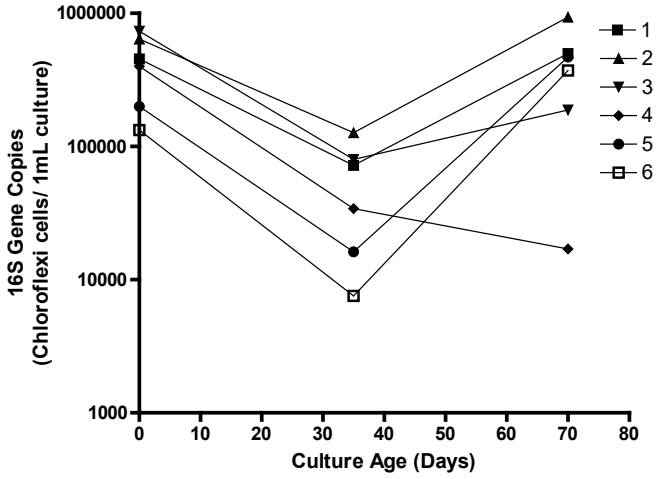


Figure 2.2 Numbers of anaerobically dechlorinating DF-1 bacteria cultures used for sediment mesocosm inoculum. A total of six cultures were applied for this purpose. On Day 57 of the cultures GAC was added to cultures no. 4, 5 and 6.

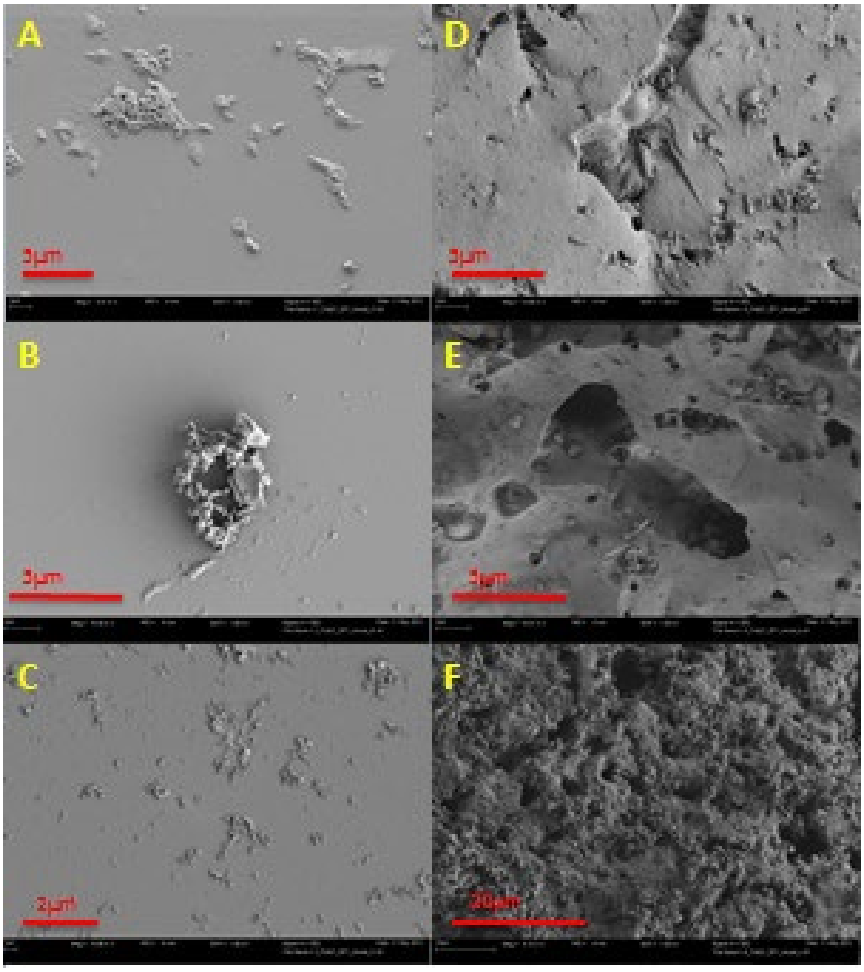
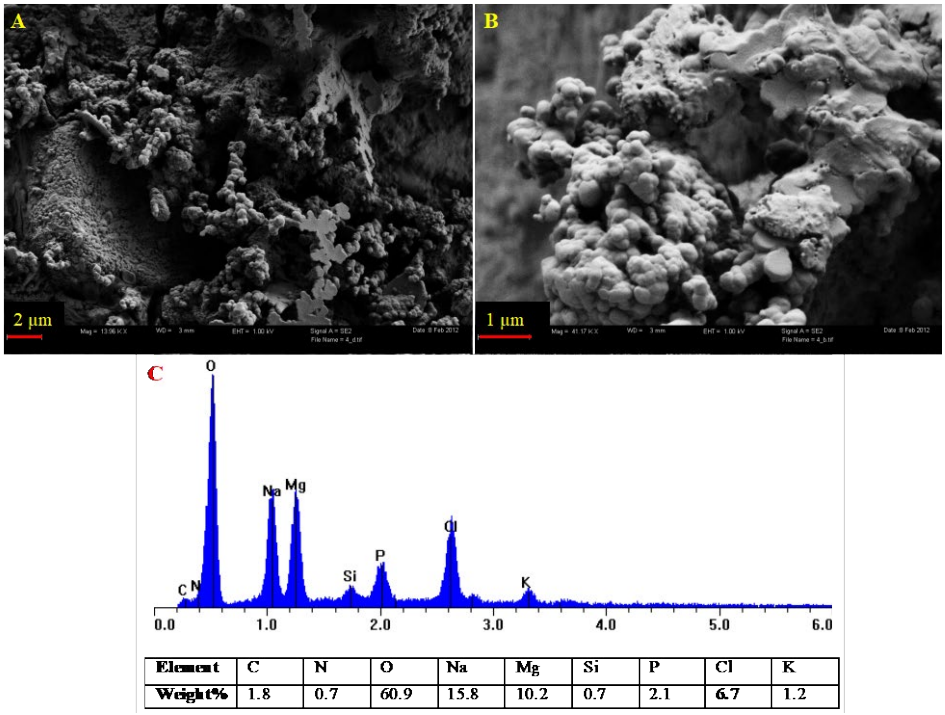


Figure 2.3 SEM of anaerobic DF1 cultures 1-3 without GAC (left: A, B, C) and 4-6 with GAC (right: D, E, F) at day 70. GAC was added to cultures 4-6 two weeks prior to imaging. Drops of cultures were dried onto silicon wafers and prepared according to SEM protocol (ICAL, MSU Bozeman, MT).



Element	C	N	O	Na	Mg	Si	P	Cl	K
Weight%	1.8	0.7	60.9	15.8	10.2	0.7	2.1	6.7	1.2

Figure 2.4 SEM image of anaerobic dechlorinating DF-1 cultures grown A) without GAC; and B) on the surfaces of the GAC in the biofilm matrix (Note: The figures show different magnifications A: ~10X and B: ~41X), C: Elemental analysis of the anaerobic dechlorinating DF-1 biofilm. The presence of the chlorine peak shows the presence of PCBs in the biofilm. Note that the chlorine content is 6.7%, whereas this content is zero for a clean surface.

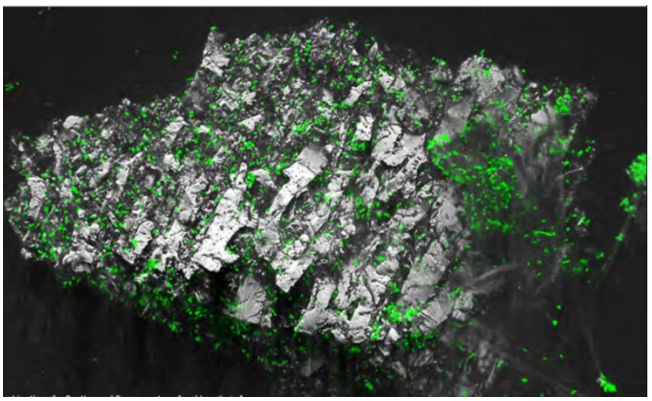


Figure 2.5 The image shows DF-1 biofilm formed on the surface of GAC particle. The bacteria were labeled with the DNA specific stain SybrGreen that only targets DNA (i.e. bacterial cell material) and not the background such as GAC and/or media components.

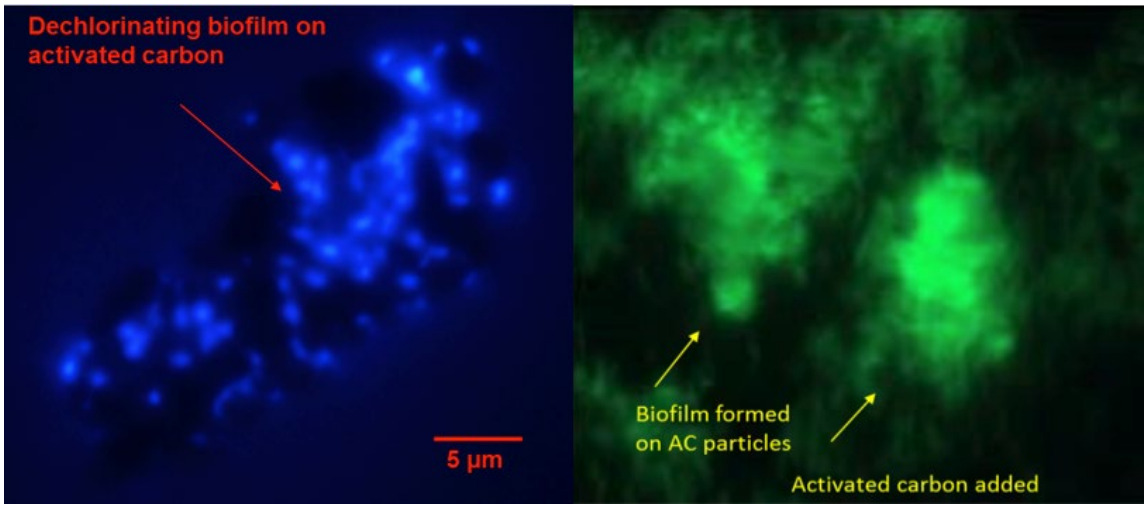


Figure 2.6 Left: DAPI staining; Right: PNA-FISH of activated carbon particles covered with anaerobic DF1 biofilm.

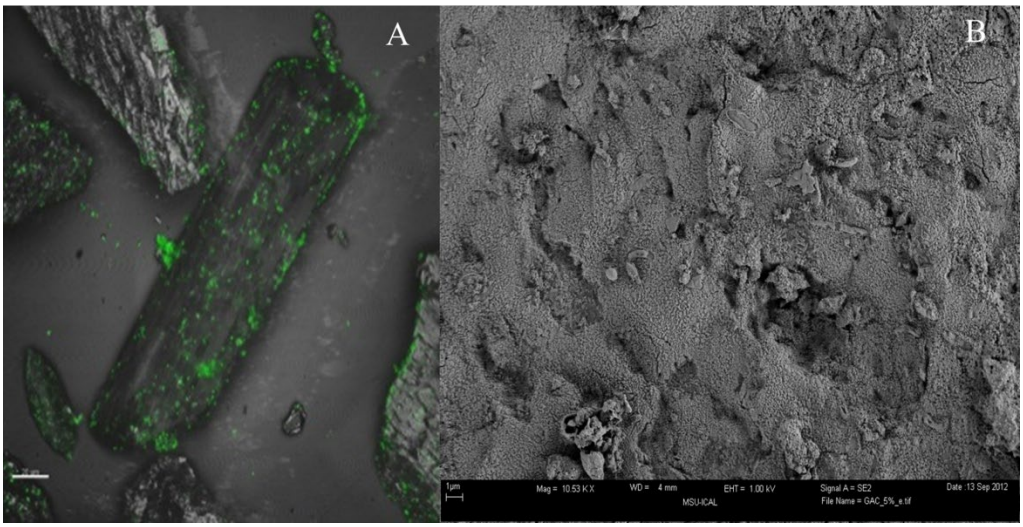


Figure 2.7 Comparative image analysis of DF-1 biofilm. A: SybrGreen confocal image (Betsy Pitts, CBE, MSU). B: SEM of the same sample of DF-1 biofilm (ICAL, MSU).

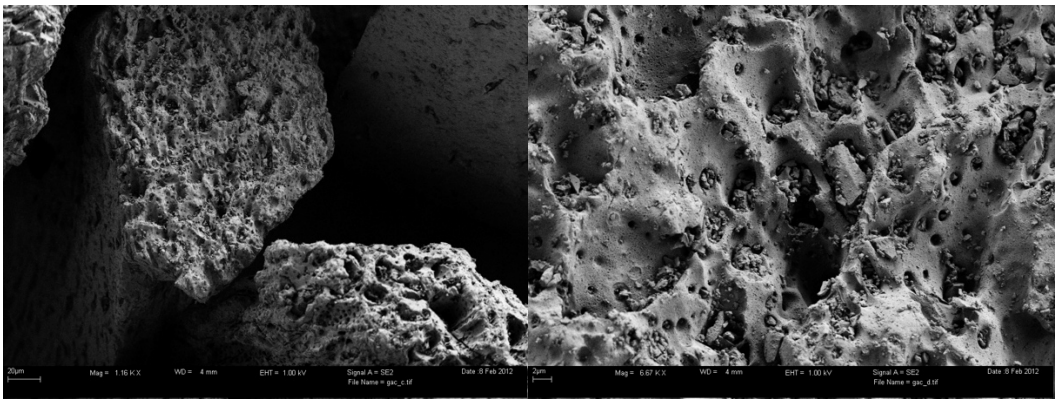


Figure 2.8. SEM images of untreated GAC surfaces that is not exposed to bacteria or culture media (Note: The figures show different magnifications, Left: 20 μm, Right: 2 μm).

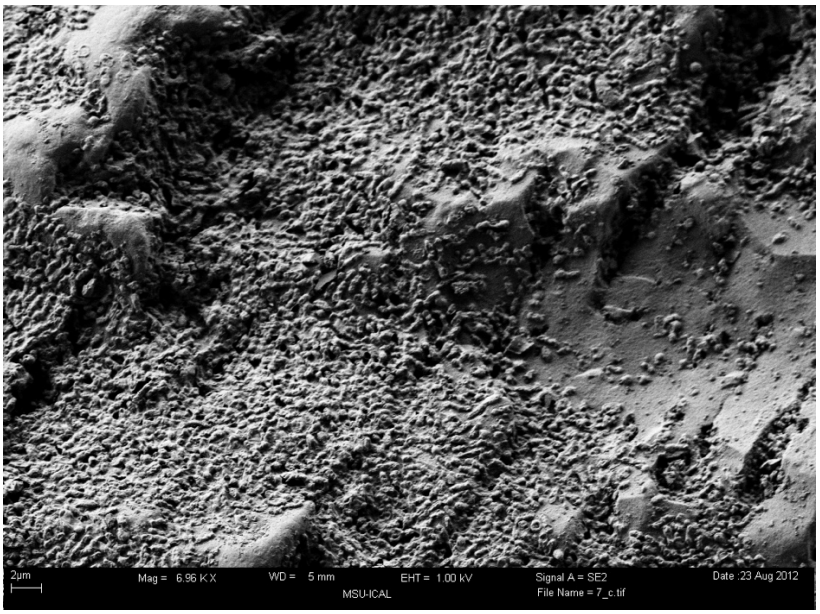


Figure 2.9 Biofilm formation of the aerobic PCB degrader *Paraburkholderia xenovorans* strain LB400 on GAC surfaces observed by SEM.

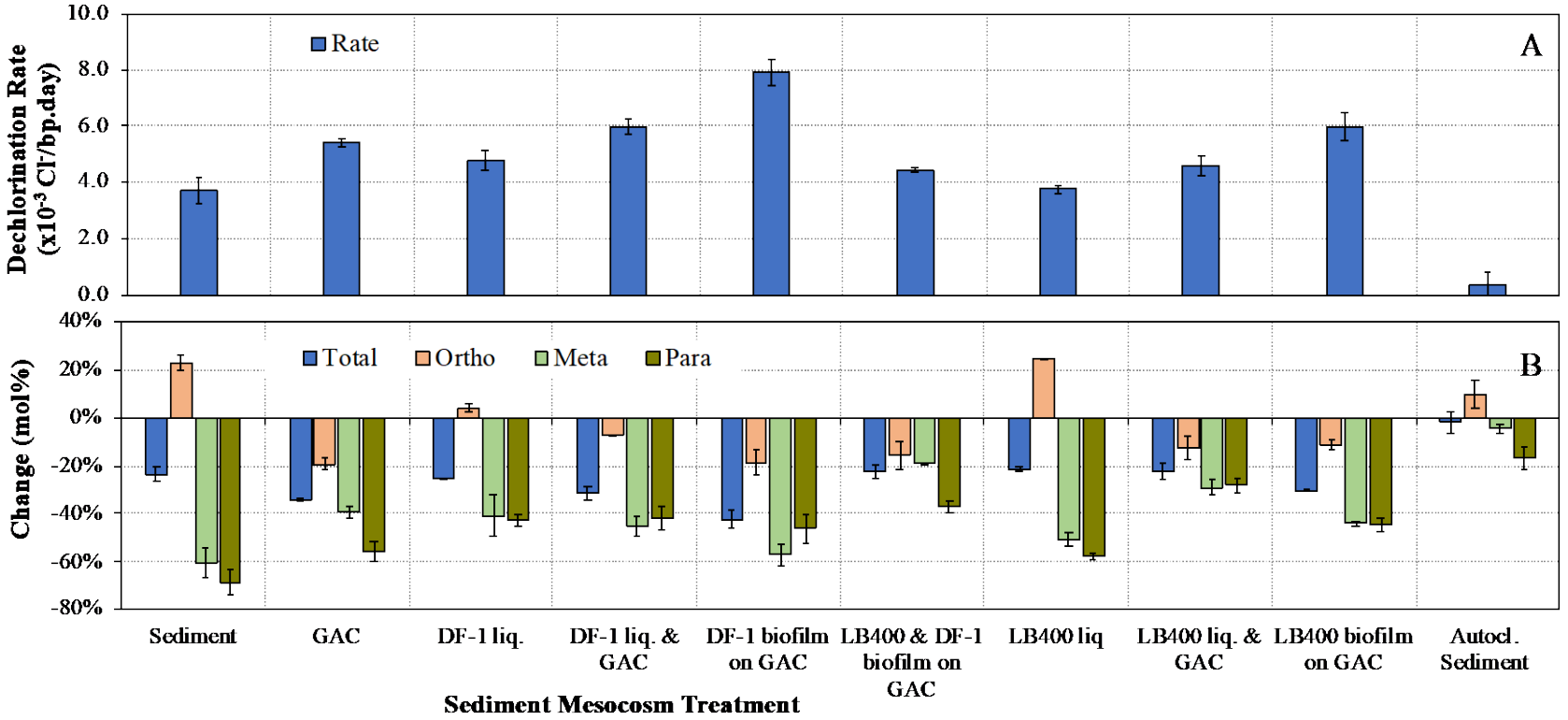


Figure 2.10. PCB dechlorination rates (A) and change in mol%, in other words, PCB dechlorination extend (B) attained during the 200 days of incubation in sediment mesocosms amended with: anaerobic bacterium DF-1 as liquid culture (with and without GAC) or biofilms formed on GAC; aerobic bacterium LB400 as liquid culture (with or without GAC) or as biofilms (with and without DF-1) formed individually on GAC; including in control mesocosms: sediment only, and autoclaved sediment only. All mesocosms were spiked with A1248 at beginning of the experiment (i.e., day 0).

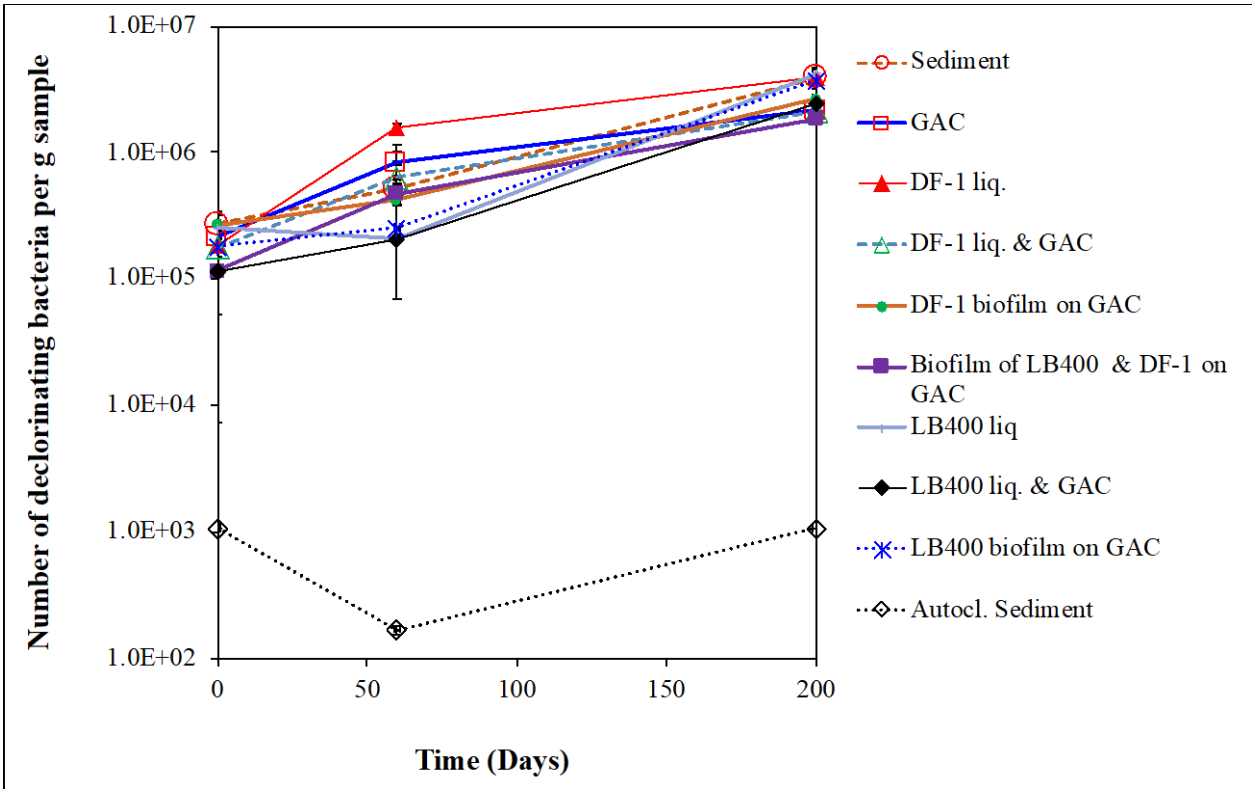


Figure 2.11. Change in the number of dechlorinating bacteria observed over the course of the experiment in each sediment mesocosms with: anaerobic bacterium DF-1 as liquid culture (with and without GAC) or biofilms formed on GAC; aerobic bacterium LB400 as liquid culture (with or without GAC) or as biofilms (with and without DF-1) formed individually on GAC; including in control mesocosms: sediment only, and autoclaved sediment only. All mesocosms were spiked with A1248 at beginning of the experiment (i.e., day 0).

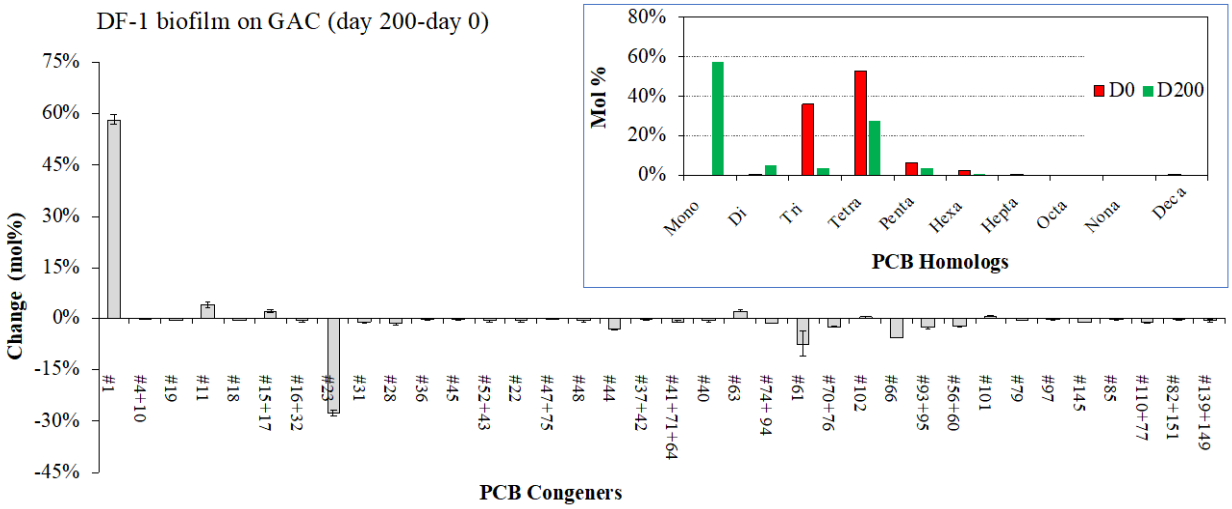


Figure 2.12. Change in PCB congeners (with a change >0.25 mol%) and homologs observed over the course of the experiment (200 days) in sediment mesocosms inoculated with anaerobic DF-1 biofilm covered GAC particles.

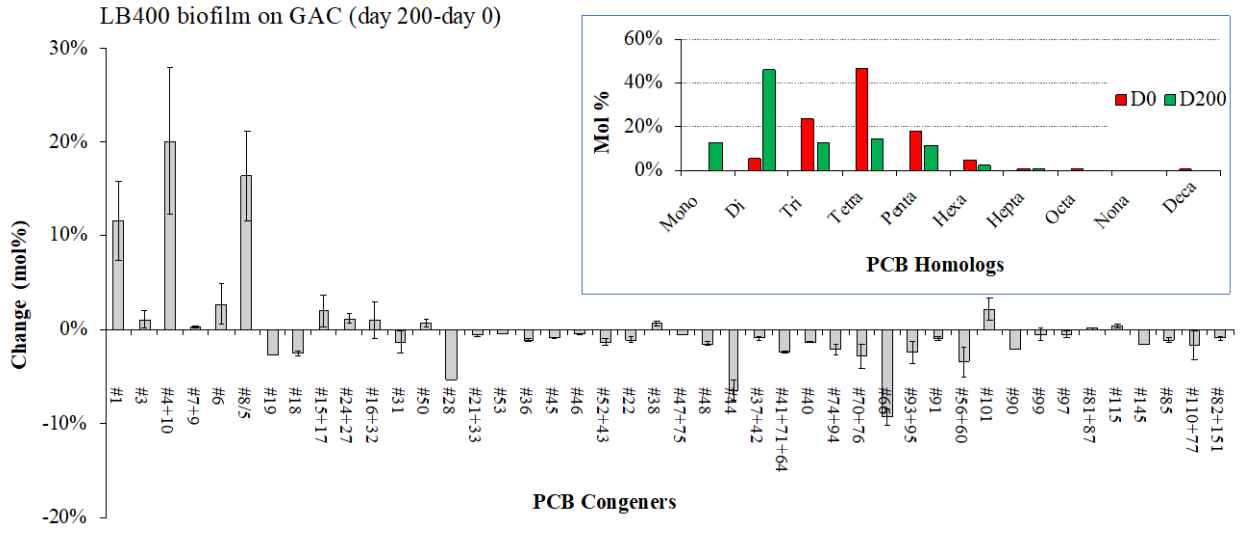


Figure 2.13. Change in PCB congeners (with a change >0.5 mol%) and homologs observed over the course of the experiment (200 days) in sediment mesocosms inoculated with aerobic LB400 biofilm covered GAC particles.

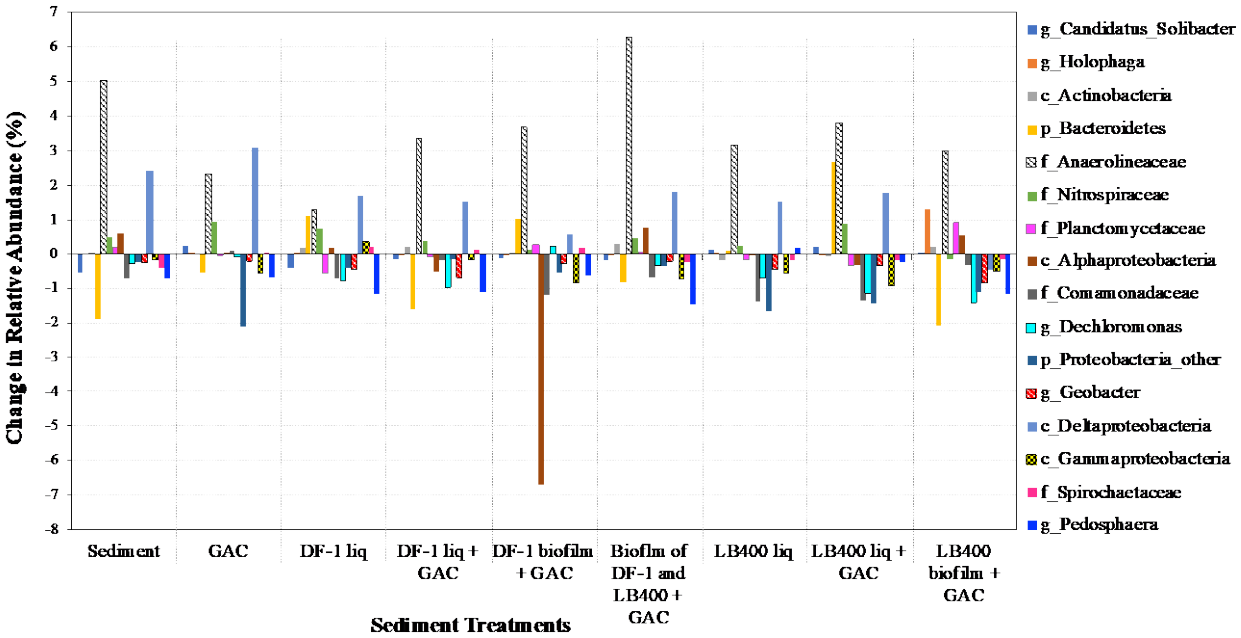


Figure 2.14. Change in major microbial communities present at the end of incubation period in each sediment treatment.

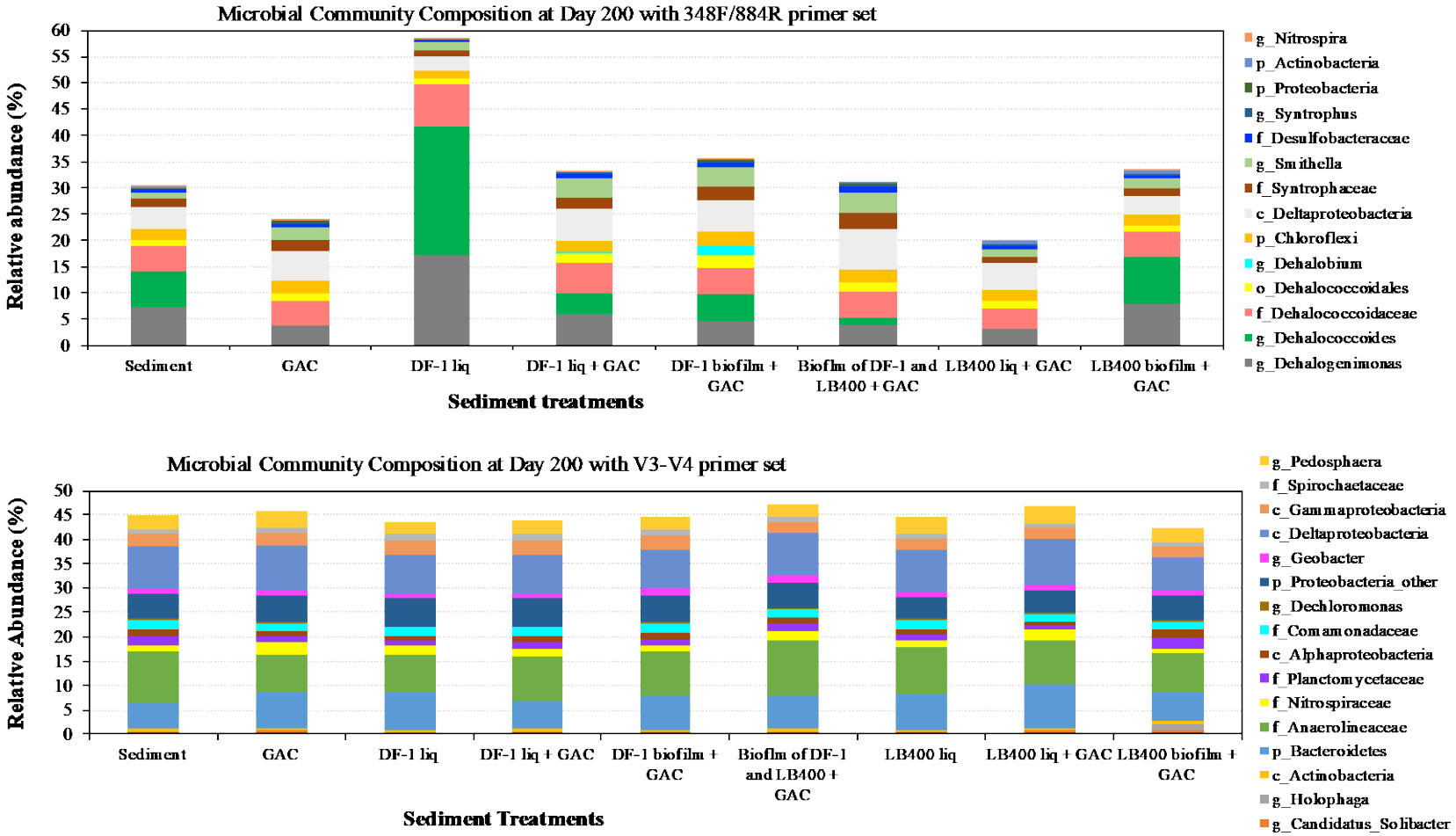


Figure 2.15 Microbial communities present at the end of the treatment in each sediment mesocosms. Please note that LB400 Liq. was sequenced with 348/884.

Supplementary Information

Table 2.S1. PCB dechlorination end-products in each mesocosms after 200 days of incubation.

terminal end product

Name	meso1	meso6	meso5	meso7	meso8	meso16	meso9-17	meso10	meso11	meso14
	D200-D0									
#1	10.4%	60.9%	19.3%	45.6%	58.2%	16.8%				
#4+10	22.0%		5.5%				25.2%	6.2%	22.7%	
#3									1.2%	
#6		3.3%	2.7%			1.2%	2.1%		3.1%	
#7+9						3.3%				
#8+5	3.9%		8.5%	1.1%		3.7%	16.6%	40.6%	19.1%	3.8%
#11			1.9%	3.6%	4.0%	5.6%				
#15+17		1.0%	1.0%		2.1%	2.8%			2.4%	
#16+32									1.5%	1.8%
#19	14.3%						15.6%			
#23						5.4%				
#24+27	1.1%		3.4%				1.5%		1.4%	
#35								2.2%		
#38			1.1%			1.4%				2.0%
#41+71+64										1.3%
#44										4.3%
#50			1.0%							
#56+60										
#63	1.0%	4.6%			2.3%					
#70+76										1.7%
#74+94										1.3%
#81+87								1.6%		
#82+151								1.0%		
#91								1.1%		
#93+95										
#99								1.4%		3.4%
#101			2.7%	1.9%			1.5%	2.9%	2.4%	
#102				1.7%		1.3%				4.3%
139+149		1.2%								

highly dechlorinated PCBs

Name	meso1	meso6	meso5	meso7	meso8	meso16	meso9-17	meso10	meso11	meso14
	D200-D0									
#3	-6.7%	-6.7%		-1.2%			-11.2%			
#4+10		-9.3%								
#8+5		-2.5%								
#15+17	-1.6%									
#16+32		-2.6%								
#18	-2.1%	-2.3%					-2.4%		-2.5%	
#19		-5.8%						-1.1%	-2.7%	
#22							-1.1%		-1.0%	
#23			-13.4%	-18.4%	-27.6%					
#24+27										
#28	-4.7%	-4.9%	-3.3%	-2.4%	-1.5%	-3.1%	-3.4%	-2.9%	-5.4%	
#31	-1.7%	-2.8%			-1.2%	-1.4%	-1.6%	-1.9%	-1.1%	
#36							-1.0%		-1.1%	
#40							-1.1%	-1.1%	-1.3%	
#41+71+64	-1.4%						-1.8%		-2.4%	
#44	-4.3%	-4.2%		-1.0%	-3.1%	-2.6%	-6.0%	-5.3%	-6.2%	
#48		-1.1%					-1.1%	-1.0%	-1.5%	
#52+43	-1.0%	-1.1%					-1.4%	-1.1%	-1.3%	
#56+60	-3.5%	-3.5%			-2.4%	-1.0%	-3.4%	-4.1%	-3.0%	-5.2%
#61			-12.3%	-12.7%	-7.4%	-10.3%				
#66	-7.3%	-6.8%	-7.5%	-6.3%	-5.7%	-7.3%	-9.7%	-11.9%	-9.1%	-12.8%
#70+76	-2.7%	-2.9%	-1.0%	-2.4%	-2.4%	-1.2%	-3.3%	-4.6%	-2.5%	
#74+94	-1.9%	-2.1%			-1.3%		-1.7%	-3.1%	-1.9%	
#79			-1.0%					-2.0%		-1.6%
#85									-1.0%	
#90	-1.3%	-1.3%	-1.3%	-1.1%			-1.9%	-2.6%	-2.2%	
#93+95	-2.6%	-2.3%		-2.6%	-2.6%	-1.7%	-2.6%	-3.3%	-2.1%	-3.8%
#97										
#101										-3.0%
#106								-1.2%		
#110+77	-1.7%	-2.1%	-2.6%	-1.1%	-1.2%	-2.1%	-1.6%	-1.7%	-1.3%	-3.2%
#139+149								-1.2%		
#145						-1.0%	-1.3%	-2.0%	-1.6%	

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Chapter 3.
Characterization of *Paraburkholderia xenovorans* strain LB400 biofilm development on various sorptive materials for potential aerobic biodegradation of organohalide contaminants.

Authors
Ana Lucia Prieto, Staci L. Capozzi, Birthe V. Kjellerup

The objective of this study was to assess the importance of the material characteristics evaluated based on a microbiological approach. Specific objectives are:

1. Characterize of materials to be tested as LB400 biofilm substratum.
2. Assess LB400 growth on sorptive materials via plate count and qPCR.
3. Evaluate microscopy techniques to quantitatively monitor biofilm formation.

Highlights
- LB400 biofilms developed on each of the tested sorptive materials.
- LB400 biofilms did not display a preference for growth on any particular sorptive carrier material.
- Advanced microscopy tools were developed and evaluated using various fluorescent dyes.

Background
Paraburkholderia xenovorans strain LB400 (LB400) are aerobic bacteria capable of degrading biphenyl as well as lesser-chlorinated polychlorinated biphenyls (PCBs). The initial study was performed using LB400, as the aerobic bacteria grow more quickly than anaerobic dechlorinating bacterium DF-1. The study focused on growing LB400 biofilms on various solid surfaces and enumerating the attached cells on each material considered via two different enumeration methods, namely plate count and quantitative polymerase chain reaction (qPCR). The goal was to determine the fundamental characteristics of different materials and to assess the growth of LB400 on these materials. Evaluating the growth on different materials promoted a better understanding of the mechanism of attachment on the surfaces and interactions between the biofilm and carrier materials that support growth and thus degradation of biphenyl and lightly chlorinated PCBs (i.e., those PCB congeners with 1 or 2 chlorines). From previous work, it has been observed that different types of activated carbon could potentially interact with organisms in detrimental ways causing inhibitory effects after long exposure (Jonker et al., 2009; Hale et al., 2013). Therefore, an array of materials was tested for the potential to forming biofilms. The methods for quantification of biofilm included the following parameters: numbers of biofilm bacteria (plating and quantitative PCR) and surface coverage. The objectives of the study were to evaluate the potential for LB400 growth on various carbonaceous materials using plate count and qPCR techniques and assess biofilm development using microscopy techniques.

Materials and Methods

Materials

Sorptive carrier materials were selected due to their differences in electrical conductivity, surface area, and adsorption/desorption isotherms and were purchased from commercial distributors. Materials include various types of activated carbon and biochar. In addition, we obtained information regarding important parameters that enabled us to evaluate the mechanism mentioned in the proposal.

A comprehensive list of materials under consideration are provided below:

- Coconut AC OLC ww 20x50
- Calgon BL (PAC)
- Calgon OLC ww 20x50
- Calgon TOG LF 80x50
- Coal AC TOG LF 80x325
- Coal AC TOG LF 80x325
- Acai pit biochar 18x50
- Bone biochar
- Hardwood activated biochar
- Peanut hull biochar 18x50
- Sand 20x50
- Clean XAD
- Graphite foil 0.254 mm
- Graphite foil 0.13 mm
- Graphite powder

A comprehensive list of parameters under consideration are provided below:

- Surface Area (total and biologically relevant)
- Electrical Conductivity
- Sorption Capacity
- Porosity
- Porosity and Pore Volume

Growth conditions

A 1.5M stock solution of biphenyl in acetone was prepared in a glass flask and let dry until the acetone was completely evaporated. A total of 30 mL of 5 mM of M-9 minimal salt solution was used as the media for growing LB400 and was transferred to the glass flask along with 1 mL LB400 culture. The flask was incubated at 30°C for 3 days to allow the bacteria to grow.

Triplicates of the batch culture and each material were prepared and sampled every 1 to 3 days. Solid and liquid portions of the triplicates were taken out of each flask and transferred into microcentrifuge tubes. The liquid was decanted and the solid material was weighed. One of the tubes was transferred to the -20 °C freezer awaiting qPCR analysis whereas M9 solution was added to the other tube. The tube was then vortexed and sonicated for 5 minutes to resuspend the cells attached to the solid material. The liquid was transferred to another microcentrifuge tube and the solid material was discarded. The sonicated liquid was used to make dilutions for the

plate counts and the remainder of the liquid was transferred to the -20 °C freezer awaiting quantitation.

Plate count

Serum bottles containing a suspension of 300 mL of M9 media and 300 mg of each of the materials were tested. Biphenyl was used as carbon source (5 mM) dissolved in acetone. One mL of LB400 was used as seed culture. The cell concentration of the seed stock solution was approximately 10^5 cells per milliliter. Each material was tested in triplicates. Samples were taken daily during the first part of the experiment (exponential growth) and then intermittently (every 1 or 2 days) until reaching stationary phase (plateau). Samples of materials (solids) and liquids were collected and analyzed at each time point. The solids were sonicated for 5 minutes in sterile buffer to resuspend the attached cells. The sonicated supernatant was stored at -20˚C for further analysis. Biofilm characterization techniques included DNA extraction, and quantitative polymerase chain reaction (qPCR) with specific 16S rDNA primers. Active biomass was measured using a plating technique for Colony Forming Units (CFU).

A total of five dilutions of a broth culture of LB400 were prepared using serial dilutions ranging from 10^{-1} to 10^{-5} dilution factors. Broth solutions were then inoculated with the dilutions from 10^{-1} to 10^{-5} by spreading 100 µL of each dilution tube using aseptic technique. The plates were incubated at 30ºC for 2 days and examined for cultures containing well-separated colonies.

DNA extraction and q-PCR of LB400

DNA was extracted from biofilm samples using InstaGene Kit (QIAGEN, Hilden, Germany). A primer set specific for LB400 *bphA* (CIOP0/CIOP1) was used to monitor LB400 by qPCR (Payne et al., 2013). A seven order-of-magnitude dilution series of plasmid DNA containing the 16S rRNA gene from LB400 was used as a standard for quantification. Both quantitation were run in triplicate on a single 96 well PCR plate. Total reaction volume was 25 µL, which included 10 µL of template, 12.5 µL SYBR Green mix (Bio-Rad Laboratories, Hercules, CA, USA), 2 µM each of the appropriate forward and reverse primers, and 0.5 µL of nuclease-free water.

Scanning Electron Microscopy

Micrographs were taken using a Scanning Electron Microscope (SEM; Supra 55VP, Zeiss, Thornwood, NY) in the Image and Chemical Analysis Laboratory at Montana State University. The samples were prepared for SEM imaging by transferring the particle materials onto glass slides, where they were dried and mounted on the SEM sample holder using a double-sided carbon tape. The loose particles on the carbon tape were removed with a gentle stream of high purity nitrogen before loading the samples in the SEM chamber. Samples were imaged at different magnifications with a primary electron beam energy of 1 keV.

Confocal laser scanning microscopy

Methods were developed and tested for staining, imaging and quantitative image analysis using a Confocal Scanning Laser Microscope (CLSM) for several types of materials. CLSM analysis was performed in collaboration with the Center for Biofilm Engineering at Montana State University. A Leica TCS SP5 II confocal laser scanning microscope (Leica Microsystems, Model SP5 SMD) equipped with 63x long distance objective was used to image the abiotic and biotic materials. CLSM is based on collecting stacks of in-focus images through a thick sample

which are then put together in software to construct a 3-dimensional image, which can be rotated in space or looked at on the side. CLSM 3-D images were compiled via Imaris x 64 software (version 8.4.0, Figure 1). Biofilm samples were placed in a petri-dish with sterile filtered water after they had been stained with fluorescent stains (30 minutes, in the dark). Images of fluorescent- stained biofilms were obtained by using a different fluorescent filters, while reflection of the material using reflected light. Images of fluorescent stained microbial biofilms and reflection of the materials were overlaid in the Imaris software (Bitplane Scientific Software, South Windsor, CT). Images were analyzed using Imaris software and the area of biofilm coverage (i.e., biosurface area) was calculated.

Results

Colonization of LB400 biofilm on sorptive materials.

Several methods were developed for characterization and quantification of biofilm growth. Batch experiments were carried out using LB400 to develop biofilm on different materials or attachment surfaces. Materials such as granular activated carbon (GAC) were selected for their high surface area and sorptive affinity to organic chlorinated compounds. In addition to GAC, this study also explored other materials that might present an alternative for improved biofilm growth. A variety of biofilm carrier materials were tested during these experiments (Table 1). Carrier materials range in physical and chemical properties such as surface area and chemistry.

Table 3.1 Overview of material characteristics that have been evaluated for LB400 biofilm formation.

Material	**Notes**	**Manufacturer**	**%Carbon**	**Surface Area** (m^2/g)	**Skeletal Density** (g/cm^3)	**Bulk Density** (g/cm^3)	**Average Pore Width** (nm)	**Pore Size** (A)
Acai Pit Biochar	none	Biochar Eng Corp	772	198	1.23	0.65	1.43328	NA
Bone Biochar	Bovine origin	Bonechar Carvao Ativado Do Brasil Ltd.	11	200	NA	0.65	NA	18406
Pine Dust Biochar (hardwood)	none	Biochar Eng Corporation	22.1	109	0.98	0.43	1.56177	NA
Peanut Hull Biochar	none	Biochar Eng Corporation	31.9	107	1.28	0.6	1.55981	NA
Granular Activated Carbon, OLC 20x50	Coconut based	Calgon	90.8	1305	1.45	0.57	1.45183	NA
Granular Activated Carbon, TOG LF 80x325	Bitumino us Coal Based	Calgon	80.9	1116	1.61	0.64	1.47923	NA
Powder Activated Carbon	Bitumino us Coal Based	Calgon	NA	NA	NA	NA	NA	NA
Graphite powder	<50um particle size	Alfa Aestar	100	Natural (5-7), Synthetic (10-20)	NA	NA	NA	NA
Graphite foil	none	Alfa Aestar	100	90	0.3-1	1.12	NA	40
Sand	General purpose, SiO_2	Quickrete	NA	0.0485	NA	2.5-2.8	NA	NA
POM	none	NA	NA	107	NA	NA	NA	NA

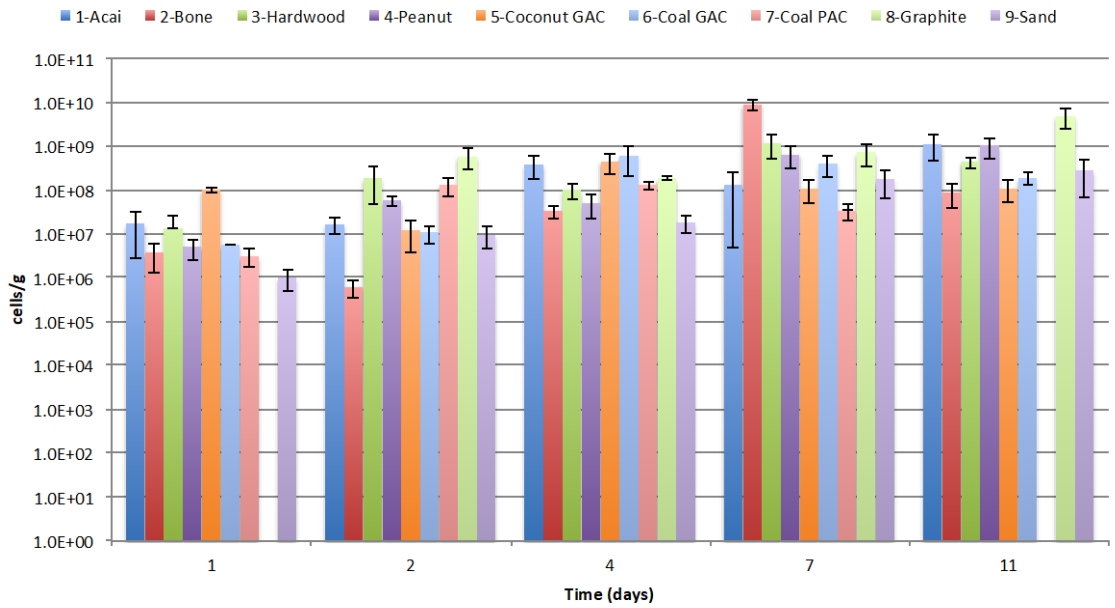

Figure 3.1 Biofilm formation of LB400 biofilms on materials after 11 days. qPCR results reporting the number of cells per gram of sample of material.

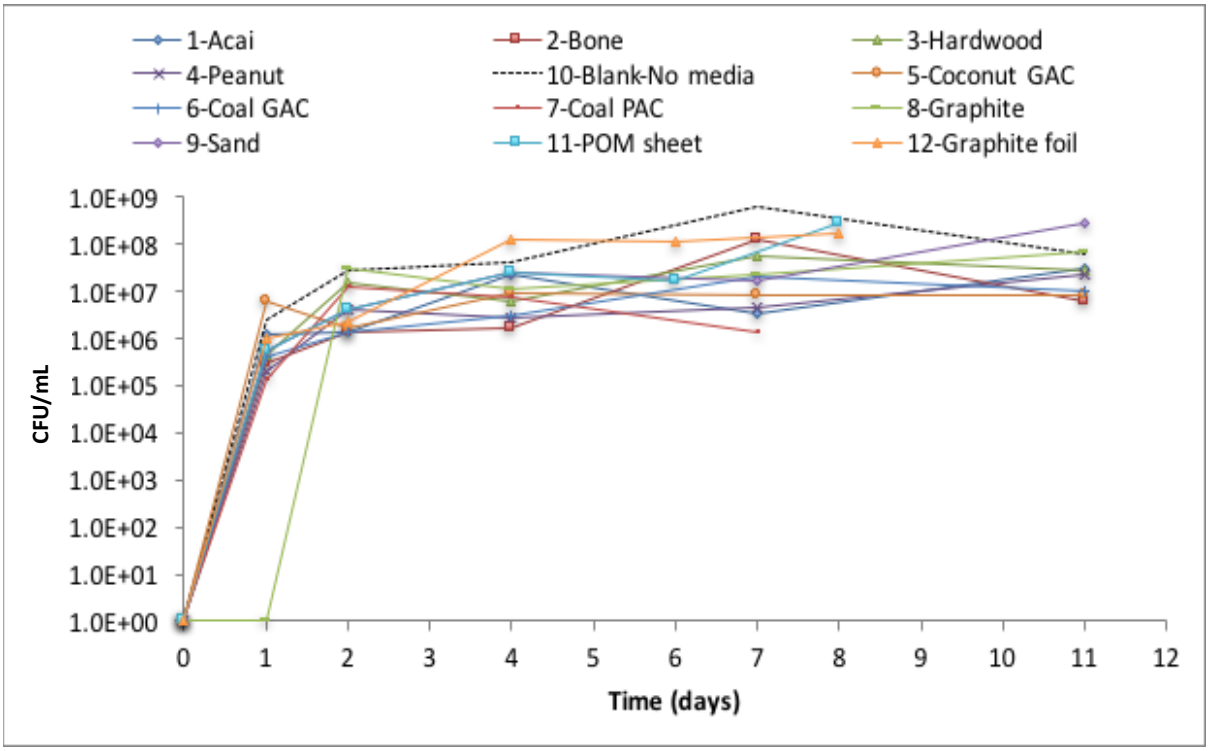

Figure 3.2 Plate counting results reporting the number of LB400 cells per gram of material.

Figures 1 and 2 summarize the biofilm development on different materials over an experimental time of 11 days. No adverse effects in biofilm growth were observed by using any of the tested materials, which was in contrast to some of the anecdotal information. For all materials, an

increasing trend of biofilm mass (reported as cells/g of material) could be observed with increasing exposure time. Significant differences of biofilm growth were observed among the tested materials. Sand, Acai and GAC appeared to yield a larger and steady number of attached bacterial cells over the experimental 11 day observation period. Acai has a high carbon content, which may have implications for higher sorptive characteristics in comparison to other explored materials. Also, GAC has a large surface area (1,116 m^2/g) in comparison to the other examined materials which provides more area for cells to attach. Sand and bone biochar may provide a smooth and even surface for cell attachment allowing for less growth compared to a rough surface. Finally, it should be noted that there was a very short lag phase for all the materials tested. The stationary phase (i.e. the level at which the biofilm mass levels off) was also similar for each material thus showing that it was the environmental growth conditions such as electron donor and acceptors that determined the biofilm growth during aerobic conditions and not the material surface characteristics. None of the materials appeared to inhibit biofilm formation.

Evaluation of microscopy techniques

Microscopy techniques were developed and tested thus allowing for both qualitative and quantitative analysis of biofilm formation over time. Scanning electron microscopy and confocal laser scanning microscopy were performed on samples of LB400 attached to several types of materials including GAC, bone biochar, sand, graphite sheet and polyoxymethylene (POM).

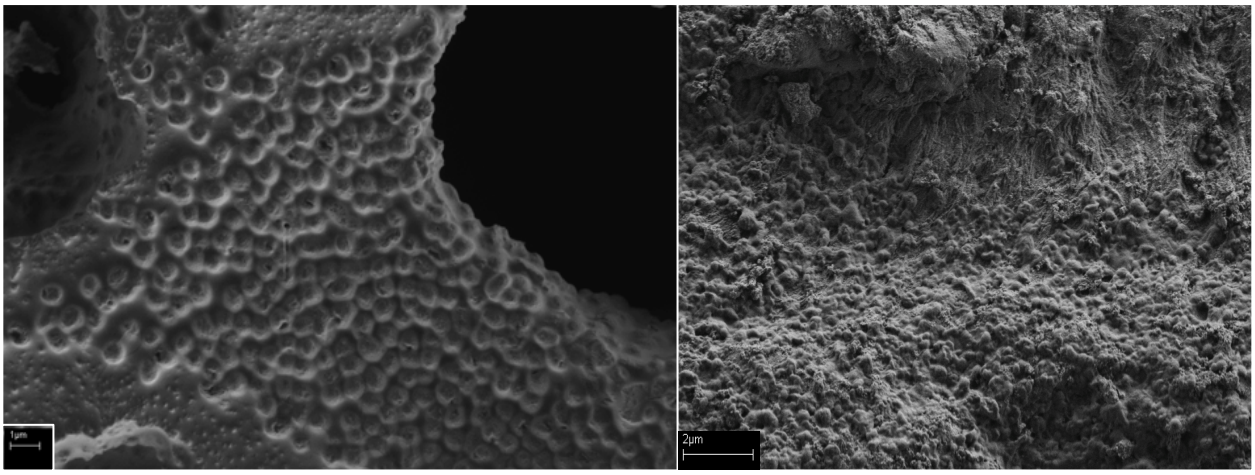

Figure 3.3 Scanning Electron Microscopy image showing LB400 formed biofilm on Bone Biochar (left) and Acai Biochar (right) (Note: The figures show different magnifications, Left: 1 µm, Right: 2 µm).

Scanning electron microscopy

Assessment of the biofilm formation with high resolution microscopy was performed using Scanning Electron Microscopy (SEM). The SEM imaging analysis was performed at Montana State University's Image and Chemical Analysis Laboratory (ICAL). SEM images were obtained in order to evaluate the biofilm formation on the surfaces of test carrier materials. Images of the LB400 biofilms attached to the surface of activated carbon is shown in Figure 3. The SEM signals that are derived from electron-sample interactions reveal information regarding external morphology, chemical composition, crystalline structure and orientation of the materials making

up the sample. LB400 bacterial cells adhered to the surface and formed robust and expansive biofilms on each carrier material tested.

Confocal laser scanning microscopy
CLSM of *in situ* biofilm samples was applied to evaluate the formation and structure of the biofilm on the surface of the materials tested. The bacteria were labeled with a fluorescent probe targeting dechlorinating bacteria and they were subsequently visualized allowing both qualitative and quantitative analyses. Methods were developed for staining, imaging and quantitative image analysis for several types of materials. Using CSLM, the surface of the adsorbent was imaged with reflection scanning, while the attached community is imaged with fluorescence. The two stacks of images were overlaid, and reconstructed digitally to develop a 3-dimensional image of the particle surface and attached microbial community (Figure 4).

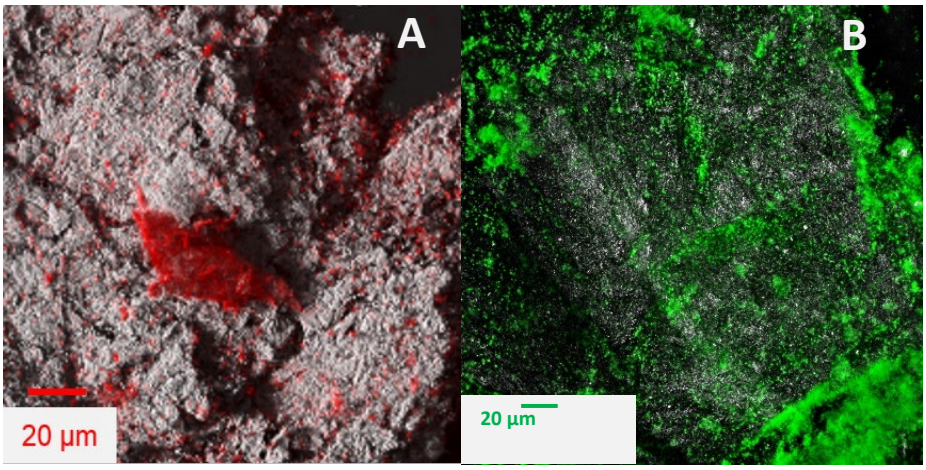

Figure 3.4 Biofilm of LB400 formed on coal GAC and stained with wheat germ agglutinin (WGA) [CLSM: Fluorescence/reflection].

Fluorescence was generated by applying non-specific nucleic acid dyes allowing for identification of bacteria and spatial relationships in the biofilm. The LB400 biofilms consisting of both bacterial cells as well and the surrounding matrix material called Extracellular Polymeric Substances (EPS) were also evaluated using several specific and general EPS stains (Table 2). The purpose was to develop staining methods that could detect functional groups present in the biofilm since this might impact the adsorption and growth of cells on the activated carbon particles. This method was used to monitor biofilm formation and determine biofilm coverage based on area/volume ratios for quality assessment of the inoculum during the experiments (Table 3). Assessment of the changes that occurred in the PCB- biodegrading biofilm populations were rapidly assessed using this comprehensive approach.

Table 3.2 Extracellular polymeric matrix stains tested on biofilms of aerobic PCB degrader LB400 on bone biochar and granular activated carbon (GAC). Qualitative results of coverage: ++ Complete; +: partial; -: none.

Stain	Target	Materials tested		Reference
		Bone biochar	GAC	
Calcoflor white	Polysaccharides (cellulose, chitin), matrix	-	-	(Neu et al., 2002)
C2-dichlorotriazine (C2D)	alcohols, polysaccharides, and amines	+	+	(Schultz et al., 2011)
m-dansylaminophenylboronic acid (D2281)	Alcohols, carboxylic acids, matrix	-	NA	(Pitts, 2015)
FM1-43	Cellular membrane dye, lipid	+	-	(Chimileski et al., 2014; Tremblay et al., 2015)
WGA lectin	Wheat germ agglutinin, lectin, matrix, cell associated	++	++	(Strathmann et al., 2002)
Sypro Ruby	Proteins, matrix	-	NA	(Baird et al., 2012)
Cell Mask Orange (CMO)	Cellular membrane dye	++	+	(Chimileski et al., 2014)
Bodipy	Lipids, membranes	-	++	(Doroshenko et al., 2014)
Nano-orange	Proteins	-	NA	(Larsen et al., 2008; Stiefel et al., 2016)
SybrGreen I	Nucleic acid	+	+	(Cerca et al., 2011)

Table 3.4 Quantification of aerobic PCB degrader LB400 biofilm area coverage (Confocal imaging and Imaris image analysis).

Material	Area coverage (%)		
	Day 1	Day 2	Day 5
Bone Biochar	0.29 ± 0.19	0.14 ± 0.09	0.70 ± 0.02
GAC	0.02 ± 0.02	0.73 ± 0.42	0.92 ± 0.98
Sand	0.08 ± 0.11	0.06 ± NA	NA
POM	0.002 ± NA	0.05 ± 0.03	NA
Graphite sheet	0.010 ± NA	0.02 ± NA	NA

Summary and Environmental Implications

Experimental results demonstrate the same level of biofilm formation and growth on each carrier material tested. These results suggest that (1) LB400 biofilm growth was determined by environmental conditions and rather than surface conditions in the aerobic system and (2) none of the tested materials were inhibitory to LB400 biofilm formation. In addition, EPS was stained and detected in the experiments using a variety of fluorescent dyes. CLSM methods were established for staining, imaging and quantitative image analysis for several types of materials (GAC, bone biochar, sand, graphite sheet and POM) in this work. Results from this study revealed a pool of sorptive materials that were used for further development of the anaerobic biofilm system. This two-phased approach is expected provide an efficient and cost-effective method for delivering microorganisms for bioaugmentation of PCB contaminated sites thus enabling complete onsite bioremediation.

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Chapter 4.
***Dehalobium chlorocoercia* DF1 maintenance, scale up and optimization**

Authors
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Objectives
The objective of this study was to test a combination of growth conditions, bacterial strains and sorptive materials to find efficient solutions for biofilm development to apply in the mesocosm experiments.

Highlights
- *Dehalobium chlorocoercia* DF1 (DF1) was scaled up, grown and maintained using tandem 20 L bioreactors.
- Tasks for scale-up method development strategies were overcome by events (OBE) due to early termination of funding.

Optimization and test of biofilm inoculum in microcosms.
A). Cultures for bacterial inoculum
We used cultures of *Dehalobium chlorocoercia* DF1 (DF1) and *Paraburkholderia xenovorans* strain LB400 (LB400) for formation of biofilm inoculum. The PCB dechlorinating bacterium DF1 was obtained from Charleston Harbor, SC and was cultured in co-culture with *Desulfovibrio* sp., since DF1 can be cultured in high numbers utilizing PCBs or other chlorinated compounds as electron acceptor. When PCBs are applied DF1 performs double flanked *meta/para* dechlorination (May et al., 2008). LB400 was cultured aerobically together with biphenyl or mono-chlorinated biphenyls that have been shown to provide efficient as electron donors (oxygen is electron acceptor). The listed bacterial cultures (Table 1) were used in this work.

B). Growth conditions
The microcosms optimization was performed in 10 or 20 ml culture tubes by using mineral medium (Sowers and Noll, 1995) with and PCE or PCB-61 as electron acceptor for DF1, biphenyl or mono-chlorinated biphenyls for LB400 (Payne et al., 2013). PCE was used as electron acceptor, since PCE and the degradation product TCA both are volatile compounds that can be flushed out prior to release of the inoculum for bioaugmentation. For the aerobic biofilm development, the solid substrate was loaded up with biphenyl and then inoculated with LB400 and cultured in an aerobic bio-slurry reactor to develop the biofilm. Biphenyl has previously been applied as a co-substrate to develop a biofilm on carbon surfaces that could degrade PCBs.

Table 4.1. Bacterial cultures and PCB degradation activities that were used for biofilm growth on AC and other sorptive materials.

Designation	Condition	PCB degradation activities	Culture status
Dehalobium chlorocoercia DF1	Anaerobic	Double flanked *meta/para* dechlorination	Isolate & co-culture
Desulfovibrio sp.	Anaerobic	Support for DF1, biocorrosive property for Fe-impregnated AC	Isolate
Paraburkholderia xenovorans strain LB400	Aerobic	Cleavage of the biphenyl ring → Mineralization	Isolate

Scale up of cultures

The goal of this task was to scale up growth of DF1 and LB400 for kinetic and biofilm microcosm studies and to provide large-scale growth of DF1 and LB400 for later mesocosm studies. In 2016 we (Sowers group) discovered that our stock cultures of *Dehalobium chlorocoercia* DF1 (DF1) would not recover when transferred. Nine months were required to resolved the issue and fully recover the culture. In the interim, methodologies continued to be developed using a surrogate PCB dechlorinating culture, strain WBC-1, until DF1 was recovered. We used multiple approaches to revive DF1. We tested a variety of medium amendments to revive growth. These included activated carbon, vitamin B12 +/- 5,6-dimethylbenzimidazole, sediment, extracts from older re-isolated *Desulfovibrio*, and concentrated *Desulfovibrio* extract, which are all reported to stimulate growth of halorespiring Chloroflexi similar to DF1.

We also transferred a number of older cultures stored in the lab, to address the possibility that something killed off our lab stocks (toxins in dishwashing soap, building water system or other external factors beyond our control). Active DF1 cultures were revived by transferring very old DF1 culture stocks (> 5-year-old DF1 cultures) into new media with PCB-61 (first transfer, Figure 1 below) and incubating for about 150 days. Dechlorination of the 1st transfer culture reached about 50% after about 150 days. This 1st transfer DF1 culture was serially transferred to fresh media with PCB61. As expected, DF1 growth rate and extent improved with subsequent transfers of the initial culture (2nd transfer and 4th transfer, Figure 1 below). Specifically, the 4th transfer culture reached about 63% dechlorination after only 23 days, consistent with previous growth rates of DF1.

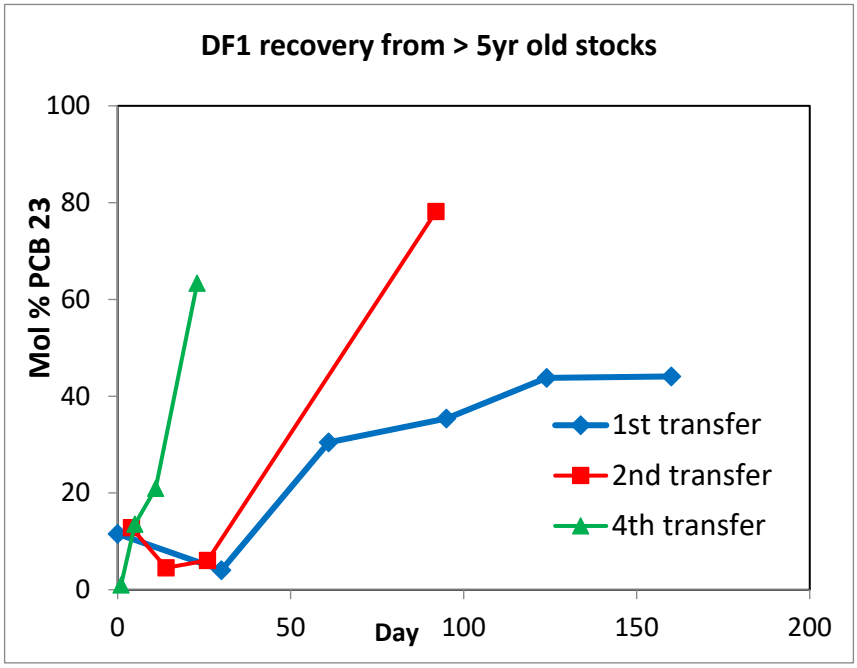

Figure 4.1 Recovery of DF1 by sequential transfer of stock cultures.

After recovery, DF1 was transferred to a 20 L fermenter with a 15 L working volume (BioFlo IV, New Brunswick Scientific) on minimal estuarine media (ECl) with 10mM Sodium Formate as the electron donor and carbon source and 200 µM tetrachloroethylene (PCE, Fisher Scientific) as the electron acceptor (Figure 2). Temperature was maintained at 30C. The headspace was N_2: CO2 (80:20). DF1 activity was monitored by dechlorination of PCE to trichloroethylene (TCE) along with trace amounts of cis- and trans-dichloroethylene (cDCE and tDCE) by GC-FID analysis of a 100 µl subsample of the headspace. Dechlorination activity was plotted as relative mole percent of the sum of the dechlorination products [(mol TCE + mol cDCE + mol tDCE)/mol PCE + mol TCE + mol cDCE + mol tDCE)]*100. When about 90% of PCE was dechlorinated to TCE+cDCE+tDCE, the vessel was sparged with N_2:CO_2 (80:20) for 20 min to prevent a build-up of toxic chloroethenes and then neat PCE was added to reach a final concentration of 200µM. Sodium formate as the electron donor was added in excess of PCE (electron donor) and was replenished periodically. DF1 growth was monitored by quantitative PCR (qPCR) of the 16S rRNA gene (using primers 348F/884R), or of a putative reductive dehalogenase (using primers SKFPat8F/SKFPat8R) and plotted as cells per ml.

The fermentor culture of DF1 rapidly dechlorinated PCE to TCE and DCEs (Figure 2). DF1 cell density of about 10^7 cells ml^{-1} was reached in 90 days, which was typical for growth of DF1. We confirmed the authenticity of the culture by comparing growth rates and dehalogenation products from PCE, PCB61 and Aroclor 1260. Thereafter we maintained two 20 L bioreactors (16 L working volume) in tandem, which provided a continuous supply of cell material for the project (Figure 3).

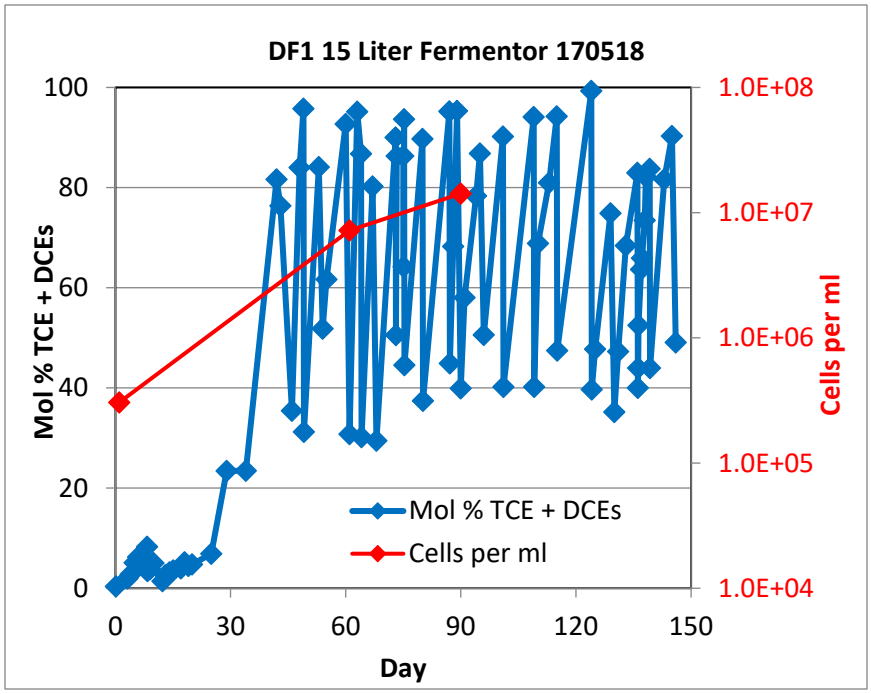

Figure 4.2. Scale up of DF1 in 20L bioreactor after recovery of the

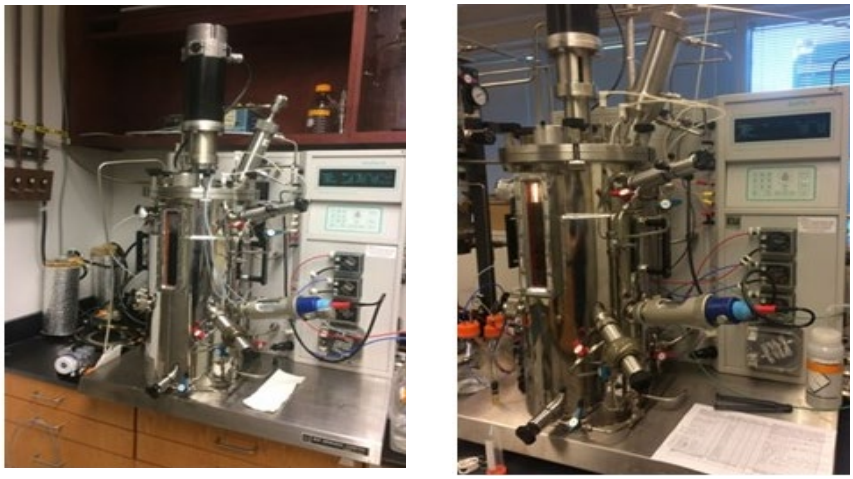

Figure 4.3 Tandem 20L bioreactors used to grow and maintain DF1

Optimizing bioreactor yields

DF1 cell density of about 8×10^{7} cells ml^{-1} was reached after 150 days in both bioreactors. Although 1×10^{8} cells ml^{-1} is normally the maximum density observed, we tested a number of approaches in an effort to increase the yield. Electron donor (formate) and electron acceptor (PCE) were ruled out as the limiting factors by repeated addition of each (data not shown). As DF1 is a co-culture with a symbiotic *Desulfovbirio* species, we tested if the *Desulfovibrio* species in the co-culture was the limiting component. The addition of 2 mM sodium sulfate stimulated the growth of the *Desulfovibrio* species but did not stimulate the growth of DF1, indicating that

the *Desulfovibrio* was not the limiting factor (data not shown). The addition of 2x Wolin's vitamins and 2x Wolfe's minerals did not stimulate the growth of DF1 (data not shown) indicating neither vitamins nor minerals were the limiting factor for growth of DF1.

Another potentially inhibiting factor is that PCE is relatively insoluble in water (Log K_{ow} = 3.40) and was often observed to accumulate on the bottom of the vessel after several sequential sparge-replenishment cycles. The other issue is that DF1 is not motile. In prior bench-scale studies, we discovered that even low rate of agitation inhibited growth of the culture. To address this limitation, we attempted to exchange PCE from the headspace at the top of the vessel to the bottom to promote mild convective mixing of the DF1 culture. Headspace containing PCE was continuously pumped (anaerobically) using a wet/dry aquarium air pump (Pentair Aquatics) from the top of the vessel into the bottom of the vessel and dispersed into the media through a fine aquarium air stone (ca. 0.1 L per minute). We observed that DF1 grew under these conditions, although recirculation of headspace containing PCE into the media had no noticeable effect on DF1 growth or dechlorination (Figure 4).

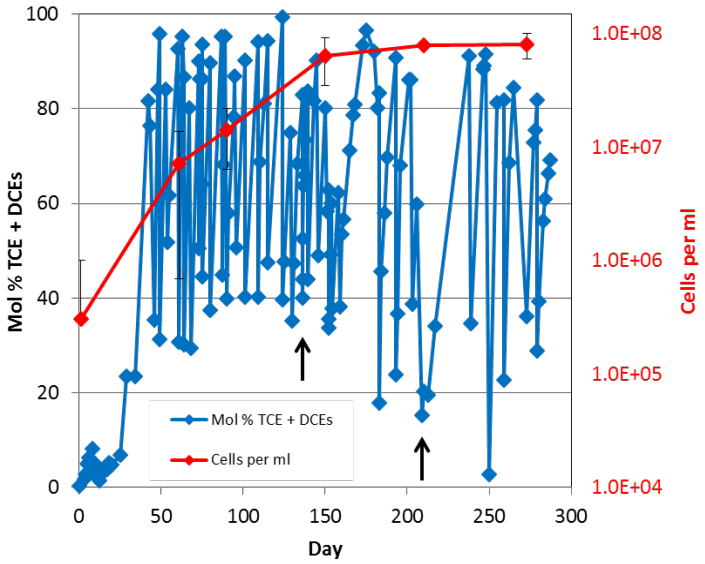

Figure 4.4 Slow recirculation of gaseous phase (ca. 0.1 L/min) was started at Day 136 (*arrow*) and maintained for another 74 days (*arrow*). Cell numbers level off at about $8x10^7$ cells per ml.

Initial attempts to grow DF1 in the fermenter with constant slow agitation (slowest setting) starting at time 0 days (50 rpm) were unsuccessful. We inferred that DF1 was unable to grow under continuous gentle agitation. Since growth under constant agitation was unsuccessful, we then tried constant slow recirculation of media. To test this, media was slowly recirculated from the bottom to the top of the 20 L vessel (hydraulic retention time of 24 hours) using a peristaltic pump (Buchler Instruments). Although DF1 grew under these conditions, recirculation of liquid media had no inhibitory effect on total cell numbers or dechlorination rate (Figure 5).

DF1 Fermentor. Liquid Recirculation Experiment

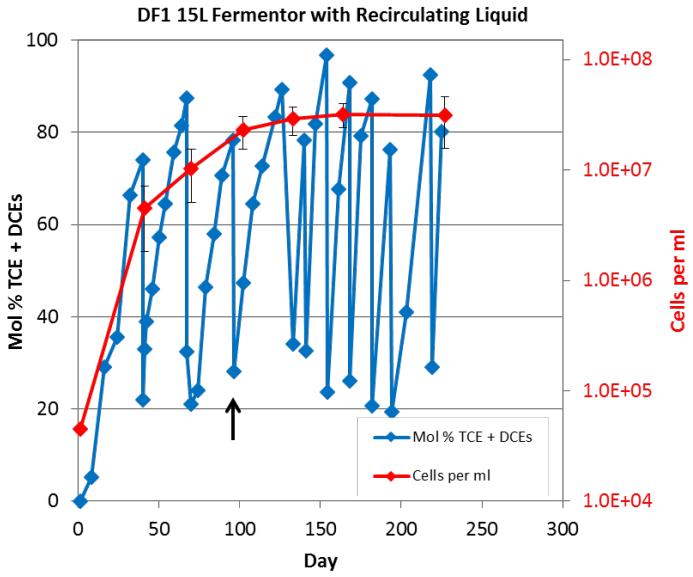

Figure 4.5 Slow liquid recirculation (hydraulic retention time of 24 hours) was started at Day 102 (*black arrow*). Cell numbers maintain at about $3x10^7$ for another 120 days.

We observed that chloroethenes were still accumulating in the bottom of the bioreactor even with recirculation. Because of the configuration of the stainless steel bioreactors with both the impeller and the gassing ring approximately 2 cm above the concave bottom the Dense Non-Aqueous Phase Liquid (DNAPL) layer was not being mixed during the recirculation process. We hypothesized that periodic stirring, rather than continuous stirring, would be effective in mixing DF1 with insoluble PCE. After DF1 reached its maximum growth yield ($4.5x10^7$ cells per ml) and maintained for 85 days, the culture was mixed at ca. 100 rpm once a day for the next 57 days. Unfortunately, under conditions of daily periodic mixing for 1 hour, DF1 cells lost 50% viability (from $4.5x10^7$ cells per ml to $2.5x10^7$ cells per ml), and showed no detectable dechlorination of PCE (Figure 6).

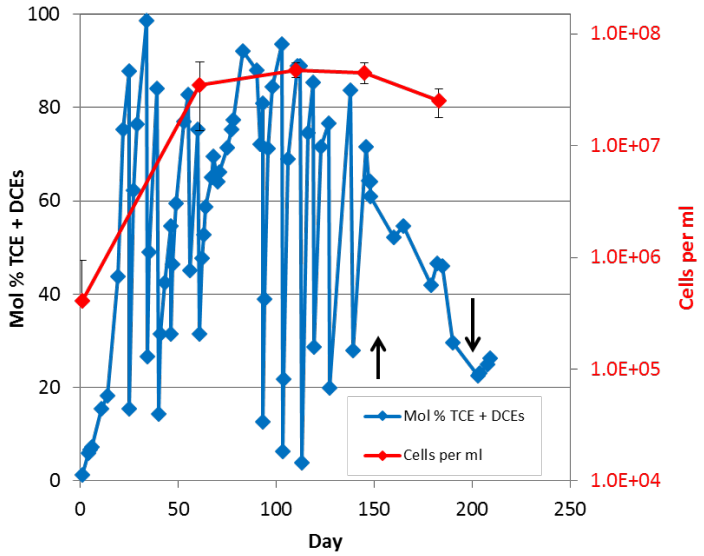

Figure 4.6 Stirring 1 hour at *ca.* 100 rpm 1x per day at Day 146 (*arrow)* and continued until Day 203 (*arrow*). A twofold *decrease* in cell numbers from $4.5x10^7$ to $2.5x10^7$ cells per ml was observed. 0.1% GAC was added at day 203. Dechlorination activity resumes after cessation of stirring and addition of GAC.

Summary of DF1 Culture and Optimization Activities

These studies provided the optimal bioreactor configuration and growth parameters for maintaining a source of DF1 culture for all subsequent experiments. The results indicated that the likely limiting factor for cell density of DF1 was the solubility of the PCE substrate as culture densities increased. Although attempts to promote dissolution rates by convection and direct stirring did not significantly increase the cell density or in the latter case actually decreased growth, we were able to demonstrate that DF1 could be maintained at densities approaching $2.5x10^7$ cells per ml for extended periods of time. This required periodical sparging of residual DCE when PCE was depleted and replenishing the PCE, and by replenishing the medium with 50% replacement of medium. The results also enabled us to design bioreactors for microcosm and mesocosms experiments.

Methods for removal of residual PCE on sorbents (OBE)

One concern with this project was the removal of residual PCE associated with AC during formation of biofilms. Current methodology for growing DF1 for use in experiments is to sparge residual PCE and dehalogenation products form the culture with nitrogen prior to using the cells for bioaugmentation.

We already began forming biofilms on AC in one of our bioreactor and the plan was to develop a method for removal of residual organochlorines using zero valent iron. The iron would serve as an additional nutrient for the bacteria and residual iron would also stimulate reductive dechlorination. A glass bioreactor containing DF1 with AC was initiated and we intended to test the effectiveness of Fe^O for dechlorinating residual PCE and its microbial products. The goal was to test different ratios of Fe^O to AC and incubation times and monitor both the chloroethene concentrations and cell viability to identify the optimal conditions for treating the AC biofilms. This task was not completed due to early termination of funding thus results are not available.

Scale-up method development (OBE)
We initiated a moving bed biofilm bioreactor in a glass bioreactor with powdered activated carbon as the substrate. To this task we designed a 13-liter glass bioreactor configure with a gassing stone and stir bar that extended to the bottom of the bioreactor (Figure 7). The glass bioreactors were set up for anaerobic growth and were configured with medium sampling and headspace sampling ports and a sparging port to purge the culture with nitrogen-carbon dioxide prior to replenishment with PCE. The bioreactors had a nitrogen-carbon dioxide (4:1) gas line connected to a fritted glass sparging stone and a 1 PSI check valve to that the chlorinated ethenes could be periodically sparged out prior to PCE replenishment. provided active DF1 as needed for the remainder of the project. To 10 L of medium we added sterile AC (0.1% final mass/mass) as well as 0.5 L of active DF1 from another culture.

In addition to monitoring dechlorination rates and growth, the Kjellerup lab would use this material to assess biofilm formation. The goal was to assess the optimal conditions for biofilm formation. This bioreactor configuration would enable us to set up multiple bioreactors for testing both fluidized bed reactor (FBR) and Sequential moving-bed biofilm reactor (SMBBR) approaches for efficient biofilm formation. The goal was to test AC, AC with Fe, porous silica and/or biochar for the efficacy of biofilm formation. This task was not completed due to early termination of funding (OBE).

Mesocosm preparation (OBE)
Mesocosm tanks already available in the laboratory from a prior project (ER-201215) were assembled and prepared for use. They consisted of sealed TLC tanks and 12 channel peristaltic pump for recirculating water from the target site to simulate anaerobic and aerobic zones similar to those found in situ. Sediments were collected and characterized for the proposed tests, which were intended to test augmentation with DF1 biofilms. This task was not completed due to early termination of funding (OBE).

Figure 4.7. DF1 growth in glass bioreactor set up for testing the MBBR and FBR approaches to develop biofilms with DF1 on different substrates.

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Chapter 5.
Kinetics of PCB microbial dechlorination explained by freely dissolved concentration in sediment microcosms

Authors
Needham, T.P., Payne, R.B., Sowers, K.R. and Ghosh, U., 2019. Kinetics of PCB microbial dechlorination explained by freely dissolved concentration in sediment microcosms.

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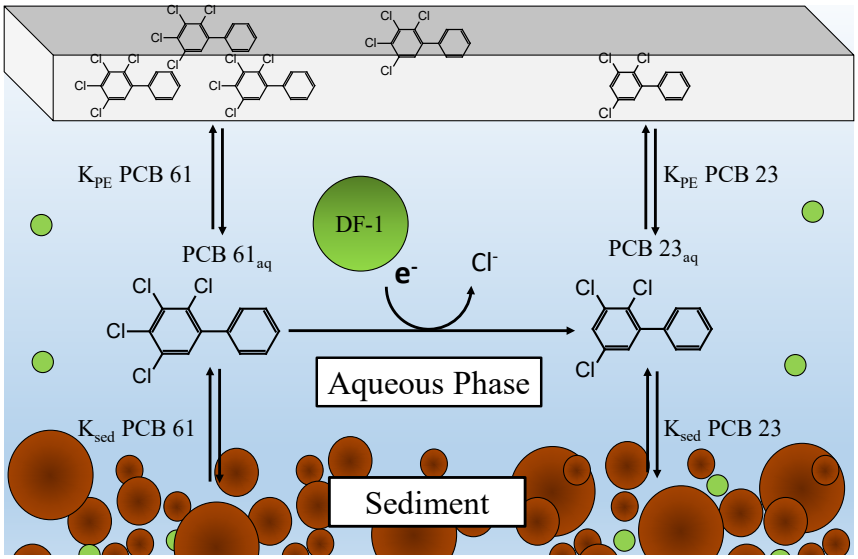
Highlights
- The rate of PCB 61 was found to be linearly dependent on the freely dissolved concentration in both sediment and in sediment-free microcosms.
- PCB microbial dechlorination kinetics can be predicted in sediments.

Abstract
While microbial dechlorination of polychlorinated biphenyls (PCBs) has been observed in sediments over the last three decades, translation to the field has been difficult due to a lack of clear understanding of the kinetic limitations. To address this issue, the present study used passive dosing/sampling to accurately measure the biological rate of dechlorination of 2,3,4,5-tetrachlorobiphenyl (PCB 61) to 2,3,5-trichlorobiphenyl (PCB 23) by an organohalide respiring bacterium, *Dehalobium chlorocoercia* (DF-1). The biological rates were measured over an environmentally relevant concentration range of 1-50 ng/L of freely dissolved concentrations with and without the presence of sediment in bench scale microcosm studies. The rate of dechlorination was found to be linearly dependent on the freely dissolved concentration of PCB 61 both in sediment and in sediment-free microcosms. The observed rate of dechlorination in sediment microcosms could be predicted within a factor of 2 based on the kinetics measured in sediment-free microcosms. A threshold for dechlorination was not observed down to an aqueous concentration of about 1 ng/L of PCB 61. We demonstrate that with a combination of accurate measurement of the aqueous phase dechlorination kinetics and an understanding of the site-specific partitioning characteristics, it is possible to predict PCB microbial dechlorination in sediments.

Graphical Abstract



Introduction

While naturally occurring anaerobic and aerobic microorganisms have been observed to slowly transform Polychlorinated biphenyls (PCBs) in the environment, these compounds have persisted in aquatic sediments and bioaccumulated in organisms (Abraham et al., 2002). *In-situ* microbial degradation is a sought-after cost-effective alternative to capping or dredging for the remediation of contaminated sediments. Microbes can either degrade PCBs by breaking the aromatic ring through oxidative degradation (Abramowicz, 1990) or dechlorinate PCBs through anaerobic reductive dechlorination known as organohalide respiration (Quensen et al., 1988; Bedard, 2008; May et al., 2008). Microbial dechlorination of PCBs was first observed in highly contaminated sediments in the upper Hudson River downstream from a General Electric transformer maintenance facility in upstate New York (Brown et al., 1984). One advantage of anaerobic dechlorination is that it reduces the number of chlorines on the PCB molecules resulting in less toxic lower chlorinated congeners that bioaccumulates less in lipid tissue due to lower hydrophobicity (Abramowicz, 1995; Kaya et al., 2017). In addition to being less toxic, lower chlorinated congeners are susceptible to oxidative degradation by aerobic microbes (Brown et al., 1987). There are different strains of anaerobic bacteria capable of dechlorinating PCBs within the group of organohalide respiring Chloroflexi, often targeting specific chlorine orientations (Sowers and May, 2013). For example, the primary organism of interest for this work, *Dehalobium chlorocoercia* (DF-1), will dechlorinate double flanked meta and para chlorines (May et al., 2008). DF-1 is also significant since it is one of the first dechlorinating strains to be isolated and grown in sediment-free medium, enabling it to be used for accurate microbial kinetics measurements and grown in large batches for bioaugmentation (Wu et al., 2002; Field and Sierra-Alvarez, 2008; May et al., 2008). Recent laboratory and pilot scale studies have demonstrated the feasibility of in-situ microbial bioaugmentation for the treatment of PCB contaminated sediments (Payne et al., 2011; Payne et al., 2013b; Payne et al., 2017a).

Microbial PCB dechlorination has been reported in sediments from a variety of sites and several isolates have been characterized in the laboratory (Abramowicz, 1995; Field and Sierra-Alvarez, 2008; Hiraishi, 2008; Kjellerup et al., 2008; He and Bedard, 2016). Despite the widespread presence of dechlorinators, the bottleneck for effective dechlorination of PCBs in the natural environment is not well understood. For example, Lake Hartwell, SC sediments were analyzed and compared from 1987 and 1998 by Pakdeesusuk et al. to assess the natural attenuation by microbial degradation of PCBs (Pakdeesusuk et al., 2005). They observed rates in 1998 that were slower than predicted rates based on the rates measured in 1987 which suggests a plateau in dechlorination activity. Similar plateau effects have been reported for the St. Lawrence River, Grasse River, and Hudson River sediments (Cho et al., 2003; Cho et al., 2009; Sokol et al., 2009). In each of these studies, the dechlorination rate was based on the bulk concentration in sediments and the aqueous concentration in the porewater was not measured. Part of the reason for our inability to understand the lack of effective dechlorination in the field despite the natural abundance of PCB dechlorinators, is the inability to measure the true rates of dechlorination in an aquatic environment where sorption to solids by these strongly hydrophobic compounds limits their potential bioavailability. Zwiernik et al. proposed that residual petroleum in sediments reduces the rate of dechlorination of PCBs by reducing the bioavailable fraction in the freely dissolved phase, however, their rate studies did not include measurements of the freely dissolved concentrations (Zwiernik et al., 1999). The only study to our knowledge that reports PCB dechlorination kinetics with respect to freely dissolved concentration in water is recent work by

Lombard et al. that developed a method to measure microbial kinetics using a novel passive dosing-passive sampling approach with polyoxymethylene (POM) sheets (Lombard et al., 2014). They found no concentration threshold for anaerobic dechlorination of PCB 61 by DF-1 at environmentally relevant aqueous concentrations (1 to 500 ng/L PCB 61_{aq}). The biological rate of dechlorination was determined to follow a first-order aqueous rate regardless of the initial aqueous concentration.

The main objectives of the present research were to 1) Measure the PCB aqueous dechlorination rate using low-density polyethylene (PE) passive sampling that is known to equilibrate faster than POM and validate the findings of Lombard et al. (2014a), 2) demonstrate the ability to predict dechlorination kinetics in a well-mixed sediment slurry based on independent measurement of the dechlorination kinetics in a sediment-free system, and 3) compare rates of dechlorination to reported rates of desorption from sediment to determine limiting factors for microbial dechlorination of PCBs in sediments.

Materials and Methods

Sediment Preparation

Sediment was collected using a petite Ponar grab sampler from a boat dock at the Smithsonian Environmental Research Center on the Rhode River, MD (38.885528 N, -76.541611 W). The site was selected due to low background levels of PCB (SI Table S1). Sediments were passed through a No. 5 (4 mm) sieve to remove pieces of shell and detritus; then homogenized with an electric paint mixer prior to use.

Passive Sampler Preparation

Each passive sampler device consisted of a 0.15 g sheet of 2 mil polyethylene (PE) which was cut into 0.015 g pieces and attached to a heavy gauge stainless steel wire following similar experimental design previously reported by Lombard et al.(Lombard et al., 2014) The PE passive samplers were then cleaned in a hexane:acetone (1:1) solution for 24 h. Cleaned PE passive samplers were then loaded with PCB 61 in a methanol:water solution for 14 days on a rotary shaker. Targeted PE concentrations were 220, 22, and 2.2 (µg PCB/g PE). PE passive samplers were then equilibrated for 24 h in deionized water to remove any methanol prior to use in sediment-free microcosms.

Sediment Free Microcosm

Sediment free microcosms were prepared anaerobically under sterile conditions consisting of 90 mL of anaerobic media (E-CL; (Berkaw et al., 1996), 10 mM sodium formate, and 0.15 g of PE loaded with PCB 61 in 125 mL pre-cleaned glass jars with PTFE lined lids. PE sheets were loaded with varying initial concentrations for a targeted equilibrium aqueous concentration of 1, 0.1, and 0.01 nM PCB 61 based on PE partitioning coefficients reported by Smedes et al.(Smedes et al., 2009) Microcosms were allowed to equilibrate in the dark for 28 days on a rotary shaker (40 rpm) in an anaerobic glove box (Coy Laboratories) prior to the addition of DF-1 culture. Five mL of DF-1 culture containing $4.6x10^7$ cells/mL (n=3, S.D.=$3.6x10^7$) were added to each microcosm with an average initial cell density of $1.6x10^6$ cells/mL (n=9, S.D.=$5.7x10^5$) estimated by quantitative PCR as described below. PE sheets were removed at days 0, 1, 7, 14, 21, 28, and 42 for analysis.

Sediment Slurry Microcosms

PCB amended sediments were prepared by adding 5, 20, and 100 µg of PCB 61 (Ultra Scientific) dissolved in acetone to pre-cleaned amber wide mouth jars. The jars were then rolled by hand in a fume hood to coat the interior surface until all solvent evaporated. Wet sediment (370 g; 27% solids) and clean PE passive samplers with no PCBs were added to each jar to achieve a targeted sediment concentration of 50, 200, and 1,000 µg/kg by dry weight. Jars were rolled for an initial period of 60 d to equilibrate the sediment and PE samplers. Microcosms were prepared in triplicate for each sediment concentration consisting of 10 g wet sediment, 0.15 g of PE passive samplers, 80 mL E-CL medium, and 10mM sodium formate in 200 mL glass jars with PTFE lined lids. Jars were maintained under anaerobic conditions on a rotary shaker (40 rpm) in an anaerobic glove box and allowed to equilibrate for an additional 14 d period prior to the addition of DF-1 culture. Ten mL of DF-1 culture containing $5.6x10^7$ cells/mL (n=3, S.D.=$3.9x10^7$) were added to each microcosm for a targeted cell density of $5.6x10^6$ cells/g wet sediment (n=9, S.D.=$4.22x10^6$). PE sheets were removed at days 0, 7, 14, 28, and 42 to measure the porewater concentration of PCB 61 and any dechlorination products. Additionally, sediments were sampled at day 0 and 42 of the experiment to measure the solid phase concentration of PCBs.

PCB Analysis

Prior to extraction, surrogate recovery standards (PCB 14 and 65) were added to assess processing efficiency. PE sheets were cleaned with DI water, wiped of any debris and placed in 40 mL glass vials. PE samplers were extracted with hexane for 24 h on a rotary shaker three times. Sediment samples were lyophilized for 24 h until dry and extracted with hexane:acetone (1:1) using an Accelerated Solvent Extractor (Dionex) following EPA method 3545 as previously reported (Payne et al., 2011). Extracts were condensed to 1 mL under nitrogen then treated with copper to remove sulfur compounds (EPA method 3630b) and a modified silica gel clean-up (EPA 3630c) before congener-level analysis by GC-ECD following modified EPA method 8082a (Lombard et al., 2014). Before analysis internal standards consisting of 30 µL PCB 30 and 204 (500 µg/L each in acetone) were added to extracts prior to analysis. A total of 129 PCB congeners were measured either as single co-eluted congeners. PCB calibration standards were composed of a mixture of Aroclor 1232, Aroclor 1248, and Aroclor 1262 that comprise the dominant congeners found in the environment. PCB 61 and the dechlorination products PCB 23 and PCB 29 standards were prepared separately.

Estimation of Dechlorination Rate

The PCB dechlorination rate (k_b) in a passively dosed system that contains sorptive materials must account for the buffering capacity of the solid phase when measuring changes in the aqueous concentration. The following equation is a modification of the equations reported by Lombard et al. to include two solid phases consisting of the PE and sediment (Lombard et al., 2014).

$$\frac{dC_w}{dt} * \left(\frac{V_w * + m_{PE} * K_{PE} + m_{sed} * K_{sed}}{V_w}\right) = C_w * k_b \qquad \text{Eq. 1}$$

Where C_w is the water concentration, V_w is the volume of water, m_{PE} is the mass of the polymer in the system, K_{PE} is the equilibrium partitioning coefficient for the polymer, m_{sed} is the mass of sediment, and K_{sed} is the equilibrium partitioning coefficient for the sediment. Based on the

experimental construct, V_w, m_{PE}, K_{PE}, m_{sed}, and K_{sed} are all known constants and the equation can be further simplified by defining k_b':

$$k_b' = k_b * (\frac{V_w}{V_w{*}+m_{PE}*K_{PE}+m_{sed}*K_{sed}}) \quad \text{Eq. 2}$$

Equation 1 can be solved as a first order rate constant:

$$\frac{C_w}{C_{w\,o}} = e^{-k_b' t} \quad \text{Eq. 3}$$

The buffering capacity (B) is represented by the inverse of the term in the parentheses of equation 2 and describes the resupply from the sorbed pool as the aqueous concentration is depleted. This term has also been called the bioavailability factor (B_f) by Zhang and Bouwer[3] and demonstrated to be applicable in this experimental design by Lombard et al. (2014a). No microbial growth was observed using comparable cell densities and PCB concentrations by Lombard et al (2014a). The rate constant k_b' was determined by normalizing the measured degradation rate (nmole d^{-1}) in the first 14 days by the initial concentration ($C_{w\,o}$). Assumptions made in this model are that the microbial population remains constant and that the mass transfer between the polymer and water is faster than the microbial dechlorination rate as observed by Lombard et al. (2014a). To maintain a high rate of mass transfer between the solids and water phase, the microcosms were continuously stirred on a rotary shaker throughout the experiment. Past work modeling uptake of PCBs in PE passive samplers in a well-mixed system shows that the polymer comes to equilibrium within a few hours (Jalalizadeh and Ghosh, 2017). In comparison, the time scale for degradation in the present experiments was about 21 days.

Sediment Partitioning Coefficient Calculation

The sediment partitioning coefficient is based on the C_{sed} and C_{PE} measured at day 0. The aqueous concentrations were calculated based on PE partitioning coefficients calculated from correlation reported by Smedes et al. (2009) using octonal-water partitioning coefficients from Hawker and Connel (Hawker and Connell, 1988; Smedes et al., 2009).

$$C_w = \frac{C_{PE}}{K_{PE}} \quad \text{Eq. 4}$$

Where K_{PE} is in L/kg, C_{PE} is in µg/kg, and C_w is in µg/L. The following log K_{PE} values were used in Eq.4: PCB 61 (5.87), PCB 29 (5.35), and PCB 23 (5.31). Based on calculated aqueous concentrations for C_w, the sediment-water partitioning coefficient K_{sed} was calculated by Eq. 5:

$$K_{sed} = \frac{C_{sed}}{C_w} \quad \text{Eq. 5}$$

Where K_{sed} is in L/kg, C_{sed} is in µg/kg, and C_w is in µg/L. Values are reported in SI Table S2.

DNA Extraction and Analyses

DNA was extracted from 0.25 ml liquid culture or 0.25 g sediment (wet wt.) aliquots with a Power Soil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA) as previously

described and modified by using a QIACube robotic workstation (www.qiagen.com; (Payne et al., 2011). Microorganisms were enumerated by real-time quantitative PCR (qPCR) using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) with primer pairs: (1)SKFPat9F/SKFPat9R specific for a putative reductive dehalogenase of **"***Dehalobium chlorocoercia"*; or (2)348F/884R specific for the 16S rRNA gene of a deep branching putative dechlorinating clade within the Chloroflexi, using conditions described previously (Payne et al., 2013b). Amplification efficiencies of standards and samples were 99±10.0% with $R^2 = 0.98$.

Results

Dechlorination in passively dosed microcosms without sediment

As shown in Figure 1, dechlorination activity in sediment free microcosms was observed immediately within the first 24 h with no lag phase similar to prior observation by Lombard et al. The loss of PCB 61 is accompanied by stoichiometric accumulation of the dechlorination product PCB 23. Dechlorination activity measured using PE samplers in this experiment was rapid in the first 21 days and was nearly complete in 48 days for all three initial starting concentrations. Also notable is that the mole percent plots show a near identical kinetic profile across initial starting concentrations of PCB 61 that span two orders of magnitude. This indicates a first order type process with a rate constant independent of starting concentration. The results of the PE passive dosing experiment validated the results previously reported by Lombard et al.[16] using polyoxymethylene (POM) as the polymer passive sampler. To date, dynamic passive dosing has been reported utilizing silicon O-rings for PAHs (Smith et al., 2012), polyoxymethylene (POM) for PCBs (Lombard et al., 2014), and now in the present study with low-density polyethylene (PE) for PCBs.

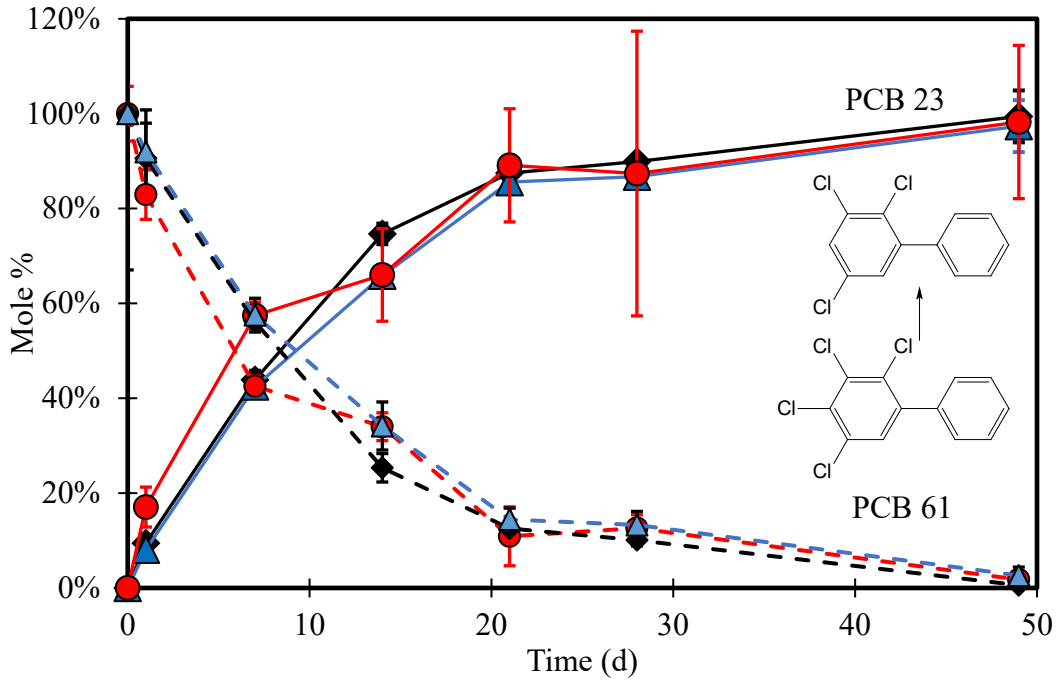


Figure 5.1 Transformation of PCB 61 (dashed lines) to PCB 23 (solid lines) measured in PE samplers at three different initial aqueous concentrations of PCB 61: 0.0043 nM (♦), 0.11 nM (●), 0.41 nM (▲).

The apparent (k_b') and true (k_b) dechlorination rate constants were calculated based on the measured accumulation of the product PCB 23 in PE (Figure S1) and are listed in Table 1. For comparison, the dechlorination rates for PCB 61 measured previously using the same organism and passive dosing with POM[16] are also listed in Table 1. The first order rate constant used to compare the dechlorination rates between the two studies is the true biological rate constant k_b for the system which accounts for the buffering capacity of the solid states as previously described (Eq. 1). In addition, the inoculum densities were different in the two studies, thus for a correct comparison, the true biological rate constants were normalized to an inoculum density of 10^6 cells/ml (last column in Table 1) assuming that the dechlorination rate is proportional to the cell density in the system. As shown in Table 1, the cell density normalized true biological rate constant is within a factor of 2 between the measurements performed using POM previously[16] and that using PE in the present study. Also notable is that the rate constant is relatively invariant across the 2 orders of magnitude range of PCB 61 initial concentrations studied. One difficulty in comparing these rates is the accuracy of qPCR to quantify viable cell numbers (due to differences in DNA extraction efficiencies and qPCR error) resulting in variability that can be in the range of a factor of two (Lombard et al., 2014). Overall, the fact that the intrinsic microbial rate constant was estimated to be within a factor of 2 in separate experiments conducted 4 years apart using different polymers, provides confidence that these rates are truly reflective of the biological process.

Table 5.1 Accumulation rates of PCB 23_{aq} for different initial PCB 61_{aq} concentrations and solid matrices.

Matrix	Initial Cell Density	PCB 61_{aq} initial (nM)	PCB 23_{aq} accum rate (nM d^{-1})	Std Error	r^2	k_b' (d^{-1})	B	k_b (d^{-1})	k_b (d^{-1}) *** (10^6 cell/mL)
POM*	1.20E+06	3.23E-01	9.19E-03	4.00E-05	0.89	0.029	960	27	23
POM*	1.20E+06	9.01E-02	3.08E-03	8.50E-05	0.97	0.034	960	33	27
POM*	1.20E+06	3.33E-02	1.20E-03	3.30E-05	0.98	0.036	960	35	29
POM*	1.20E+06	8.56E-03	2.78E-04	3.00E-05	0.84	0.032	960	31	26
PE	1.60E+06	4.10E-01	1.01E-01	5.30E-02	0.97	0.25	309	76	48
PE	1.60E+06	1.10E-01	2.90E-02	1.30E-02	0.99	0.26	309	81	51
PE	1.60E+06	4.30E-03	9.00E-04	5.80E-05	0.78	0.21	309	65	40
PE+Sed.	5.60E+06	1.34E-01	1.77E-02	4.60E-03	0.91	0.13	1830	242	43
PE+Sed.	5.60E+06	1.50E-02	2.00E-03	5.60E-04	0.99	0.12	1830	215	44
PE+Sed.	5.60E+06	5.10E-03	4.00E-04	1.00E-04	0.97	0.08	1830	144	26
PE+Sed.**	5.60E+06	1.34E-01	1.77E-02	4.60E-03	0.84	0.17	1830	302	54
PE+Sed.**	5.60E+06	1.50E-02	2.00E-03	5.60E-04	0.93	0.15	1830	269	54
PE+Sed.**	5.60E+06	5.10E-03	4.00E-04	1.00E-04	0.92	0.10	1830	179	32

* Values reported in Lombard et al.[24]

** Includes a 20% unavailable fraction.

*** k_b is normalized to an initial cell density of $1x10^6$ cells/mL in order to compare between microcosms. It is assumed that the dechlorination rate is proportional to the cell density.

Dechlorination in Sediment Microcosms

Background PCB concentration in Rhode River sediment used in this study was 22 µg/kg dry weight total PCBs and the full congener list can be found in SI Table S2. The aqueous concentration of the PCB 61 and products PCB 23 and PCB 29 were below quantitation limits (<0.1 ng/L using 0.01 g PE). The sediment consists of 28% sand (>250 microns), 56% fine sand (63-250 microns) and 16% fines (<63 microns). The total organic carbon content of the sediment was 2.9% by dry weight (n=3, STDEV=0.04). Rhode River was selected due to the relatively low background levels of PCBs and an assumption was made that the native population of organohalide respiring bacteria would also be low. However, after equilibrating the sediments with PCB 61 for 60 days prior to DF-1 inoculum, dechlorination products were observed in all sediments. The indigenous PCB respiring bacteria removed chlorine from both the *meta* and *para* positions resulting in a <10% formation of nearly equal amounts of PCB 23 (2,3,5 trichlorobiphenyl) and PCB 29 (2,4,5 trichlorobiphenyl). In contrast, DF-1 selectively dechlorinates double flanked *para* chlorines (Figure 2). The estimated cell density of indigenous Chloroflexi based on qPCR analysis of the 16s rRNA gene was 7.58 $x10^5$ cells/g sediment (n=12, S.D.=8.5$x10^5$) assuming 1 gene copy per cell. It is important to note that not all organisms within Chloroflexi are capable of dechlorinating PCBs (Watts et al., 2005). These population levels are lower than levels traditionally observed in some contaminated sediments such as Grasse River (3$x10^8$ cells/g), Anacostia River (2.2$x10^8$ cells/g) (Kjellerup et al., 2008), but closer to Baltimore Harbor sediments (5$x10^4$ cells/g) (Fagervold et al., 2007). The halogenated electron acceptor utilized by the native dechlorinating organisms could not be identified, however, it is possible that the population is sustained on a naturally produced chlorinated organic compound (Gribble, 2003).

Dechlorination activity was observed in each dosed sediment evidenced by the presence of both PCB 23 and PCB 29. The dominant dechlorination product, however, in the DF-1 amended sediments was PCB 23 which is attributed to dechlorination by DF-1 (Figure 2 and SI Figure S2). Similar to observations in the sediment-free microcosms, PCB 61 was dechlorinated rapidly without a detectable lag phase and stoichiometric amounts of the products were formed. About 90% of the initial PCB 61 was dechlorinated to PCB 23 and PCB 29 in 42 d with a majority (85%) going to PCB 23.

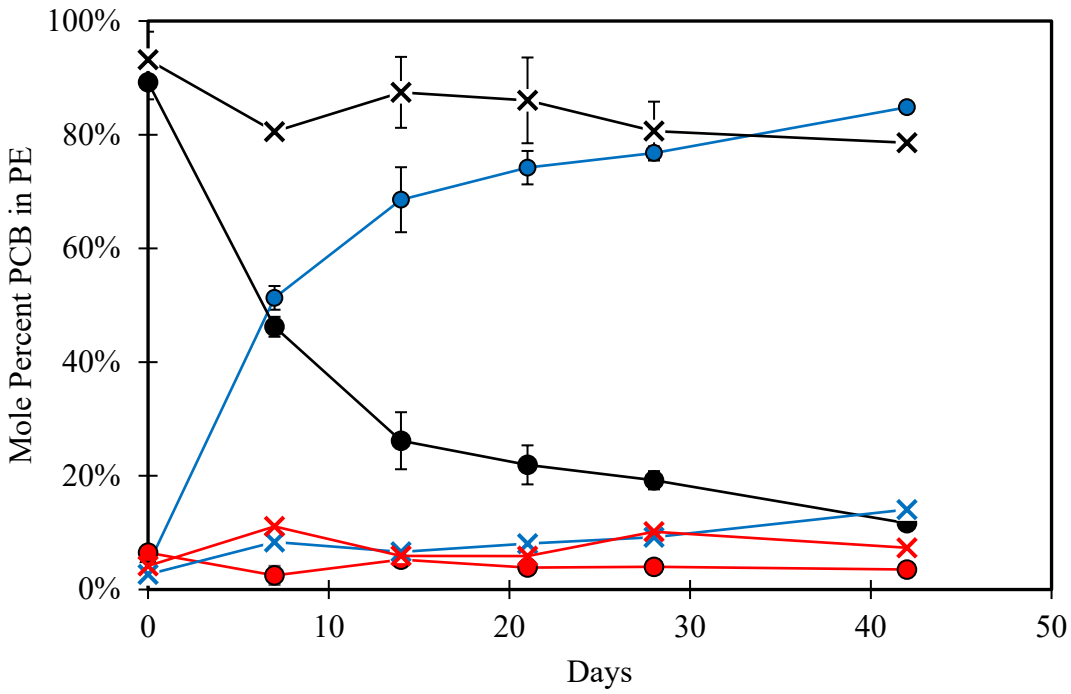


Figure 5.2 Comparison of PCB 61 dechlorination measured in unamended control sediment (x) containing a native microbial community and in DF-1 bioamended sediment (●). PCB 61 (black), PCB 23 (blue), and PCB 29 (red).

Predicted and measured dechlorination rate in sediments

To validate the hypothesis that the biological rate measured by passive dosing/sampling is the true biological rate constant applicable to any buffered system, including sediments, three different concentrations of PCB 61 were added to clean sediments to achieve a comparable range of freely dissolved concentrations tested in the sediment-free microcosms. Sediments were equilibrated to target environmentally relevant freely dissolved concentrations below saturation ($5x10^{-3}$-0.1 nM or 1.5-39 ng/L). As shown in Figure 3, the normalized profiles of the loss of PCB 61 and the accumulation of PCB 23 in the sediment microcosms were similar for all three starting concentrations. Accumulation rate of PCB 23 (nM/d) is shown in Figure S3. Interestingly, for the sediment microcosms, the extent of dechlorination to PCB 23 is about 80 – 85% compared to nearly 100% for the sediment-free microcosms over the same period of time. It is possible that a small fraction of the PCB 61 is bound strongly to sediment and is released too slowly for dechlorination during the study duration. In addition, the native organisms are transforming some of PCB 61 to PCB 29 making less PCB 61 available for the DF-1. Using the average biological rate constant (k_b=46 d^{-1}) measured in the sediment-free microcosm using PE, we estimated the accumulation rate of PCB 23 and depletion of PCB 61 in the aqueous phase based on Eq. 3 (Figure 3). The modeled accumulation of PCB 23 is in reasonable agreement with the measured aqueous concentration calculated from PE but predicts a higher extent of dechlorination (99%) than observed (80-85%). The predicted kinetics of dechlorination can be improved by assuming that 20% of the PCBs are either sorbed strongly and is limited by desorption rates much slower than the microbial kinetics or transformed by the native organisms.

When this small unavailable fraction is applied, the prediction is improved, especially with respect to the residual concentration as shown by the solid lines in Figure 3. Thus, dechlorination of the available PCBs is limited by microbial rate kinetics with respect to the freely dissolved concentration in water.

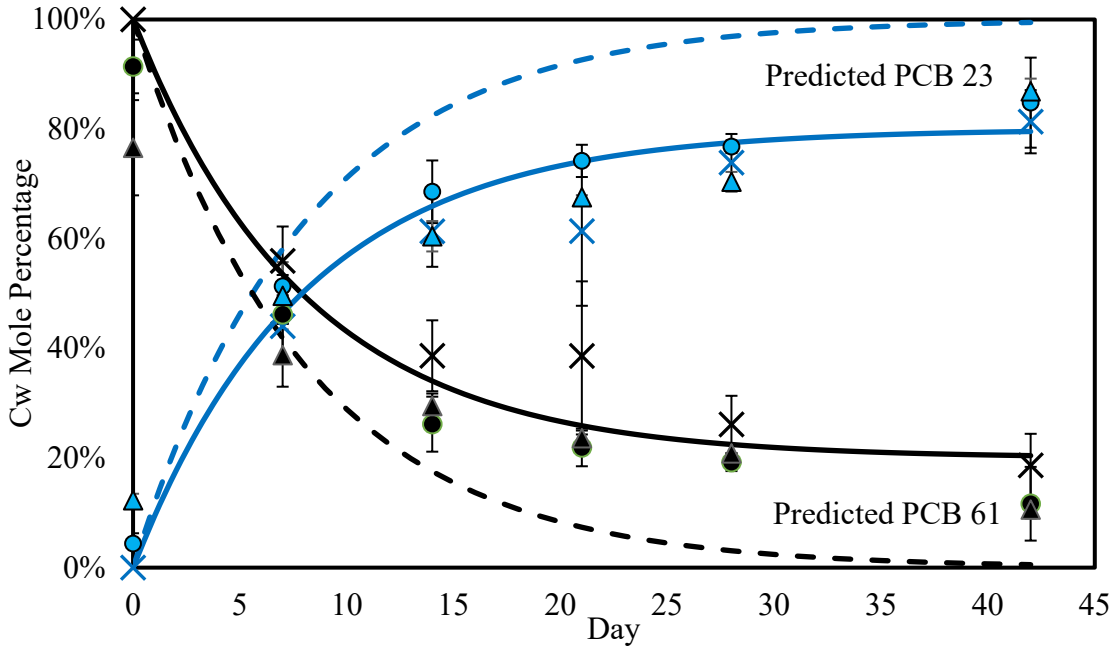


Figure 5.3 Accumulation of PCB 23 (blue) and depletion of PCB 61 (black) at three different aqueous concentrations in spiked sediment: 0.005 nM, (x), 0.015 nM (●), 0.134 nM (▲). Dashed lines are predictions based on the first order biological rate constant measured in a sediment-free system. Solid lines are predictions that include a slow desorption fraction of 20%.

Assuming a 20% unavailable fraction of PCB 61 in sediments, we calculate the average biological rate constant (k_b) to be 47 d^{-1} for the sediment slurries based on the measured accumulation rate of PCB 23, and normalized to a cell density of 10^6 cells/mL (Table 1). These results confirm that PCB dechlorination in sediment closely follows the first order rate (k_b=46 d^{-1}) measured in the sediment-free system. Without accounting for the unavailable fraction, the average biological rate constant (k_b) is 37 d^{-1}, also close to the sediment-free rate. These results also confirm that PE is a suitable substitute for sediment to measure the aqueous rate of dechlorination that is applicable to other buffered systems when desorption rate is not limiting. It is important to note that in static sediments the mass transfer of PCBs can be further hindered by slow inter-particle diffusion requiring additional mass transfer limitations to be incorporated in the overall kinetic model (Zhang et al., 1998).

The PCB dechlorination trend measured in the PE samplers is also reflected in the sediments measured at the conclusion of the experiment. Total molar concentrations in the dosed sediment was preserved throughout the experiment with no statistical difference between the initial and final values as shown in Figure 4. The loss of PCB 61 is accompanied by stoichiometric

accumulation of the primary dechlorination product PCB 23 and a small amount of PCB 29 in all bioactive sediment microcosms.

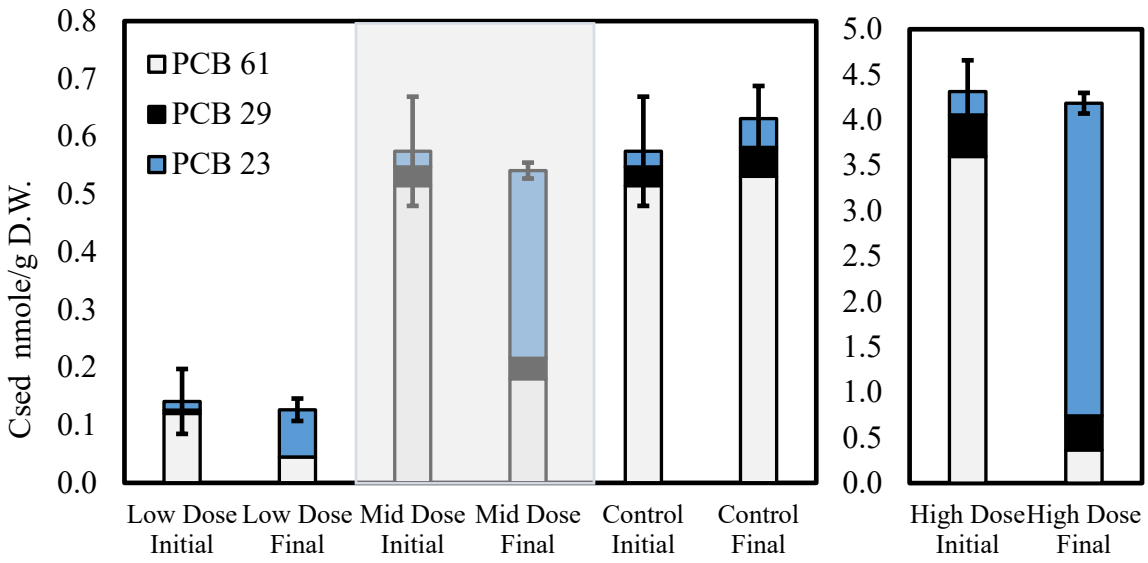


Figure 5.4 PCB concentration in sediments at day 0 (initial) and day 42 (final) for sediments dosed at three initial PCB 61 concentrations (Low Dose, Mid Dose, and High Dose, each inoculated with DF-1) and control sediment that was not inoculated with DF-1. PCB 61 initial concentrations in the sediments were: low dose sediments 35 µg/kg, mid dose sediments 150 µg/kg, and high dose sediments 1,050 µg/kg. The control sediments were spiked to a concentration of 150 µg/kg.

Effect of desorption rate on dechlorination

PCB desorption from contaminated sediments can be described as a two-compartment model in which there is a fast and slow desorbing fraction (Ghosh et al., 1999; Sun and Ghosh, 2007):

$$\frac{C_{sed}}{C_{sed\,o}} = f * e^{(-k_f * t)} + (1 - f) * e^{(-k_s * t)} \qquad \text{Eq. 6}$$

Where, C_{sed} is the PCB concentration in sediment at time t (d^{-1}), $C_{sed\,o}$ is the initial PCB concentration in sediment, f is the fast desorbing fraction, k_f is the fast desorption rate (d^{-1}), and k_s (d^{-1}) is the slow desorption rate. In the present study, the sediments were freshly spiked and equilibrated for 14 days, and therefore, the sorbed PCBs will be primarily associated with the fast desorbing fraction in the sediment. As discussed earlier, the extent of PCB 61 dechlorination in sediments was high at 90% in 42 days, indicating availability of the spiked PCB for dechlorination. The remaining 10% of the PCB 61 in sediments that did not dechlorinate in 42 days is likely attributable to the small slowly desorbing fraction in this spiked sediment. In field-weathered sediments, a higher fraction of PCBs may be associated with the slowly desorbing pool (Ghosh et al., 1999).

For example, in a previous study using sediment from Grasse River, NY it was found to contain 66% fast desorbing PCBs and 33% slow desorbing PCBs (Sun and Ghosh, 2007). For Grasse River sediment, the fast desorption rate (k_f) for PCB 61 (log k_{ow} =6.04)(Hawker and Connell, 1988) is estimated to be 1.2 d^{-1} and the slow desorption rate (k_s) is estimated to be 0.010 d^{-1} based on reported K_{ow} correlations.(Ghosh et al., 1999) The region between slow and fast desorption rates for PCB 61 in Grasse River is shown by the grey shaded region in Figure 5a. Additionally, the estimated dechlorination rates based on measured biological rate constant for DF-1 (k_b=46 d^{-1}) and cell densities ranging from 10^3-10^7 cells/g sediment are shown in Figure 5a. The buffering capacity of the sediment was estimated using reported K_{sed} values(Werner et al., 2010), 30% solids content, and 5.2% TOC as previously reported for Grasse River sediment (Sun and Ghosh, 2007).

Comparing the predicted microbial rates for different cell densities with the shaded region indicating the bracket between slow and fast desorption rates, we observe that at cell densities of 10^5 cells/mL and smaller, the microbial kinetics is slower than both the fast and slow desorption rates. At cell density of 10^6 cells/mL, the microbial rate is faster than the slow desorption rate but slower than the fast desorption rate. At this cell density (used in the present microcosms), the fast desorption fraction will dechlorinate at a rate controlled by the microbial rate as observed in the present study. However, the slow desorption fraction will remain apparently recalcitrant and only become available for dechlorination at the rate of the slow desorption. This is an important observation for determining optimal cell densities for microbial bioremediation treatments and also provides an explanation for the leveling off or plateau in dechlorination activity previously reported in other studies (Abramowicz et al., 1993; Cho et al., 2003; Magar et al., 2005; Pakdeesusuk et al., 2005). With increasing cell density, dechlorination activity should increase proportionately but will reach a point at which the microbial rate is no longer limiting due to the slow desorption from sediment. At this point, the bioavailability of residual PCBs can no longer support the high cell densities.

As the hydrophobicity (K_{ow}) of the PCB increases for the higher chlorinated congeners, the desorption rate becomes slower. For example, 2,2',3,3',4,4',5,5' octochlorobiphenyl (PCB 194, log K_{ow}=7.8) is susceptible to dechlorination by DF-1 and has the same chlorination pattern on both phenyl rings as PCB 61. The estimated fast and slow desorption rate for PCB 194 in Grasse River sediments is 0.065 and 0.0021 d^{-1} respectively. As shown in Figure 5b, PCB 194 will demonstrate greater recalcitrance than PCB 61 even if the intrinsic microbial dechlorination rate for the two PCB congeners are similar.

Since PCB contamination in sediments is primarily the result of Aroclor mixtures that contain a range of PCB congeners, the net PCB dechlorination will follow a two-stage model in which lower chlorinated congeners may be dechlorinated first due to fast desorption and increased bioavailability followed by slower dechlorination of higher chlorinated congeners. This trend was observed in Grasse River sediment slurry microcosms spiked with Aroclor 1248 (Cho et al., 2003). Their measured dechlorination rates for different PCB congeners varied between 1.036 and 0.006 d^{-1}. Evaluating their results based on log K_{ow} of the PCB congeners a general trend is observed in which log k_b decreases as log K_{ow} increases (R^2=0.62) (Figure S4).

Explanation of previously reported threshold concentrations for PCB microbial dechlorination
Two kinds of kinetic thresholds for PCB dechlorination have been reported in the literature. The first type is the observation that even under the most optimal conditions for dechlorination, the process slows down after the residual concentration falls below a threshold value. Previous kinetic studies of Lake Hartwell sediments (Magar et al., 2005; Pakdeesusuk et al., 2005) and St. Lawrence River sediments (Sokol et al., 2009) observed such a plateau effect but could not provide an explanation for the observations, attributing this effect to a kinetic threshold for PCB dechlorination. Our results do not support the view that a threshold concentration for dechlorination in sediments exists. Rather, we attribute this apparent threshold to the mass transfer limitation from sediment particles to the bioavailable aqueous phase. We demonstrate in the present study that even at a low aqueous concentration of 1 ng/L, microbial dechlorination is active and only limited by the availability of the PCB in the aqueous phase. The second type of threshold discussed in the literature pertains to PCB concentration in sediment that is unable to support the growth of the dechlorinators to reach levels that can substantially reduce sediment concentrations within a reasonable period of experimental observation. Past kinetic studies using sediment slurries have suggested kinetic thresholds for PCB dechlorination as high as 40 mg/kg (Rhee et al., 2001; Cho et al., 2003) which is three orders of magnitude higher than our lowest concentration tested at 35 μg/kg. Rhee et al. (2001) correctly point out that their threshold observation is most likely related to a growth threshold (Rhee et al., 2001). Our experiments were able to avoid a growth threshold by augmenting the sediments with DF-1 that was grown separately and introduced into the sediments.

Study implications and the case for bioaugmentation
Results from the present study and those published by Lombard et al (2014a) were unable to identify a kinetic threshold for dechlorination within the detection limits of the instrumental analysis (Lombard et al., 2014). The present work suggests that the low PCB dechlorination rates observed in weathered sediments are due to either low native cell densities and/or slow desorption kinetics (Cho et al., 2003; Pakdeesusuk et al., 2005; Sokol et al., 2009). By comparing measured dechlorination rates and known desorption rates, we show that it is possible to make an assessment of whether low abundance of PCB dechlorinators is the rate-limiting factor for PCB dechlorination in the environment. For example, native abundance of active dechlorinating bacteria at 10^3 or 10^4 cells/g would require a timespan of decades to significantly reduce PCB levels as shown in the kinetic profiles in Figure 5 (Krzmarzick et al., 2012). Our observations are in general agreement with that of Rhee et al. (2001) who postulated that "the biomass of dechlorinating microorganisms may indicate the dechlorination potential of a given site"(Rhee et al., 2001). Based on this observation is should be possible to break the bottleneck of growth threshold for dechlorinators by engineering bioaugmentation with dechlorinators. Such bioaugmentation has recently been demonstrated in laboratory and pilot studies (Kjellerup et al., 2008; Payne et al., 2013a; Payne et al., 2017b). In such instances, where the native population of dechlorinators is low and the PCB concentration in sediment/porewater is also below the threshold for growth, bioaugmentation of PCB dechlorinating organisms would enable faster dechlorination than the rate of natural attenuation. However, even in bioaugmented systems, we show that there is an optimal level of augmentation, and the final extent of dechlorination will be controlled by the kinetics and proportion of the slowly desorbing pool. In organic rich anaerobic sediment environments where PCBs are often found, there should be sufficient availability of electron donors which should not be limiting.

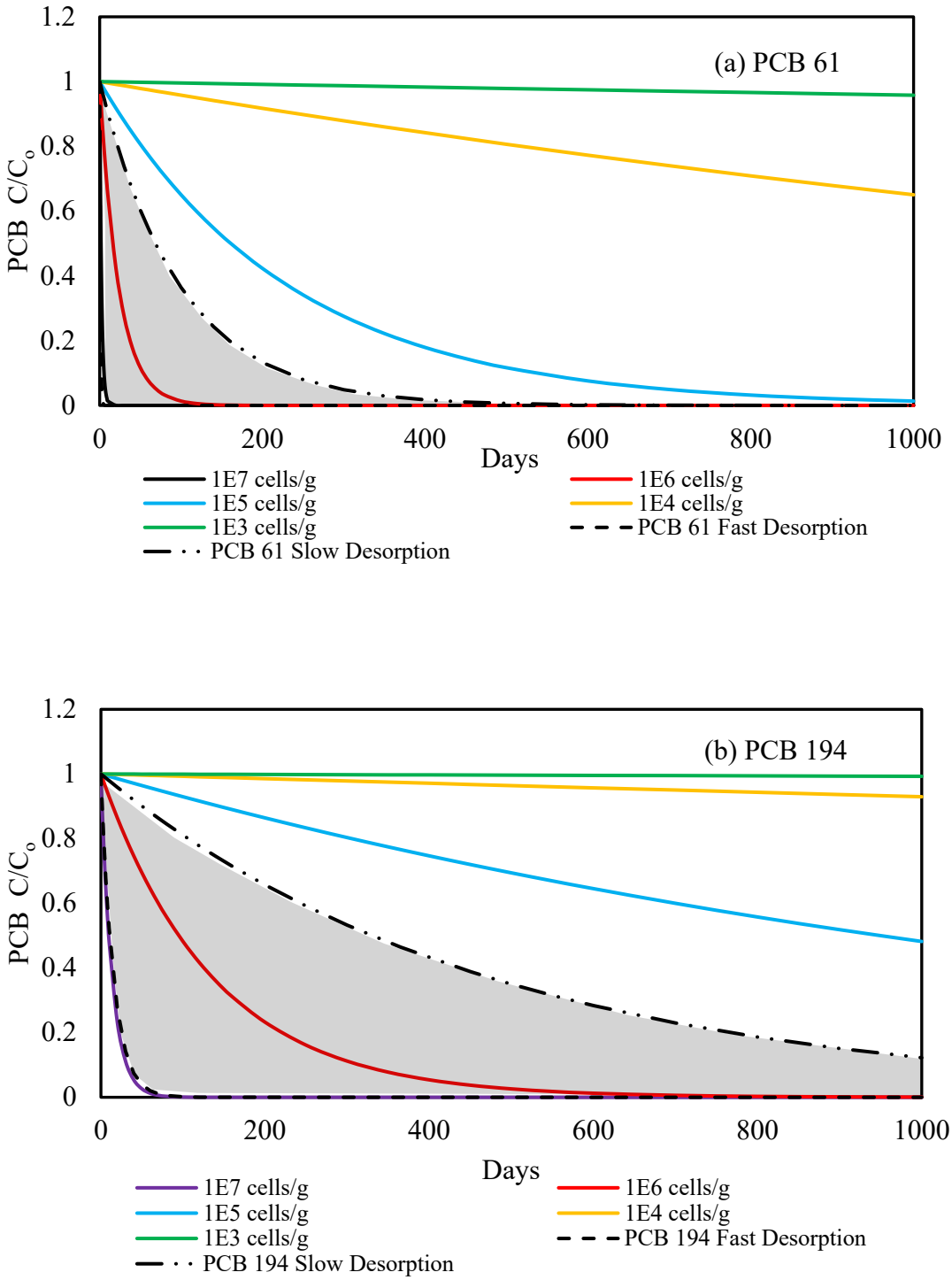


Figure 5.5 Effect of cell density of DF-1 on dechlorination rate compared with the rate of desorption of PCB 61 (a) and PCB 194 (b). Shaded region depicts the range between fast and slow desorption rates estimated for Grasse River sediment.

Based on a comparison of microbial and desorption rates in Figure 5, a targeted cell density between 10^5-10^6 cells/g sediment appears to be optimal. Payne et al. (2011) observed approximately 80% dechlorination in a Baltimore Harbor sediment mesocosm bioaugmented with 10^5 cells/g DF-1. Payne et al. (2013b) also saw approximately 80% total degradation in mesocosm studies bioaugmented with 10^5 cells/g DF-1 and the aerobe LB400 (Payne et al., 2013b; Payne et al., 2017a) (Payne et al., 2011). In the 2017 study, re-amending the mesocosm with more organisms after 1 year did not result in further degradation (Payne et al., 2017a). The effects of slow and fast desorption have been documented for PCB bioavailability to macro-organisms in bioaccumulation studies and similar results are expected for a microbial dechlorinator (Sun and Ghosh, 2007). Once the pool of fast desorbing PCBs in sediment has been exhausted by a microbial population, it is possible that the population would decrease as less PCBs are available in the aqueous phase and are replenished by the slow desorbing pool. This population "die-off" in bioamended sediments has been observed recently in both microcosm and pilot studies in which the cell density decreases by an order of magnitude after one year (Payne et al., 2013b; Payne et al., 2017a).

There is renewed interest in bioaugmentation of PCB degrading bacteria based on recent successful laboratory and pilot studies (Payne et al., 2011; Payne et al., 2013b; Payne et al., 2017a; Sowers et al., 2018). By considering the geochemical properties of the sediment, porewater concentrations, and cell density of dechlorinating bacteria; bioremediation through bioaugmentation or monitored natural attenuation can be better modeled and optimized for specific sediment sites. This work provides a framework to evaluate the effects of indigenous dechlorinating microbial communities, bioamendments, and additional factors that may affect dechlorination rates in PCB impacted sites. This approach will further enhance our understanding of microbial dechlorination in contaminated sediments and enable us to develop efficient engineering approaches for effective remediation.

Supplemental Information.
Additional information is available below on estimation of microbial rate constants, kinetic profiles at different initial concentrations, and native PCB concentration in sediment.

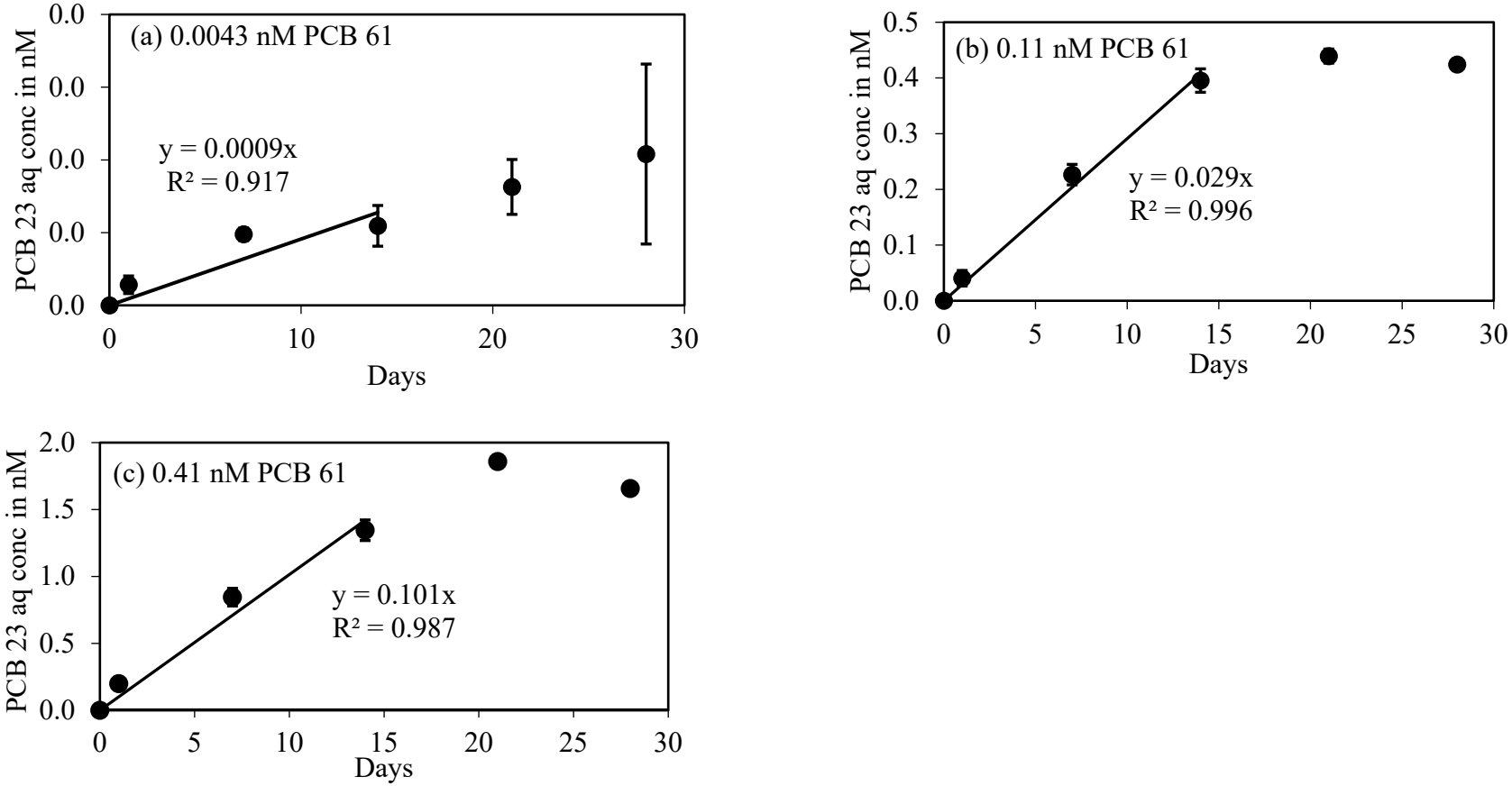


Figure 5.S1. PE Dechlorination Rates: Rate of accumulation of PCB 23 (nmole day^{-1}) measured at specific initial aqueous concentrations (a) 0.0043 nM. (b) 0.11 nM, (c) 0.41 nM, The first 14 days were used to calculate the first order rate constant to capture the linear portion of the rate.

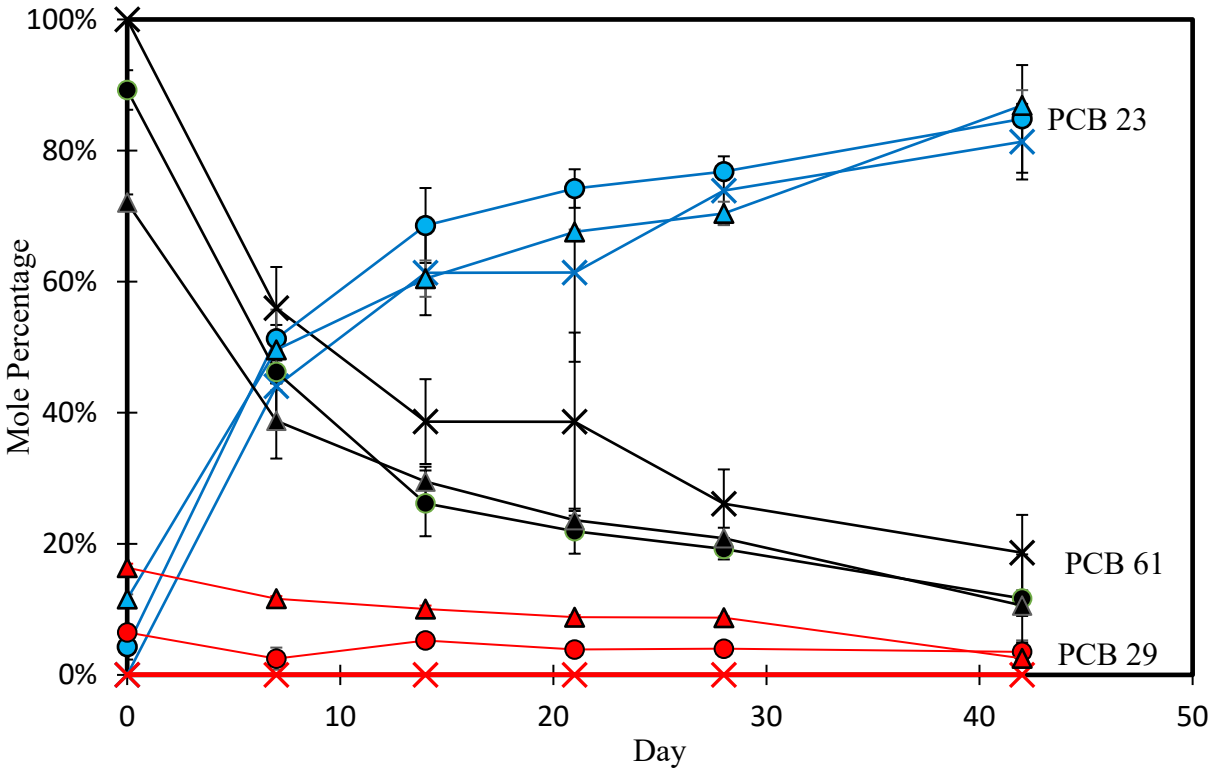


Figure 5.S2 PCB dechlorination in a sediment slurry: PCB 61 (black) with dechlorination products PCB 23 (blue) and PCB 29 (red). Initial PCB 61_{aq} concentrations shown are 0.005 nM (♦), 0.015 nM (●), and 0.134 nM (X).

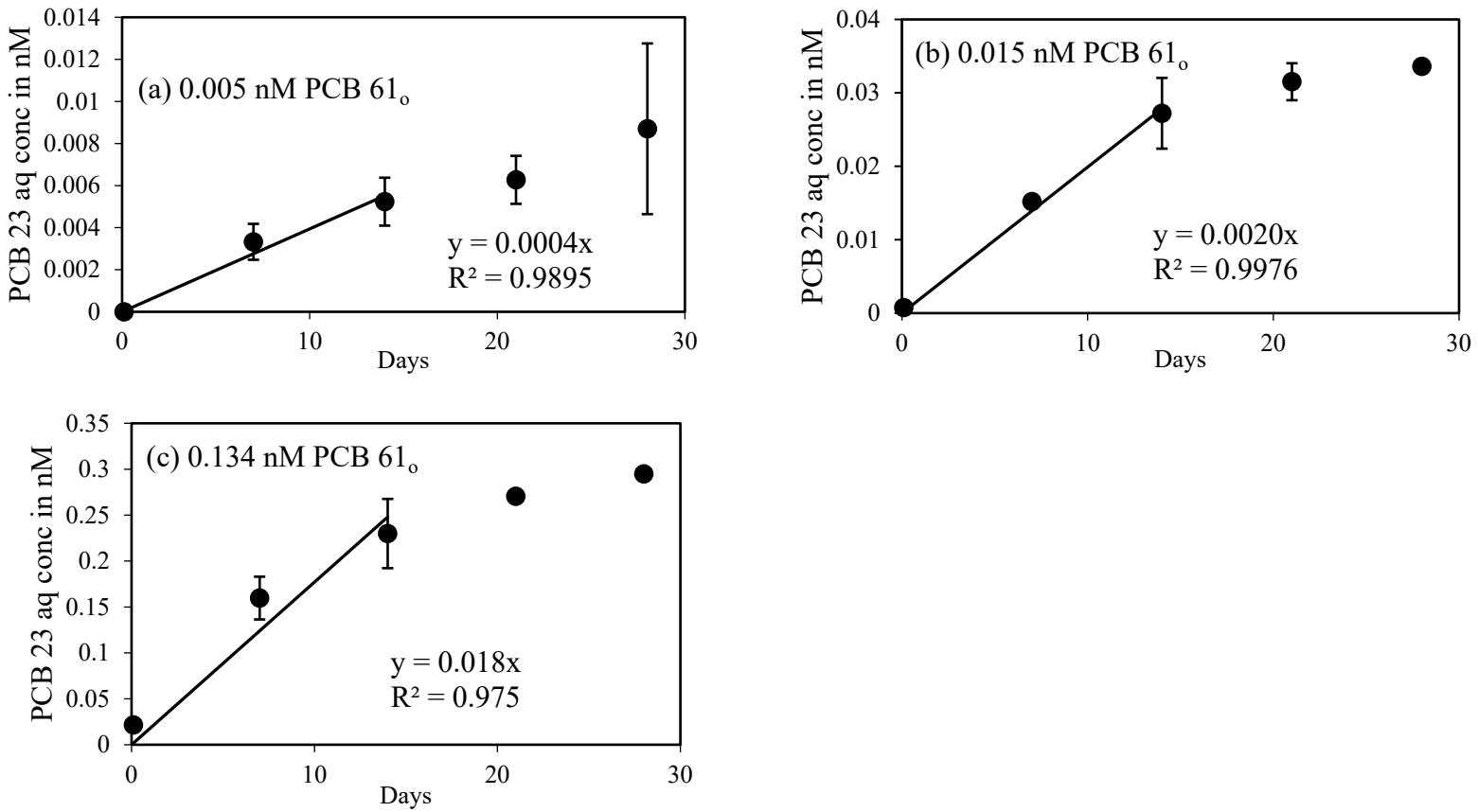


Figure 5.S3 Sediment Dechlorination Rates: Rate of accumulation of PCB 23 (nmole day^{-1}) measured with the respective initial aqueous concentrations (a) 0.005 nM, (b) 0.015 nM, (c) 0.134 nM. The first 14 days were used to calculate the first order rate constant to capture the linear portion of the rate.

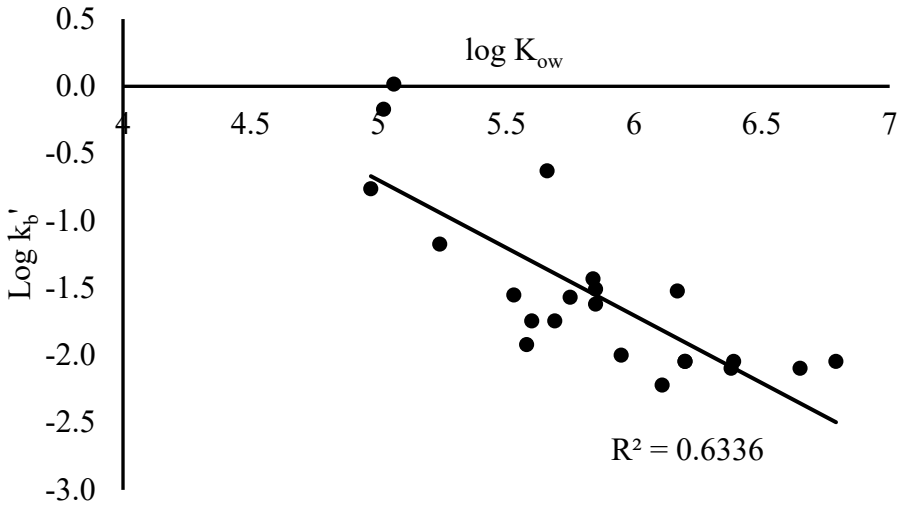


Figure 5.S4 Log of measured dechlorination rate constants (k_b') in Grasse River sediment for individual congeners in Aroclor 1248 reported by Cho et al. (2003) plotted against log K_{ow}.(Hawker and Connell, 1988; Cho et al., 2003). Coeluting congeners reported by Cho et al. (2003) were not included in this correlation (Cho et al., 2003).

Table 5.S1 Background Rhode River sediment PCB levels

PCB	**Chlorine Position**	**µg/kg dry weight**
(1)	2	0.000
(3)	4	0.000
(4+10)	22' , 26	0.000
(7+9)	*24 25*	0.000
(6)	23'	0.000
(8+5)	24' 23	0.000
(19)	22'6	0.000
(12+13)	34 , 34'	0.000
(18)	*22'5*	0.000
(15+17)	22'4	0.000
(24+27)	236 , 23'6	0.000
(16+32)	22'3 , 24'6	0.000
(26)	23'5	0.560
(25)	23'4	0.000
(31)	24'5	0.000
(28)	244'	0.000
(21+33+53)	234 , 2'34 , 22'56'	1.774
(51)	22'46'	0.684
(22)	234'	0.000

(45)	22'36	0.663
(46)	22'36'	0.000
(52+43)	22'55' , 22'35	0.000
(49)	22'45'	0.319
(47)	22'44'	0.000
(48)	22'45	0.000
(44)	22'35'	0.000
(37)	344'	0.000
42	22'34'	0.000
(41+71)	22'34 , 23'4'6	0.000
(64)	234'6	0.000
(40)	22'33'	0.000
(100)	22'44'6	0.000
(63)	234'5	0.000
(74)	244'5	0.000
(70+76)	23'4'5 , 2'345	0.000
(66 + 95)	23'44'	0.000
(91)	22'34'6	0.000
(56+60)	233'4' , 2344'	0.499
(92+84+89)	22'355' , 22'33'6 , 22'346'	0.000
(101)	22'455'	2.237
(99)	22'44'5	2.472
(83)	22'33'5	0.160
(97)	22'3'45	0.066
(81+87) 81 negligible	344'5 , 22'345'	0.638
(85)	22'344'	0.000
(136)	22'33'66'	0.000
(77+110) 77 negligible	33'44' , 233'4'6	0.799
(82 + 151)	22'33'4	0.000
(135+144+147+124)	22'33'56' , 22'345'6 , 22'34'56 , 2'3455'	0.000
(107)	233'4'5	0.000
(123+149)	2344'5 , 22'34'5'6	0.000
(118)	23'44'5	3.770
(134)	22'33'56	0.201
(114+131)	2344'5 , 22'33'46	0.000
(146)	22'34'55'	0.000
(153)	22'44'55'	0.210
(132)	22'33'46'	0.000
(105)	233'44'	0.014
(141)	22'3455'	0.000

(137+176+130)	22'344'5 , 22'33'466' , 22'33'45'	0.321
(163+138)	233'4'56 , 22'344'5'	0.272
(158)	233'44'6	0.000
(178+129)	22'33'55'6 , 22'33'45	0.000
(175)	22'33'45'6	0.000
(187+182)	22'34'55'6 , 22'344'56' , 22'33'45'6	0.000
(183)	22'344'5'6	0.000
(128)	22'33'44'	0.000
(185)	22'3455'6	0.000
(174)	22'33'456'	0.021
(177)	22'33'4'56	0.000
(202+171+156)	22'33'55'66' , 22'33'44'6 , 233'44'5	0.748
(157+200)	233'44'5' , 22'33'45'66'	0.000
(172 + 197)	22'33'455'	0.000
(180)	22'344'55'	0.282
(193)	233'4'55'6	1.273
(191)	233'44'5'6	0.000
(199)	22'33'4566'	0.000
(170+190)	22'33'44'5 , 233'44'56	0.142
(198)	22'33'455'6	0.000
(201)	22'33'4'55'6	0.068
(203+196)	22'344'55'6 , 22'33'44'56'	0.000
(208+195)	22'33'455'66' , 22'33'44'56	1.188
(207)	22'33'44'566'	0.083
(194)	22'33'44'55'	0.985
(205)	233'44'55'6	0.000
(206)	22'33'44'55'6	0.559
(209)	Deca	0.936
Total		21.943

Table 5.S2 Calculated log K_{sed} values for the three initial sediment concentrations**.**

	0.005 nM	**0.015 nM**	**0.134 nM**	**Average**
PCB 23	4.33	4.56	4.08	4.32
PCB 29	---------	4.46	4.18	4.32
PCB 61	4.37	4.53	4.43	4.48

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Chapter 6.
Colonization and growth of dehalorespiring biofilms on carbonaceous sorptive amendments

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Highlights
- Dehalorespiring biofilms formed on all carrier materials tested with a preference for sorptive carbonaceous materials
- Biofilm-based inoculum can be an efficient approach for bioaugmentation of PCBs in sediment

Abstract
Removal of polychlorinated biphenyls (PCBs) from contaminated aquatic sediments is a priority due to their ability to enter the food chain, where they result in harmful effects. Recent success with reduction of PCB bioavailability due to adsorption onto activated carbon has led to the recognition of *in situ* treatment as a viable remediation approach for contaminated sediment. In this study, the reduced bioavailability and subsequent break-down of PCBs in dehalorespiring biofilms was investigated using *Dehalobium chlorocoercia* DF-1. DF1 formed a patchy biofilm ranging in cellular biomass thickness from 3.9 to 6.7 µm with an average of 4.6 ± 0.87 µm, while the biofilm coverage area varied from 5.5% (sand) to 20.2% (activated carbon) indicating a preference for highly sorptive carbonaceous materials. Quantification of DF1 bacteria in the biofilms showed abundances from 1.2 to 15.3 x10^9 bacteria per g material. After 22 days, coal activated carbon, bone biochar, polyoxymethylene, and sand microcosms had dechlorinated 73%, 93%, 100%, and 83% respectively. These results show that biofilm-based inoculum for bioaugmentation of weathered PCBs in sediment can be an efficient approach.

Introduction
Polychlorinated biphenyls (PCBs) have for decades contaminated soil and sediment and continue to be a problem due to their toxic and bioaccumulative characteristics as well as their persistence in the environment (Erickson, 1997). Dehalorespiration with PCBs under anaerobic conditions in sediments is a critical process for the transformation to innocuous end products. Few bacterial species within the bacterial group *Chloroflexi* have been confirmed to respire with PCBs including *Dehalococcoides mccartyi* strain 195 (formerly *D. ethenogenes*) (Fennell et al., 2004; Zhen et al., 2014), *Dehalobium chlorocoercia* DF-1 (Payne et al., 2013a), and o-17 (Cutter et al., 2001; May et al., 2006). Natural *in situ* reduction of PCBs through dehalospiration has been demonstrated (Wiegel and Wu, 2000), but has not been widely adopted as a remedial alternative due to the slow process, low bioavailable concentrations of PCBs and frequently low abundances of indigenous dehalorespiring microorganisms (Sowers and May, 2013a). Studies have shown that dehalorespiring microorganisms cultured in the form of biofilms can achieve higher cell

abundances and maintain activity for longer periods of time (Mukerjee-Dhar et al., 1998; Borja et al., 2006).

The preferred mode of growth of most microorganisms in the environment is in the form of biofilms where bacteria are attached to solid surfaces and this sessile lifestyle is favored due to many benefits (Costerton et al., 1995; Jefferson, 2004a). Biofilm-embedded microorganisms possess several advantages over their free-floating counterparts. One advantage is the ability of the EPS to capture and concentrate nutrients, such as carbon, nitrogen, as well as organic contaminants such as PCBs (Borja et al., 2006; Carrel et al., 2018). Another advantage is that a sessile lifestyle increases the resistance to removal caused by sudden changes in environmental conditions such as increased shear forces, pH changes, or grazing from other organisms (Allison, 2003; Hobley et al., 2015). The biofilm approach has been widely applied in the environmental engineering field. In drinking water systems, activated carbon (AC) has been applied for decades as the carrier material for biofilms for removal of nutrients and pathogenic bacteria (Camper et al., 1985, 1986). Recent research has examined the potential role of biochar and activated carbon in accelerated biodegradation of organic contaminants and focused efforts on identifying the mechanism behind the enhanced degradation and microbial community shifts (Oh et al., 2012; Yu et al., 2015; Lefèvre et al., 2017; Song et al., 2017; Chen et al., 2018) and only few studies have considered the role of biofilms (Borja et al., 2006; Kjellerup et al., 2014b; Frankel et al., 2016). Previous studies suggested that biofilms can more effectively biodegrade PCBs in comparison to suspended cells (Mukerjee-Dhar et al., 1998; Borja et al., 2006). Furthermore, the approach of bioaugmentation with biofilm on carbonaceous materials promote greater removal of organic contaminants (Kjellerup et al., 2014b; Frankel et al., 2016).

Evaluation of biofilms has often been performed using confocal laser scanning microscopy (CLSM) (Lawrence and Neu, 1999; Neu and Lawrence, 2014; Schlafer and Meyer, 2017). Quantitative approaches have been applied to monitor biofilms in drinking water treatment systems (Waller et al., 2018) and in extreme environments such as on glacial surfaces (Smith et al., 2016). Recently, CLSM has been used to monitor biofilm thickness during the biodegradation of organic pollutants using a combined biofilm-biochar approach (Frankel et al., 2016). Frankel et al. (2016) investigated the potential of a mixed aerobic enrichment culture to biodegrade naphthenic acid in the presence of metal contaminants over a 30-day laboratory experiment. The biofilm-biochar approach was effective at removing napthenic acids and promoting metal sorption throughout the experiment. Other studies showed that biochar and activated carbon were beneficial for reducing the toxicity of PCBs (Zimmerman et al., 2005; Sun and Ghosh, 2008; Choi et al., 2009; Ghosh et al., 2011) and these carbonaceous materials can successfully serve as microbial carriers in sediment beds (Hale et al., 2015).

The objective of this study was to assess the colonization and growth of PCB-dehalorespiring biofilms on different materials that potentially could be used for bioremediation of PCBs in sediment. The use of biofilms is a novel application in bioremediation of contaminated sediments and the goal of the work is to investigate active dechlorinating biofilms. The importance of adsorption capacity, surface area/porosity and electrical conductivity on *D. chlorocoercia* DF-1 biofilm formation was investigated in laboratory experiments using the following materials: coal AC, bone BC, polyoxymethylene (POM), and sand.

Materials and methods

Growth of bacterial cultures

D. chlorocoercia DF1 biofilms were grown on coal activated carbon (Calgon Carbon Corporation, Pittsburg, PA), bone biochar (Bonechar Carvao Ativado Do Brasil Ltd., Brazil), polyoxymethylene (*POM) (Sigma-Aldrich, Saint Louis, MO) and* sand (Thermo Fisher Scientific, Waltham, MA) (Table 1). For each material, three replicates along with a negative control were assembled in an anaerobic chamber (5% H_2, 15% CO_2, 80% N_2) (Coy Laboratory, MI). The replicates consisted of 0.1 gram of material (AC, BC, POM, and sand), 0.1 gram of autoclaved Grasse River sediment (Massena, NY), 1 mL of DF-1 co-culture (approximately $1x10^6$ of cells per ml), 9 mL of estuarine minimal medium (Berkaw et al., 1996c), 1 mL of autoclaved *Desulfovibrio* extract (1% v/v), 10 mM degassed sodium formate (Thermo Fisher Scientific, Waltham, MA) (electron donor) and 10 ppm 2,3,4,5- tetrachlorobiphenyl (PCB 61) (Accustandard, 100% purity, CAS 33284-53-6) dissolved in high performance liquid chromatography (HPLC) grade acetone (Fischer Scientific, 99% purity, J.T. Baker Chemical Company) was used for stock solutions of PCB 61. The final concentration of acetone in the stock solution of PCB 61 was 0.1% (v/v). Controls were assembled in a similar fashion without materials, one with sediment and one without. Negative controls were assembled for each material treatment without addition of culture. The experiment was kept shielded from light in an anaerobic chamber, unshaken at 30°C. Samples were collected at days 22, 133, and 159 for CLSM and DNA analysis and dechlorination activity was confirmed.

Table 6.1 Characteristics of materials used as amendments in biofilm quantification experiments (Celzard et al., 2002; Kongkhlang et al., 2008; Chun et al., 2013; Klüpfel et al., 2014; Saquing et al., 2016; Endo et al., 2017). Materials were chosen in effort to vary one parameter at a time.

Material	Total surface area (m^2/g)	Sorption capacity Log(K_d)	Conductivity % Carbon	Avg. Pore Width (nm)	Commercially Available
Bone Biochar	200	N/A	11.0	6.25	Bonechar Carvao Ativado Do Brasil Ltd.
Coal Activated Carbon	1,116	(PCB-61) 8.70	80.9	1.48	Calgon TOG LF 80x325
Sand	0.05	N/A	0.0	$2x10^4$	Thermo Fisher Scientific
Polyoxymethylene	107	(PCB-44) 5.65	40.0	N/A	Sigma-Aldrich

Quantitative biofilm measurements

A Leica TCS SP5 II confocal laser scanning microscope (Leica Microsystems, Model SP5 SMD) equipped with a 63x 0.9 NA long working distance (2.2 mm) water dipping objective (Leica Microsystems, Exton, PA, USA) was used to image the abiotic and biotic materials. CLSM 3-D images were compiled via Imaris x 64 software (version 8.4.0, Figure 1). Biofilm samples were placed in a petri-dish with sterile filtered water after they had been stained with SybrGreen I nucleic acid stain (30 minutes, in the dark), which was supplied as 10,000x concentrate in

dimethylsulfoxide (Thermo Fisher Scientific, Waltham, MA) and diluted to a 40x working concentration in samples. Images of SybrGreen stained biofilms were obtained by using a fluorescein isothiocyanate (FITC) filter, while reflection of the material using reflected light. Fluorescent and reflection images were captured at an excitation wavelength of 488 nm and SybrGreen emission wavelength collection was 500–595 nm. Images of SybrGreen stained microbial biofilms and reflection of the materials were overlaid in the Imaris software (Bitplane Scientific Software, South Windsor, CT). Imaris software is a program for quantification of three-dimensional biofilm structures. For each biofilm sample a total of 10 images were collected at randomly chosen fields of view. Z-stacking images were captured by vertical sectioning through the biofilm with a z-step increment of 1 μm. Images were analyzed using Imaris software and the area of biofilm coverage (i.e., biosurface area), biofilm volume (i.e., biovolume), and biofilm thickness measurements were calculated. Biomass volume was defined as the number of biomass pixels in all images of a stack and multiplied by the voxel size. Cellular biomass thickness measurements were calculated by finding the average height of the biomass volume and area of the fluorescent images in the Imaris software. Negative controls were imaged ($n = 5$) and showed biofilm coverage $<0.05\%$ for all materials. CLSM imaging of the samples demonstrated the selective binding of the SybrGreen nucleic acid stain to the bacterial cells and not unspecific staining of the amendment materials (Figure S1).

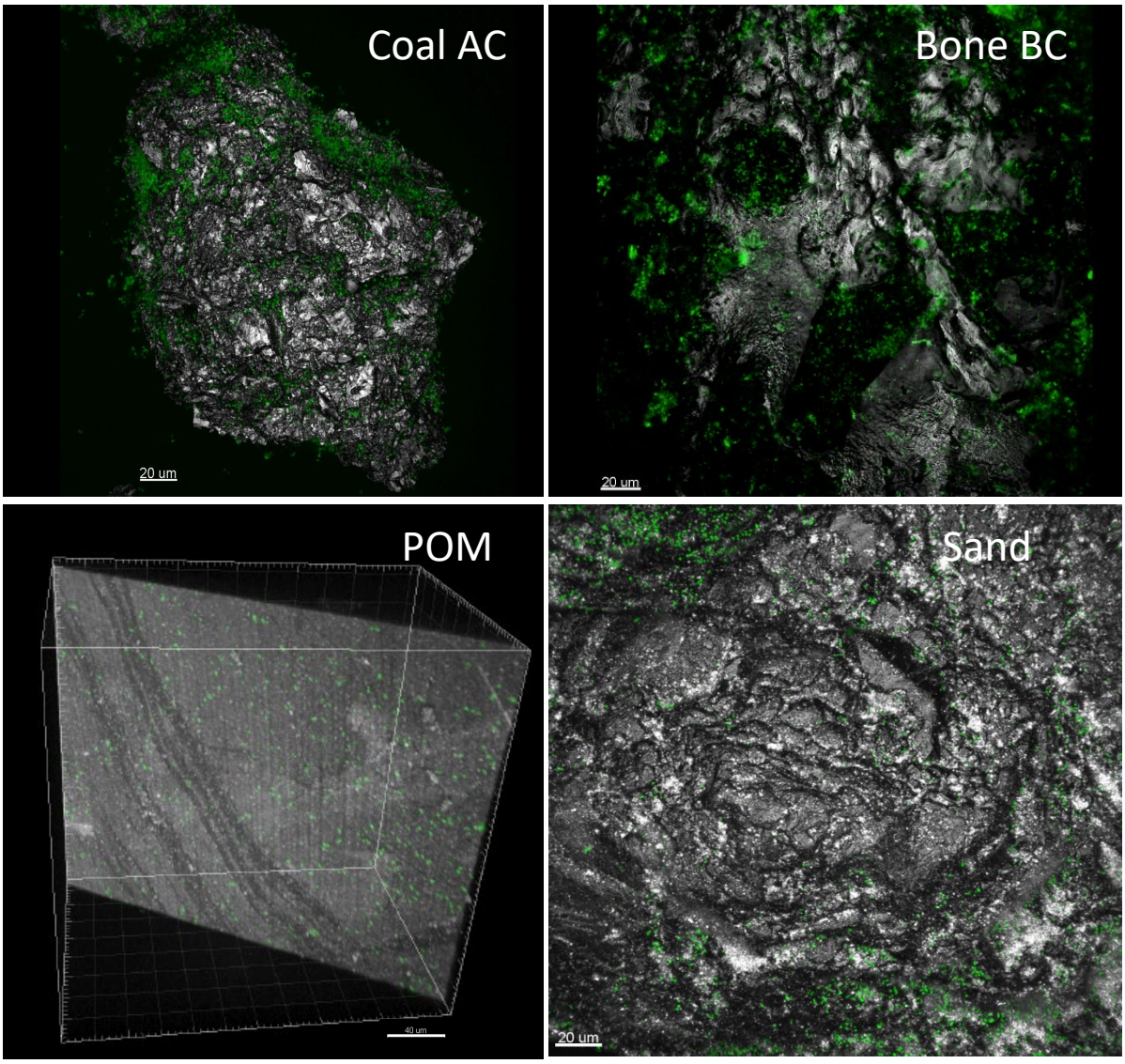

Figure 6.1 Confocal Laser Scanning Microscopy with SYBR Green stain of *Dehalobium chlorocoercia* DF-1 biofilm formed on the surface of coal activated carbon (Coal AC), bone biochar (Bone BC), polyoxymethylene (POM), and sand. From left to right: The scale bar is 20 µm for Coal AC, 20 µm for Bone BC, 40 µm for POM, and 20 µm for Sand.

Parameters for biofilm coverage characterization

Surface area (μm^2) calculation function in the Imaris software was used to find surface area for each channel imaged. The surface area of the biofilm coverage was determined by calculating the total area covered with biofilm in a selected field of the fluorescent image compared to the total area of the same selected field calculated in reflection mode (Smith et al., 2016). The surface area calculation parameters were optimized to each sample. DF1 biofilms were often less than 10 µm thick making light penetration of the entire biofilm possible. Thresholding was based

on a background subtraction method to avoid bias measured featured of the biofilm and all voxels greater than zero were collected.

DNA extraction and quantitative polymerase chain reaction (q-PCR)
Enumeration of DF1 cells was performed using quantitative PCR. A total of three microcosm replicates for each treatment were included in the study along with appropriate controls. DNA was extracted from biofilm samples using DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). A 10 order of magnitude dilution series of plasmid DNA containing the 16S rRNA gene was used as a standard for DF1 quantification (triplicate). DF1 biofilm samples were also run in triplicate. The total reaction volume was 25 µL, which included 4.5 µL Ultra-Pure Water (Gentrox, Claremont, CA, USA), 6 µL of template, 12.5 µL of iTaq Universal SybrGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 1 µL each primer [dehcl – 348F: 5' GAG GCA GCA GCA AGG AA 3'] and [dehcl – 884R: 5' GGC GGG ACA CTT AAA GCG 3'] (Fagervold et al., 2005a). The thermocycler program was as follows: 2 min at 95°C, 40 cycles of (95°C for 45 s, 55.7°C for 45 s, 72°C for 30 s). The 40 cycles were followed by an extension step of 72°C for 30 s after which melting curves were generated by increasing temperature from 60°C to 95°C in 0.5°C per cycle increments. The melting curves confirmed that a single PCR product was synthesized in all reactions (BioRad CFX Connect Realtime Systems).

Dechlorination in cultures
PCB 61 dechlorination was evaluated for all DF1 cultures in the experiment. Samples were collected (0.1 mL) and transferred to a clean glass amber vial. Next, 3 mL of hexane (Fischer Scientific, >99% purity, ACROS Organics) was added as well as a mass recovery standard (tetrachloro-m-xylene, TCMX) (Accustandard, >97% purity, CAS 877-09-8). The samples were vortexed for two minutes and rested for 10 minutes for separation of the organic and aqueous phases. The organic layer was transferred to a clean amber vial and the process was repeated twice. The sample was then concentrated via nitrogen evaporation (model N-EVAP 111, Organomation Associates, Massachusetts) and transferred to a clean vial. PCB 61 (100% purity, CAS 33284-53-6) and 2,3,5- Trichlorobiphenyl, (PCB 23, 99% purity, CAS 55720-44-0) were purchased from Accustandard (New Haven, CT). PCB 61 and PCB 23 were quantified via a five-point calibration curve (triplicate) using the internal standard (2,4,6-Trichlorobiphenyl, PCB 30, 100% purity, CAS 35693-92-6, Accustandard, CT) method of quantification (Lombard et al., 2014a). DF1 can dechlorinate PCB congeners with double flanked *para* or *meta* chlorines and does not dechlorinate PCB congeners without doubly flanked chlorines. Therefore DF1 can solely dechlorinate PCB 61 to PCB 23 (Lombard et al., 2014a). Surrogate recoveries ranged from 66-114% with an average of 88%. All samples were analyzed on an Agilent 7890B gas chromatograph (GC) equipped with an Electron Capture Detector (ECD) using DB-5 (5% diphenyl dimethyl polysiloxane) capillary column (60m × 0.25mm i.d. × 0.25µm film thickness). The temperature program for the DB-5 column was as follows: the initial oven temperature of 70°C was increased at a rate of 7°C min^{-1} to 180°C, followed by 1.05°C min^{-1} to 225°C, then 5.75°C min^{-1} to 285°C, and 11.5°C min^{-1} to a final temperature of 300°C, holding for 20 min. Samples in 1 µL were injected in splitless mode using helium as a carrier gas and nitrogen as a make-up gas. The injector and detector temperatures were 250 and 300°C, respectively. PCB 61 was dechlorinated by DF1 biofilms in microcosms containing materials and the average level (mol%) of daughter products as a sum of parent and daughter products was calculated over the observational period.

Statistical analysis
Biofilm coverage and cellular biomass thickness were examined via t-test (assuming unequal variances) to determine whether there were significant differences in coverage at the 95% confidence level. Statistical analysis was performed using Microsoft Excel Data Analysis ToolPak (version 15.26).

Results
Biofilm formation on sorptive surfaces
Biofilm growth and the relationship with selected surface characteristics was examined via CLSM (Figure 1). Results showed that the biofilm coverage increased during the experiment for all materials (Figure 2). Biofilm coverage between sampling time-points was examined via t-test to assess growth over time. There was a statistically significant difference in biofilm coverage for materials between 22 days and 133 days and between 22 days and 159 days ($p < 0.05$), thus showing significant biofilm formation over time for all materials tested. This indicated that the biofilm actively developed on the materials. Although there was more biofilm coverage on coal AC and bone BC than both POM and sand for all time points (Figure 2), there is no statistically significant difference between biofilm coverage on the materials tested. Evaluation of the biofilm coverage on the sediment particles from Grasse River showed little biofilm formation after 133 days (2.2% ± 0.03%) thus preferential attachment and subsequent biofilm formation on the amendment materials occurred. Furthermore, the DF1 biofilm biovolume (i.e., the volume of the cellular biomass attached to the material surface) after 133 days for coal AC, bone BC, POM, sand, and Grasse River sediment were $9.87x10^3 \pm 7.76x10^3$ μm^3, $1.18x10^4 \pm 6.41x10^3$ μm^3, $3.14x10^3 \pm 6.55x10^2$ μm^3, $5.69x10^3 \pm 2.54\ x10^3$ μm^3, and 628 ± 112 μm^3, respectively.

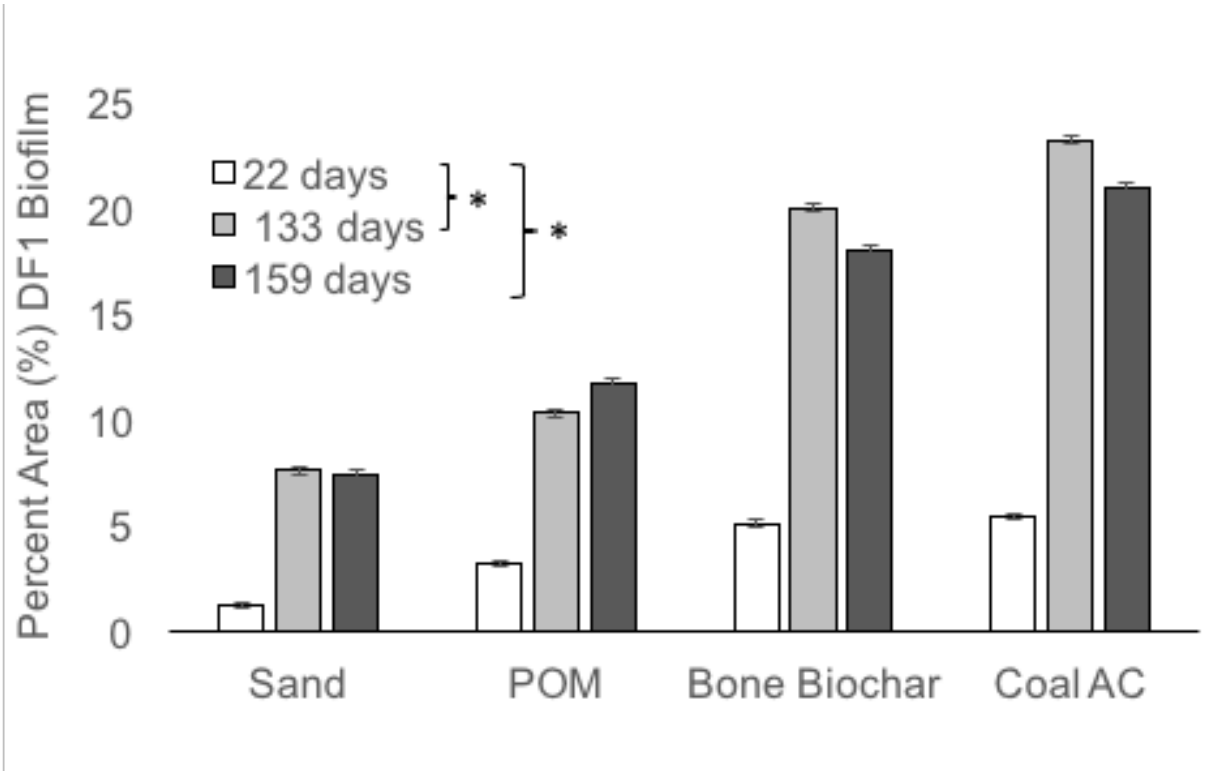

Figure 6.2 Biofilm area coverage of DF1 biofilm on evaluated materials. Error bars show standard deviation (n = 10 images). The asterisk indicates a statistically significant difference between time-points.

To further evaluate the importance of the material characteristics on the biofilm coverage, the data were compared to numerical material characteristic obtained from the manufacturer (Figure 3). For this purpose, the difference in coverage was calculated over a 137-day observation period for each material making it possible to observe trends resulting from sorption capacity on biofilm development. The results showed that the biofilm coverage increased with increasing sorption capacity as DF1 formed more extensive biofilms on coal AC and bone BC. Evaluation of the biofilm cellular thickness in DF1 biofilms showed that the biomass remained constant on coal AC and averaged approximately 4.4 ± 0.56 µm over 159 days (Figure 4). DF1 biofilm cellular thickness averaged 5.1 ± 0.55 µm on bone BC, however thickness decreased after 133 days. Lastly, DF1 biofilm cellular thickness increased on both POM and sand over the experiment and averaged 5.2 ± 0.31 µm and 4.8 ± 0.95 µm over 159 days, respectively. A t-test revealed that there was no statistically significant difference in cellular biomass thickness between the materials tested.

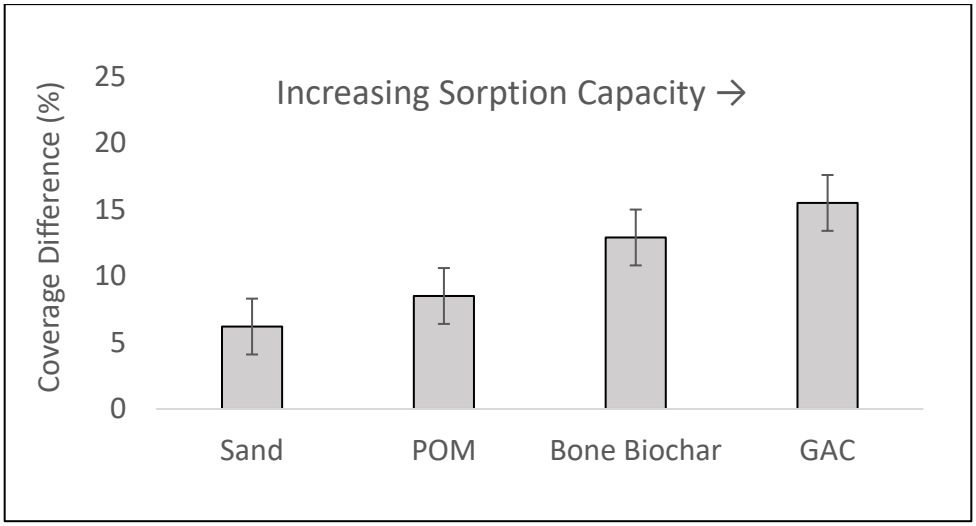

Figure 6.3 Difference in the surface area coverage of DF1 biofilms on evaluated materials. The plot is organized by increasing value of sorption capacity. Error bars show standard error (n = 10 images).

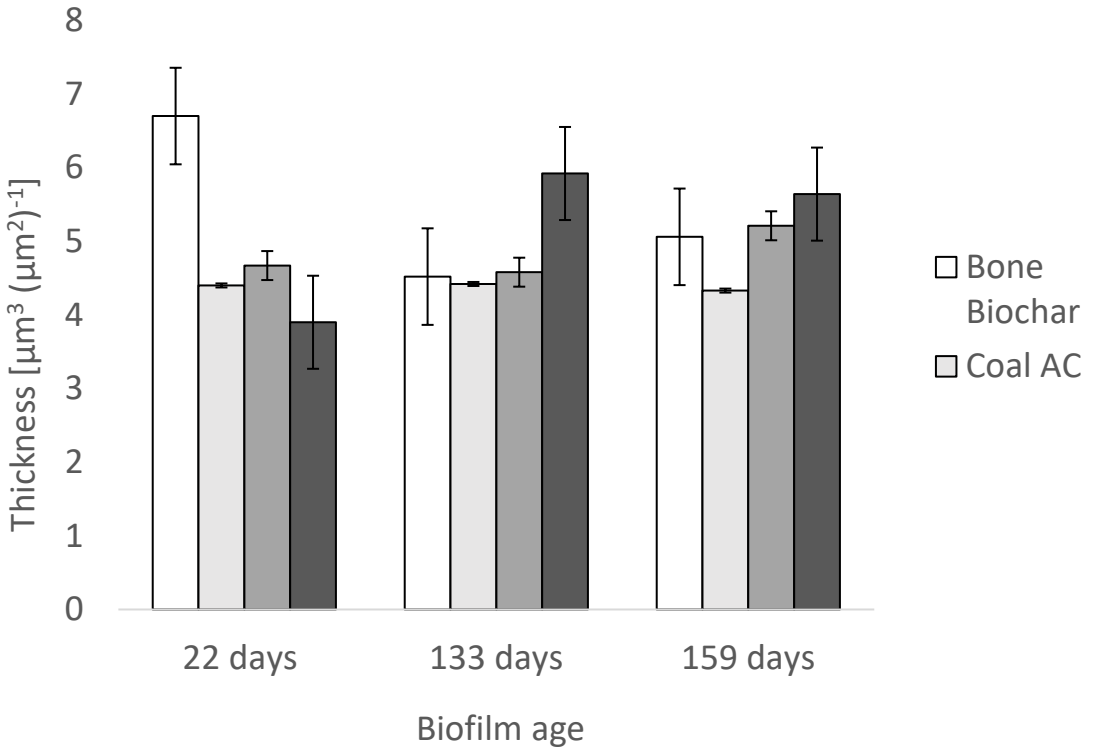

Figure 6.4 Thickness of DF1 cellular biomass on evaluated materials. Error bars show standard error.

Quantification of DF1 presence in dehalorespiring biofilms

Biofilm growth was also examined via enumeration of the bacteria in the biofilm via qPCR. In all experiments, a larger fraction of the total number of bacteria was associated with the materials than the liquid (>99%). Therefore, the observed dechlorination was performed by DF1 in the biofilms. After 22 days, coal AC, bone BC, POM, and sand microcosms had dechlorinated 73%, 93%, 100%, and 83% respectively. Simultaneously, the abundance of DF1 bacterial cells in the biofilms increased for AC, BC, POM and sand throughout the experiment (Table 2).

The evaluation of DF1 biofilm with microscopy and molecular analyses corresponded since the biofilm coverage and the abundance of DF1 cells increased during the experiment. However, the number of bacteria did not increase linearly as the biofilm coverage increased. The reason for this was likely that the biofilm was made up by a combination of bacterial cells and EPS that embed the bacteria in the biofilm. In this study, observations showed that an early biofilm formed by day 22 as indicated by the sparse biofilm coverage on each material tested. Although EPS is as an integral part of biofilm, this study did not identify the components of the extracellular material.

Table 6.2 Quantification *Dehalobium chlorocoercia* DF-1 biofilms formed on evaluated materials using quantitative-PCR. Values are reported as number of bacteria per gram of material dry weight with standard deviations in brackets.

Bacteria/g	22 days	133 days	159 days
Coal AC	8.7×10^6 (3.53×10^6)	3.1×10^9 (1.42×10^9)	6.1×10^9 (2.85×10^9)
Bone BC	3.6×10^7 (8.30×10^7)	2.1×10^{10} (1.29×10^{10})	1.5×10^{10} (2.07×10^{10})
POM	2.9×10^6 (3.74×10^6)	2.1×10^{10} (4.48×10^{10})	1.2×10^9 (1.54×10^9)
Sand	1.3×10^6 (7.43×10^6)	3.4×10^9 (1.45×10^9)	3.5×10^9 (5.93×10^9)

Discussion

Carbonaceous materials such as AC and BC have been applied to treat PCB-contaminated sediments and such technologies include *in situ* capping of a layer of the carbon amendment or mixing the carbon into contaminated sediments (Gomes et al. 2013, Rakowska et al. 2012). This study demonstrated the capability of *D. chlorocoercia* DF-1 biofilms to effectively form on a variety of materials including coal AC, bone BC, POM and sand. Sorptive materials such as coal AC and bone BC served as efficient growth surfaces and active PCB dechlorinating biofilms formed. Materials with sorptive characteristics can adsorb the PCBs and over time established an equilibrium with a steady forward and reverse transfer of the electron acceptor (i.e., PCB). The combination of the equilibrated carbon and formation of a biofilm (bacteria and EPS) aids in desorption of PCB that acts as electron acceptor for DF-1 and the biofilm matrix will assist in providing a 'protected' region with a carbon source and electron donor. Biofilms provide a durable structure and thus protection of the microorganisms from the surrounding environment (Edwards and Kjellerup, 2013a). Microorganisms living within a biofilm are safeguarded from predation, physical and chemical stresses, and can support multiple microbial species with diverse metabolic requirements and preferences (Edwards and Kjellerup, 2013a).

Macedo et al. (2005) demonstrated the complexity of the biofilm matrix using CLSM, which contained aerobic and anaerobic niches within mature biofilms. In the latter study, a microbially diverse biofilm community was grown on Aroclor 1242 transformer oil and the results showed biodegradation of lightly chlorinated congeners as well as dehalorespiration of higher chlorinated congeners (Macedo et al., 2005). In addition, the results from this study were supported by Mercier et al. (2013) that observed aerobic biofilm development within two months on activated carbon in a mixed AC-sediment laboratory study. The ability of indigenous PCB-degrading microorganisms to grow as biofilms on AC in the presence of sorbed PCBs was demonstrated. PCB adsorption, and a mixture of AC and aquatic sediments did not influence adsorption and PCB adsorption did not influence biofilm development (Mercier et al., 2013). Additionally, sorption of PCBs to biofilm did not show interferences with PCB biodegradation (Borja et al., 2006; Vasilyeva et al., 2010). Borja et al. (2006) reported a PCB removal of 80-92% in one day.

However, the development of expansive biofilms on AC might affect the affinity of the carbonaceous material for sorption of PCBs (McDonough et al., 2008a).

Utilizing a biofilm-based inoculum is an encouraging option for bioremediation of PCB-contaminated sediments. Moreover, bioaugmentation with carbonaceous amendments and simultaneously utilization as a microbial delivery vehicle can provide an efficient and cost-effective method for delivering microorganisms for *in situ* bioremediation while concurrently sequestering toxic pollutants. A biofilm-based delivery system compared to traditional liquid bioaugmentation benefits from: 1) bacteria growing at a high density at the surface of adsorbent material thus preventing washing away, 2) actively growing bacteria that can dechlorinate at deployment instead of passing a lag phase experiencing new conditions, 3) adsorption of PCBs onto the material enables the bacteria to align in close proximity to the PCBs as electron acceptor thus ensuring a constant supply, 4) the biofilm community has a larger cell density than would be possible to obtain in free-floating systems, where the chances for direct interaction between PCBs and PCB dechlorinating bacteria are rare, and 5) the dehalorespiring bacteria are fixed to the surface of the particles so they will not be washed away or consumed by other microorganisms in the environment, increasing the potential for successful long-term bioaugmentation. Finally, examining biofilms via CLSM techniques allows for the close monitoring of biofilm formation which may aid in quality assessment of the biofilm inoculum for prospective bioremediation applications.

Supplemental Information

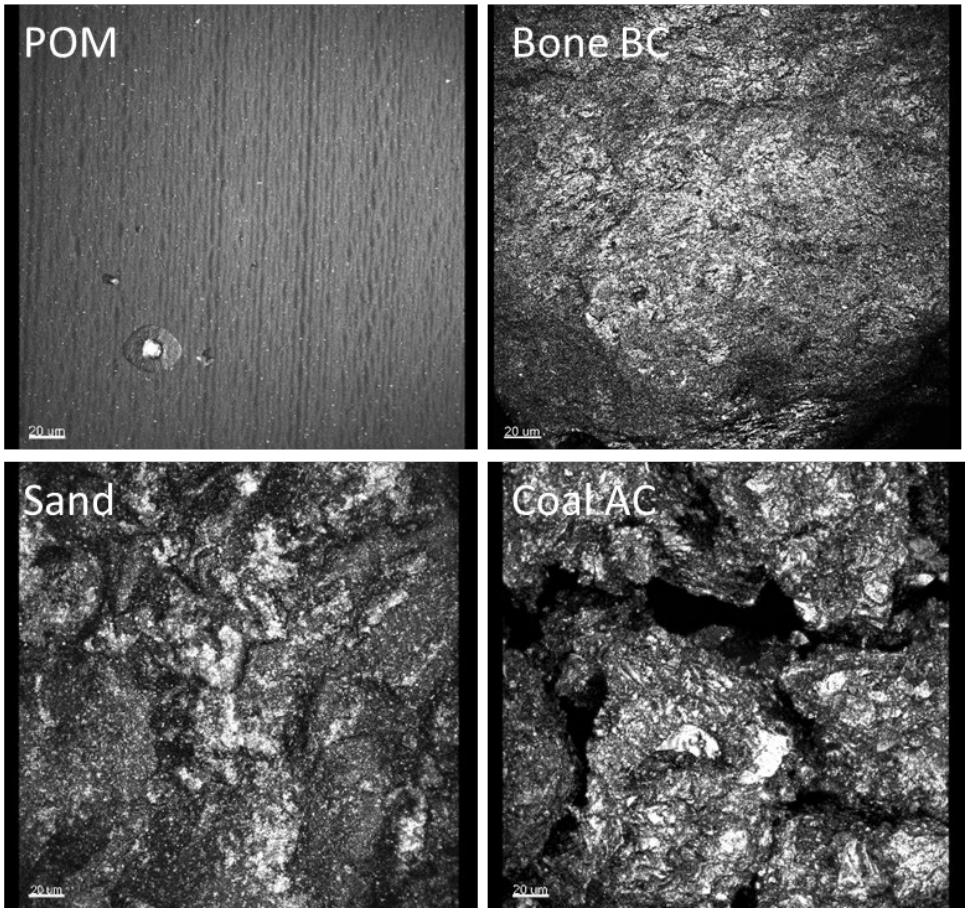

Figure 6.S1 CLSM imaging of the negative controls of the applied materials: POM, Bone BC, sand and coal AC. The results showed that non-selective binding of SYBR Green nucleic acid stain to cellular materials and materials did not occur. Scale bar = 20 µm.

SEM Images

Images were taken using a cryo field emission scanning electron microscope (Supra 55VP, Zeiss, Thornwood, NY) in the Image and Chemical Analysis Laboratory at Montana State University. The samples were prepared for SEM-cryo imaging by cutting them ~1mm thick with stainless steel razor, where they were mounted on SEM-cryo sample holder and frozen in liquid Nitrogen. Frozen sample holder with samples were transferred to the SEM-cryo stage that was cold to -193ºC. After loading the holder to the stage and pumping down the instrument, the temperature was set to -80ºC (vapor pressure of ice) to sublimate the ice on the samples and the stage. After sublimation, the temperature was set to -193ºC again and the images were taken with a primary electron beam energy of 1keV at different magnifications.

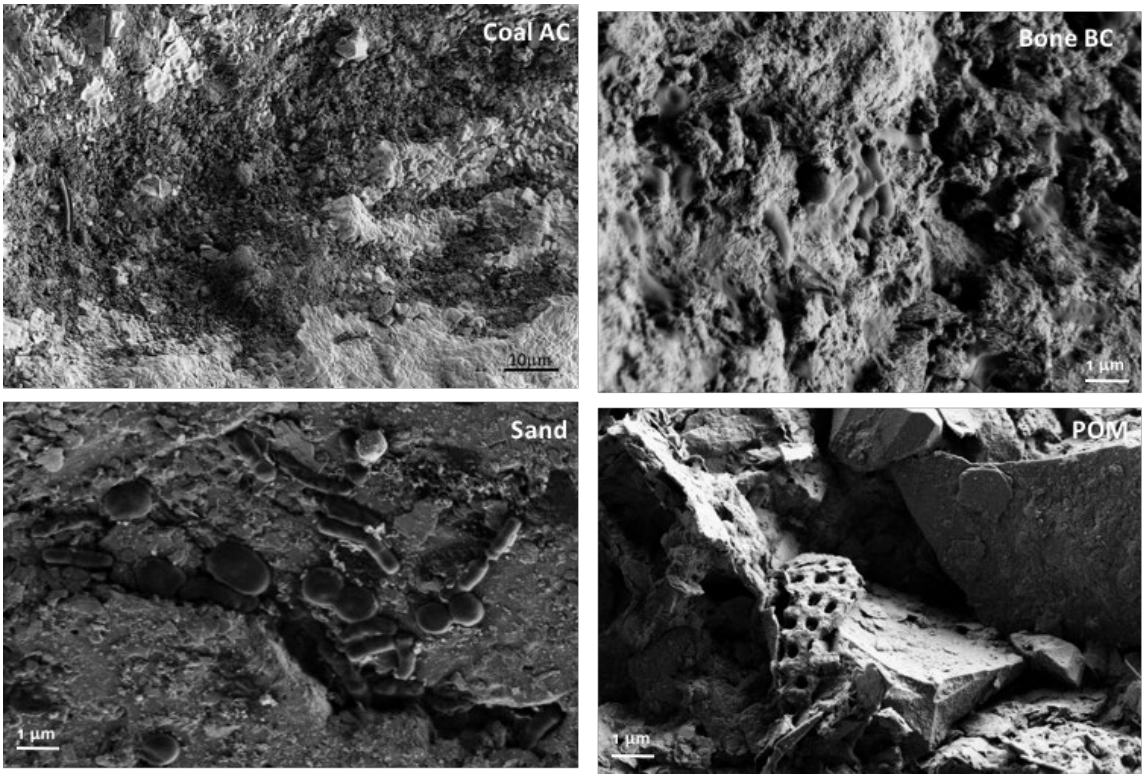

Figure 6.S2 Scanning Electron Microscopy image showing formed biofilm *Dehalobium chlorocoercia* DF-1 on the surface of coal activated carbon (Coal AC). Images from Left to Right: The scale bar is 10 µm for Coal AC, 1 µm for Bone BC, 1 µm for Sand, and 1 µm for POM.

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Chapter 7.
Application of Biofilm-based Inoculum Delivery System for Organohalide Respiration of Polychlorinated Biphenyls (PCBs) in Sediments

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Highlights
- Dechlorination of weathered PCBs in Grasse River sediment occurred in the presence of biochar, liquid bacterial inoculum and biofilm formed on biochar. All treatments improve the dechlorination efficiency, but the dechlorination rate was highest for the biofilm inoculum.
- Contaminated sediment that was bioaugmented with biofilms formed on biochar showed enhanced microbial community stability thus making this approach advantages for field applications of bioaugmentation.

Abstract
This study aims to utilize organohalide respiring bacteria as a biofilm on surfaces of pinewood biochar as an enhanced bioaugmentation approach. Polychlorinated biphenyl (PCB) degrading biofilm on surfaces of adsorbents has the potential to enhance the PCB degradation capacity. The formation of the biofilm can provide a higher cell density as compared to that of a traditional free-floating bacterial delivery system in addition to simultaneously sequestering PCBs from the sediment matrix. High sorption capacity of pinewood biochar can adsorb PCB molecules, so they are in close proximity to the biofilm resulting in increased interaction between the organohalide biofilm and the hydrophobic and adsorbed PCBs. In this study, biofilms made up by the organohalide-respiring bacteria *Dehalobium chlorocoercia* DF-1 were formed on the surface of pinewood biochar. The biochar-biofilms were subsequently applied to PCB contaminated sediment from the Grasse River, NY, USA. The goal was to evaluate the organohalide respiration of the PCB contaminated sediment in the absence/presence of the biofilm and free-floating bacterial inoculum. This approach provides an efficient method for utilizing organohalide respiring bacteria for PCB bioaugmentation thereby increasing the potential for long-term and sustained organohalide respiration in sediment.

Introduction
Polychlorinated biphenyls (PCBs) are a group of persistent organic pollutants (POPs) and they are of serious environmental concern (Cheng and Hu 2010, Jing, Yasir et al. 2019). They bioaccumulate in the food chain due to their lipophilicity and remain in the environment for a long period, when they are released from anthropogenic sources. Over the past decade, PCB remediation technologies such as bioremediation, phytoremediation, dehalogenation by a chemical reagent, thermal methods, and PCB removal by pinewood biochar have been widely studied (Wu, Chen et al. 2012, Passatore, Rossetti et al. 2014, Jing, Fusi et al. 2018). However, some of these remediation technologies are disruptive and unsustainable. The current effort is to identify an alternative and sustainable remediation approach for PCBs. An enclosed system such as mesocosms is an important experimental tool for the laboratory scale organohalide respiration of PCBs from contaminated soils or sediments (Zhang, Gannon et al. 2010). However, a normal mesocosm system of a weathered PCBs remediation study, has a limited performance due to

their low concentration and low bioactivities of natural organohalide-respiring microorganisms (Jing, Fusi et al. 2018). To overcome these challenges, this study utilized a method to concentrate a culture of organohalide-respiring bacteria i.e., *Dehalobium chlorocoercia* DF-1 onto surfaces of the absorbent materials (here pinewood biochar). Such a PCB-degrading biofilm on surfaces of adsorbent can enhance the PCB degradation capacity (Edwards and Kjellerup 2013, Kjellerup, Naff et al. 2014). The formation of biofilm can provide a higher cell density as compared to that of a free-floating bacterial delivery systems (Jefferson 2004). High sorption capacity of pinewood biochar particles enables adsorption of PCB molecules in close proximity to the bacteria result in interaction between organohalide-respiring microorganisms and hydrophobic PCBs (McDonough, Fairey et al. 2008, Liu, Zhang et al. 2015).

Studies indicated that *Dehalobium chlorocoercia* DF-1 (originating from Charleston Harbor, SC) can survive in contaminated soil was also capable of dechlorinating the highly chlorinated PCBs (May and Sowers 2016). More specifically, PCB organohalide respiration of a DF-1 biofilm inoculum has been shown in the recent bioaugmentation studies (Nuzzo, Negroni et al. 2017). Payne et al. (2011) implemented a bioaugmentation study in 2-L mesocosms with *Dehalobium chlorocoercia* DF-1 and 1.3 ppm of weathered Aroclor 1260 contaminated sediments from Baltimore Harbor, MD (Payne, May et al. 2011). The total *penta*-chlorinated PCBs decreased by 56% in the bioaugmented mesocosms after 120 days. The results of the study indicated that bioaugmentation with *Dehalobium chlorocoercia* DF-1 can enhance the dechlorination of doubly flanked chlorines of weathered PCBs. In another study, Kjellerup et al. (2012) evaluated the effects of anaerobic bioaugmentation of soil samples (Mechanicsburg, PA) contaminated with 4.6 to 265 ppm of Aroclor 1260 by using *Dehalobium chlorocoercia* DF-1 (Kjellerup et al. 2012). A change of the PCB homolog distributions indicated that the dechlorination of weathered PCBs occurred. The proportion of the hepta- and octa chlorinated congeners decreased by 14% and 5%, respectively.

Biofilms have a complex architecture in which microorganisms can exist in communities, where they are protected from external shear forces and predators (Stoodley, Sauer et al. 2002). A complex structure can provide efficient access to nutrients thereby resistance and tolerance towards changes in environmental conditions such as pH, redox and nutrient changes (Flemming and Wingender 2010). In addition, a highly organic porous surface such as the one present at pinewood biochar particles have high affinities for simultaneous attraction of biofilm forming organohalide respiring microorganisms and adsorption of PCB molecules (McDonough et al., 2008). Both the biofilm and the absorbent with a highly porous surface are essential components for bioaugmentation of weathered PCBs in sediments using a biofilm based approach (Capozzi, Bodenreider et al. 2019).

One of the objectives in this study was to evaluate biofilm formation of organohalide-respiring microorganisms i.e., *Dehalobium chlorocoercia* DF-1 on the surface of pinewood biochar. The organohalide reduction performance of the DF-1 biofilm covered pinewood biochar on the PCB contaminated sediments was also evaluated and compared with that of the liquid *Dehalobium chlorocoercia* DF-1 appliction. This biofilm based delivery system could be essential for future application to PCB contaminated sites with low-concentration of weathered PCBs. In addition, this approach has the potential to provide an efficient and sustainable approach for delivery of

microorganisms for PCB bioaugmentation thereby increasing the potentials for successful long-term bioaugmentation.

Materials and methods

Inoculation of Dehalobium chlorocoercia DF-1 culture and biofilm

The *Dehalobium chlorocoercia* DF-1 culture was inoculated with 75 ml of E-Cl medium in a 160 ml of serum bottle spiked with 2 ppm PCE. The protocol of the E-Cl medium was prepared as described in the previous study (Miller et al. 2005). A propionate stock solution was prepared in 100 ml of boiling water with a final concentration of 1 mM in a fume hood under N_2 flow as a mix of carbon sources. 1 ml of propionate (1 M) was then added to the culture as the carbon source at a final concentration of 2.5 mM (Wu et al., 2000). All the DF-1 cultures were inoculated in an incubator without shaking at 30℃ for 30 days. After 30 days, the headspace of DF-1 cultures was flushed under a N_2 gas in a flume hood to remove residual PCE and the dechlorinated degradation products. After identifying the bioactivity of the DF-1 culture, 20 ml of DF-1 culture was then transferred to 80 ml of fresh E-Cl medium with 3 g of pinewood biochar particles and 56 ppm PCE (liquid phase concentration). Finally, the E-Cl medium inoculated with DF-1 cultures and pinewood biochar particles were inoculated in an incubator without shaking at 30 ℃.

Assessment of biofilm growth on pinewood biochar

In this study, a GC-FID (Agilent Technologies, Inc, Santa Clara, California) was used to analyze the potential PCE degradation products (i.e., Trichloroethylene (TCE), 1,2-Dichloroethene (DCE), and vinyl chloride (VC)) from the headspace of the DF-1 culture by utilizing a hydrogen flame to oxidize organic molecules and produce ions. To measure the gas components, 5 mL of gas from the headspace of the inoculation bottles was extracted by using a 1 ml gas syringe (Valco Instruments Company Inc., Houston, Texas). The GC program used in this study was shown as the following: a carrier gas (N_2) flow of 3 ml/min1, column temperature of 180 °C, an injection volume of 60.0 µL with a splitless mode; a temperature program starting at 40 °C, increasing to 75°C at 20 °C/min, holding for 0.75 min, then increasing to 150 °C at 45 °C/min and holding for 0.35 min, and an injector temperature of 200 °C. A blank sample of nitrogen was also taken and measured through the GC system.

Mesocosm setup

Mesocosms were conducted with DF-1 biofilm-based inoculum with sediments collected from the Grasse River, NY, USA. For each mesocosm, 170 -180 g of sediment samples with 150 ml of E-CL medium and DF-1 biofilm inoculum were added into 250 ml of serum bottles (Thomas Scientific inc., Swedesboro, New Jersey) in triplicates. The bacterial abundance for both DF-1 biofilm and liquid inoculum was quantified using qPCR assays with 348f/884r primers specific for the 16S rDNA of organohalide-respiring bacteria. The DF-1 biofilm and liquid inoculum were then added into the mesocosm sediments to maintain an abundance of 1.5 to 1.6×10^7 gene copies/g sediments. The PCE accumulated in the biofilm covered pinewood biochar were purged out by using N_2 gas in a fume hood before adding them to the mesocosms to avoid the interference of the organohalide respiration of the weathered PCBs by PCE as the electron acceptor. The bottles were sealed with Teflon septa and secured with aluminum crimp caps and incubated in the dark at 30℃. A negative control was set up in triplicate to determine the organohalide respiration of the indigenous bacteria in the sediment. In addition, an abiotic

control experiment with and without pinewood biochar were also set up under similar conditions but with autoclave killed-sediment samples. To avoid exposure of the cultures to air, all serum bottles were set up in an anaerobic chamber (Coy). The mesocosm experiments of biofilm inoculum and liquid inoculum are shown in Table 1. Finally, a gas chromatography (GC) analysis for the individual mesocosms was established to assess whether a statistically significant transformation of the weathered PCBs occurred.

Table 1 Mesocosm experiments with DF-1 biofilm and liquid inoculum

Mesocosms	Experimental setup
Negative control	Grasse River sediment
Abiotic control	Grasse River sediment with autoclaved
Biochar adsorption group	(Grasse River sediment + pinewood biochar)×3
Bioaugmentation group 1	(Grasse River sediment + liquid DF-1 culture)×3
Bioaugmentation group 2	(Grasse River sediment + DF-1 biofilm)×3

DNA extraction

For each mesocosm, DNA was extracted in triplicate by using the MoBIO PowerSoil DNA Extraction Kit (Qiagen Sciences Inc., Germantown, Maryland). 0.25 g of sediment samples in triplicate were collected and added into the Zirconia/Silica Beads tubes. After that, all samples in the tubes were horizontally shaking on a beat beater at speed "4.5" using a FastPrep120 (Q-Biogene, Inc., California) for 10 min. The rest of the extraction steps involving proprietary reagents and spin filters were performed according to the protocol provided by the manufacturer. The final extracts were eluted in 100 µL of solution C6 and stored at -80°C. Finally, the extracted DNA samples were analyzed on the nano-drop (Fisher Scientific, Hampton, New Hampshire) to measure the concentration of the DNA and the purity. Each DNA had a A260/280 ratio of ≥1.6 and an A260/230 ratio of ≥2.0 (Payne et al., 2017).

Identification and enumeration of Dehalobium chlorocoercia DF-1

Polymerase chain reaction (PCR) was applied for the evaluation of the presence and abundance of *Dehalobium chlorocoercia* DF-1 in the mesocosm samples. In this study, the DF-1 biofilm samples with the pinewood biochar particles were centrifuged at 10000 g for 3 minutes. The pellets were then collected into a 1.5 ml tube and sonicated at 1000 Hz for 60 minutes to separate the biofilm and pinewood biochar particles. The DNA for each mesocosm sample was extracted in triplicate as described above. After that, 25 µl PCR reactions were set up with 2 µl of the extracted DNA, 1 µl of forward primer, 1 µl of reverse primer, 12.5 vl of DreamTaq Green PCR Master Mix (Fisher Scientific, Hampton, New Hampshire), and 8.5 µl of DNA free water. The applied PCR program was the following: initial denaturation at 95°C for 2 min, 95°C for 45 sec, 58°C for 45 sec, 40 cycles at 72°C for 60 sec, 72°C for 30 sec. A PCR primer set (348F/884R) was designed to select 16S rRNA gene amplicons of all putative PCB-*Dechloronating Chloroflexi*. The specificity of the PCR products was confirmed by performing gel electrophoresis at a voltage of 100 V for 40 minutes on an ethidium-bromide-stained agarose gel to confirm that the size of the product band was 536 bp.

In this study, a quantitative PCR (qPCR) assay specific for the 16S rRNA of DF-1 was used to assess the abundance of the biofilm coated pinewood biochar particles. Enumeration was performed by using iQ SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, California) and primers specific for the 16S rRNA gene of *Chloroflexi* (348F/884R) as described previously. The qPCR program was applied as the following: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 s, 55 °C for 25 s, and 72 °C for 25 s. The detection limit of all qPCR reaction was 3×10^2 gene copies/g sediment (Payne et al., 2013). The final gene copy numbers for each sample were determined by standard curves based on five dilutions of DNA samples with known abundance of microorganisms using the same qPCR procedure as described for the biofilm inoculums.

Microbial community analysis of mesocosms

16S rRNA amplicon sequencing (Miseq paired-end) was performed for all samples by using universal primers covering all bacteria (using V3-V4 primers). In addition to that, samples were sequenced by using putative PCB dechlorinating Chloroflexi specific primers (using 384F/884R). Preprocessing of the raw sequence data included read merging, quality trimming, artifact and contaminant removal. Next, high-quality passing sequences were clustered into OTUs de novo with the curated SILVA database.

Imaging using Scanning Electron Microscopy

A Zeiss Supra 55 field emission scanning electron microscope (Carl Zeiss AG, Oberkochen, Ostalbkreis) was used for SEM analysis of DF-1 biofilm samples after 45-days of inoculation and an abiotic control (Pinewood biochar) without biofilm inoculation. Elemental analysis with EDX was conducted with the Scanning Auger Electron Nanoprobe- Physical Electronics 710 (Physical Electronics, Inc., Chanhassen, Minnesota). Firstly, the pinewood particles covered with DF-1 biofilm and the abiotic control was placed on double-sided carbon glue tape and dried in air. The samples were then coated with a thin film of gold on the surface of the sample to prevent charging and put into a vacuum chamber for image analysis. SEM imaging analysis was conducted with 1 keV primary electrons with the focus to detect the presence of biofilms and individual bacteria located at the samples. Imaging was performed at x3000, x10000, x15000, x30000, and x65000 magnifications at a working distance of 5-8 mm. EDX analysis was conducted using Integrated Auger Nanoprobe. Four to five different areas were selected for each sample and the elemental distribution of each region was mapped for both DF-1 biofilm covered pinewood particles and the abiotic control. In addition, EDX spectra from the selected area of each region were obtained to determine the elemental composition of the surface coverage (i.e. biofilm) of that location.

PCB sampling, extraction, and analysis

Microwave Assisted Extraction (MAE) was used to extract PCBs from the sediment samples from the mesocosms. 3 grams of sediment (dry weight) samples were carefully transferred to the extraction vessels. 3 grams of clean sea sand (Merck) was used as a blank control and 30 ml of analytical grade hexane-acetone (1:1) was added to the vessels. Prior to extraction, 20 µL of the mixed solution of surrogates (0.5 µg mL-1 2,4,6-trichlorobiphenyl (PCB-30) and 2,2’,3,4,4’,5,6,6’-octachlorobiphenyl (PCB-204)) were added into each sample to account for losses during the extraction process. After that, MAE was performed at 115°C for 10 min at 1000 W. After extraction, the vessels were allowed to cool to room temperature before they were

opened. The extracts subsequently settled and the supernatant was collected using a glass Pasteur pipette. The residues were washed with hexane, hexane-acetone (1:1), acetone all of analytical grade quality, respectively. The supernatants were collected and concentrated to less than 1 mL by using nitrogen blowdown. Finally, hexane was added to dissolve the extracts to exactly 1.0 ml and stored at -20°C until further analysis.

The protocol for cleanup of the PCB extracts was conducted as follows: Copper was washed with sulfuric acid, dried under N2 flow and stored in a desiccator. Pasteur pipettes packed with glass wool covered with a layer of sodium sulfate, followed by a layer of mixture of copper and florosil (1:4), then a layer of sodium sulfate on top was used for purification of the extract. The columns were washed with analytical grade hexane prior to purification of the extracts. The extracts were transferred into the purification column and the effluent was collected and subsequently reduced in volume to 1.0 ml. Internal standards (20 µl of the mixed solution of 50 ng mL-1 4-bromobiphenyl and 2,2',4,5,5'-pentabromobiphenyl) for sediments at day 0, 50 ng mL-1 Tetrachloro-m-xylene (TCMX) and 2,2',4,5,5'-pentabromobiphenyl for sediments at day 466, respectively) were added for further analysis. Finally, the samples were analyzed by gas chromatography (Agilent 7890B) with an electron capture detector (GC-ECD) (Agilent Technologies, Inc., Santa Clara, California). Agilent J&W HP-5ms column (60 m × 250 µm × 0.25 µm) was used. The carrier gas was Nitrogen, and the injection was in splitless mode with a volume of 1 µL. The temperature of the oven was raised from 70°C to 180°C at 7°C min^{-1}, from 180°C to 225°C at 1°C min^{-1}, from 225°C to 285°C at 5.8°C min^{-1} with isothermal hold at 285°C for 20 min, then ramped to 300°C at 11.5°C min^{-1} and held at 300 °C for 10 min.

Data Analysis

Recoveries of surrogate standards were in the range of 60% to 120%. Five-point calibration curves for 209 PCBs ($R^2 > 0.98$) were used for quantification. For samples, where outliers were detected due to issues with the extraction and/or analysis procedure, these results were evaluated separately to assess why they were outliers and which influence they would have on the overall analysis. An average value of the total PCB concentration calculated by all the samples at day 0 was also considered and used the total PCB concentration for the negative control.

As a results of DF-1 dechlorination, chlorine atoms located at meta and para positions will be removed from the biphenyl molecule. Chlorine per biphenyl was used in this study to estimate the dechlorination efficiency. Dechlorination extent, calculated as the percentages of chlorine atom that were removed from the biphenyl in the sediment samples, were also calculated.

Chlorine per biphenyl was calculated by formula 1.

$$TCB = \sum_{i=n}^{209} \left(\frac{M_i}{TM}\right) \times CB_i \quad (1)$$

Where i (i = 1··· 209) described the individual PCB congeners, TCB stands for the calculated total chlorine per biphenyl for the sediment sample; M_i is the corresponding molar mass of the individual PCB congener; TM indicates the total molar mass of 209 PCB congeners in the sediment sample.

Dechlorination extent was calculated by formula 2.

$$DE = 1 - (\frac{TCB_{d0}}{TCB_{d466}}) \quad (2)$$

Where DE indicates dechlorination extent of the sediment sample; TCB_{d0} and TCB_{d466} stand for total Cl per biphenyl at day 0 and day 466.

To test whether significant differences between the biofilm covered biochar and other experimental parameters occurred, ANOVA tests and multiple comparisons were performed. The PCB concentrations were measured twice in time series thus chlorine per biphenyl data were calculated from PCB concentrations, repeated-measures ANOVA was used to test the differences between day 0 and day 466. One-way ANOVA was applied to test the reductions of the concentrations from day 0 to day 466, and dechlorination extents among biochar group, liquid group, and biofilm group. Multiple comparisons by Holm's Method were applied to test concentration changes, chlorine per biphenyl changes and dechlorination extents for every two groups. Statistical significance of all tests was defined as 0.05. Abiotic and negative controls were not all set up in triplicate thus ANOVA tests and multiple comparisons were not applied to these controls.

Results and Discussion

Characterization of D.chlorocoercia DF-1 (liquid culture) and bioactivity of PCE dechlorination

DF-1 liquid culture of six inoculation bottles indicated that dechlorination of PCE occurred after 10 days (Figure 7.1). The results of the headspace measurement indicated the PCE decreased from 94% ± 1.2% to 32% ± 29.5% after 10 days. Bottle 6 exhibited the most biodegradation in which 85% of PCE had been dechlorinated. As a result, 69% TCE, 12% DCE, and 10% VC were generated. After 20 days of incubation, approximately 100% of PCE were removed. Correspondingly, 14% ± 4.3% TCE and 81% ± 4.7% DCE were generated. After 30 days of inoculation, the PCE was dechlorinated to 15% ± 2.7% TCE and 81% ± 10.6% DCE. Correspondingly, an average abundance of 5.53×10^8 gene copies/mg for each culture was achieved after 30 days. Similar growth was also observed in the inoculation of PCB-respiring *Dehalococcoides mccartyi* strains with PCE after 30 days (1.2×10^8 to 1.3×10^8 cells/ml) reported by Wang et al. (2014). In contrast, only 5.9×10^6 to 10.4×10^6 cells/ml were obtained after inoculation with Aroclor 1260 for 150 days.

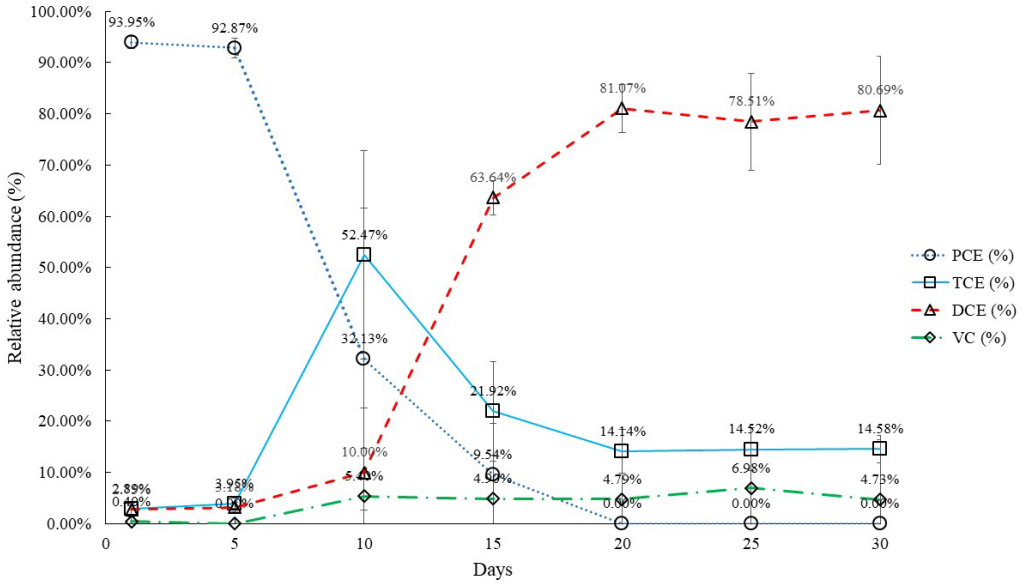

Figure 7.1. PCE dechlorination of DF-1 liquid culture. After 10 days of inoculation, DF-1 culture indicated the dechlorination activities in which PCE biodegraded to DCE.

Characterization of D. chlorocoercia DF-1 (biofilm) and bioactivity of PCE dechlorination
In a second study, *Dehalobium chlorocoercia* DF-1 was initially inoculated with 56 ppm of PCE in the presence of biochar. The biodegradation products (i.e., TCE, and DCE) were detected in the headspace of the mesocosms. After 21 days of incubation, one of ten mesocosms did not show dechlorination activity. According to Figure 7.2, the mole percent of the total PCE in the headspace decreased from 100% to 70.4% ±17.6% for the remaining nine mesocosms, which suggested that the *Dehalobium chlorocoercia* DF-1 biofilm converted PCE to TCE. After another five days, approximately 41.2% of PCE were removed. The mole percent of TCE from PCE increased from 29.6 % ± 17.4% to 37.6% ± 15.4%. Moreover, DCE (3.6% ± 5.9%) was firstly detected during the inoculations. VC was not detected in the headspace of any of the mesocosms during the inoculations. Analysis of the biofilm with PCR also confirmed that biofilm had formed on the biochar particles (Figure 7.3).

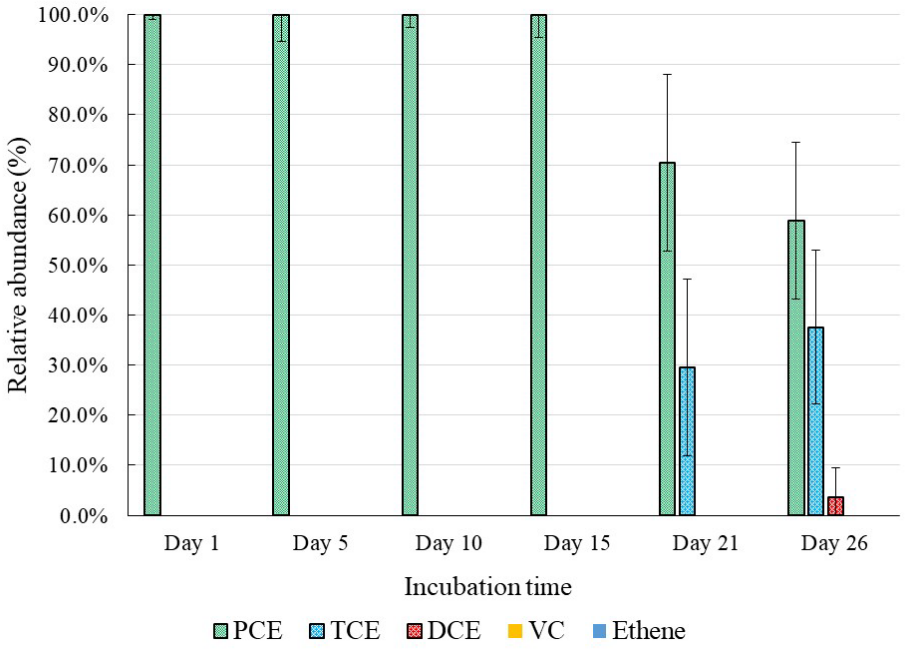

Figure 7.2 PCE dechlorination of DF-1 biofilm in mesocosms. 29.6% of the PCE were transferred to TCE in nine mesocosms after 21 days of incubation.

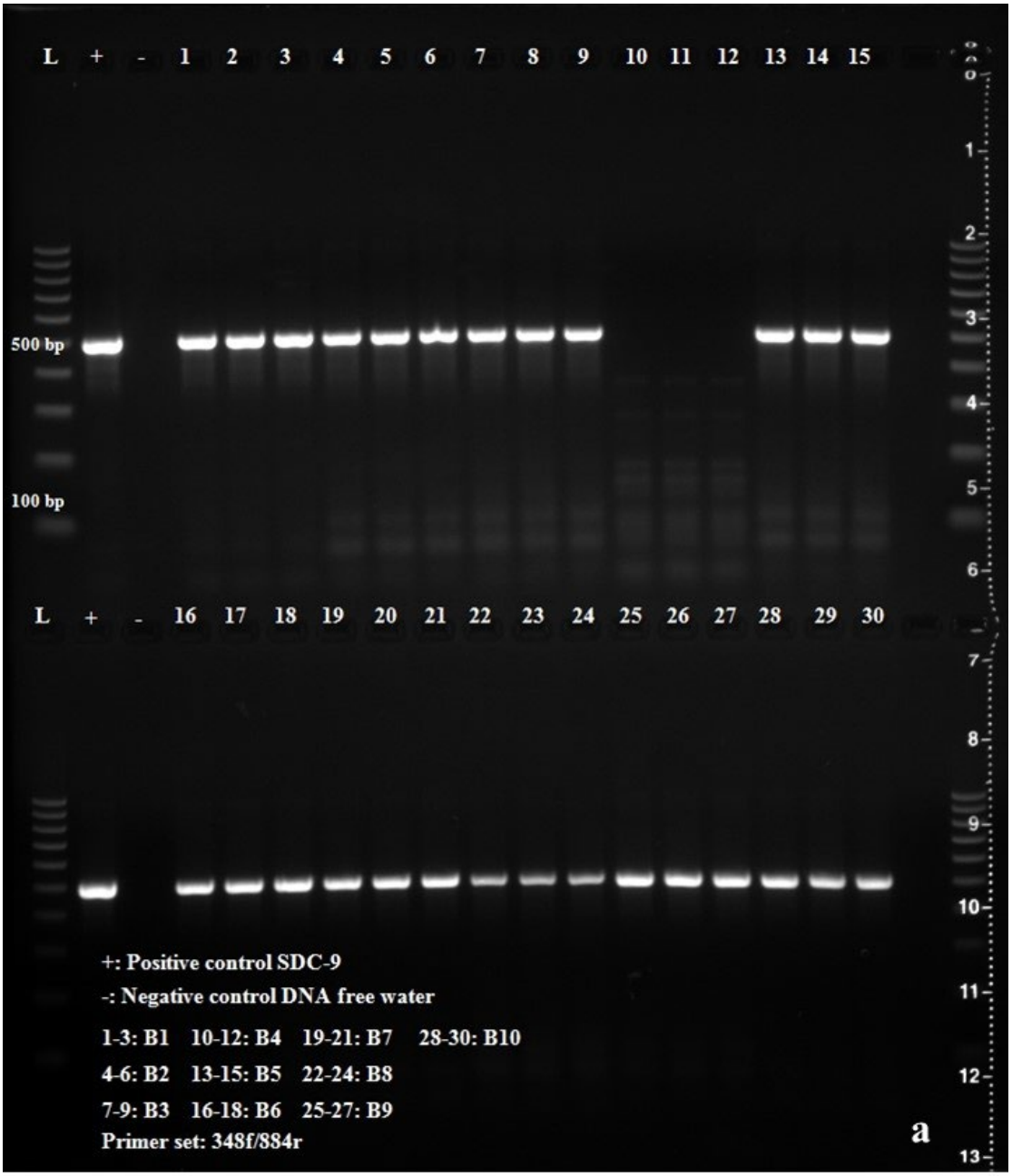

Figure 7.3 PCR of DF-1 biofilm with the 348f/884r primer set. The PCR products of the DF-1 biofilm on pinewood biochar were positive. The results of the qPCR assay indicated that the *Dehalobium chlorocoercia* DF-1 biofilm were successfully inoculated on the surface of pinewood biochar particles during 35 days of incubation.

Table 7.2 Results of q-PCR for DF-1 biofilm with the 348f/884r primer set

	Gene copies of DF-1 biofilm/g pine wood biochar	Mean	Stdev
B1	5.94×10^8 6.61×10^8 5.61×10^8	6.05×10^8	5.11×10^7
B2	5.88×10^8 5.70×10^8 5.80×10^8	5.79×10^8	8.89×10^6
B3	6.95×10^8 7.09×10^8 8.18×10^8	7.41×10^8	6.72×10^7
B5	6.75×10^8 7.08×10^8 6.83×10^8	6.89×10^8	1.70×10^7
B6	1.74×10^8 2.07×10^8 2.02×10^8	1.95×10^8	1.76×10^7
B7	2.21×10^8 2.19×10^8 2.25×10^8	2.22×10^8	3.12×10^6
B8	6.79×10^8 8.09×10^8 7.30×10^8	7.39×10^8	6.52×10^7
B9	6.39×10^8 7.03×10^8 7.03×10^8	6.82×10^8	3.72×10^7

Scanning electron microscopy and mapping of D. chlorocoercia DF-1 biofilm on biochar
This is the first report of the analysis of micro-morphology of *D. chlorocoercia* DF-1 biofilm based on high resolution microscopic analyses using SEM and EDX. The results of micro-morphology for pinewood biochar particles (abiotic control) and *D. chlorocoercia* DF-1 biofilm are shown in Figures 7.4 (a1-a3) and 7.4 (b1-b3), respectively. A large number of densely packed hive-structures can be seen on the biochar surface (Figure 7.4 (a1)). The micro-morphology of the outer surface of the hive-structures showed a striated cuticle layer (Figure 7.4 (a2)). In addition, the results shown in Figure 7.4 (a3) suggested that the hive-structures had a similar size with microporous structures located on the inside of each hive-structure. According to chemical composition analysis, the major elements of the pinewood biochar surface include carbon, oxygen, magnesium, and calcium. According to Figure 7.4 (b1-b3), an extracellular matrix deposition was present at the surface of the pinewood biochar. This is likely made up by extracellular polymers produced by the bacteria in the biofilm that over time has formed a matrix shielding the bacteria, which has led the *D. chlorocoercia* DF-1 cells attach to and grow on the biochar surface. Moreover, another evidence of extracellular polymers formation on the surface of pinewood biochar was shown in the chemical composition analysis indicating the deposition

of phosphorus and sodium. The phosphorus and sodium are usually considered as the essential elements for the cell membrane (Kaiser, Krieger et al. 2007).

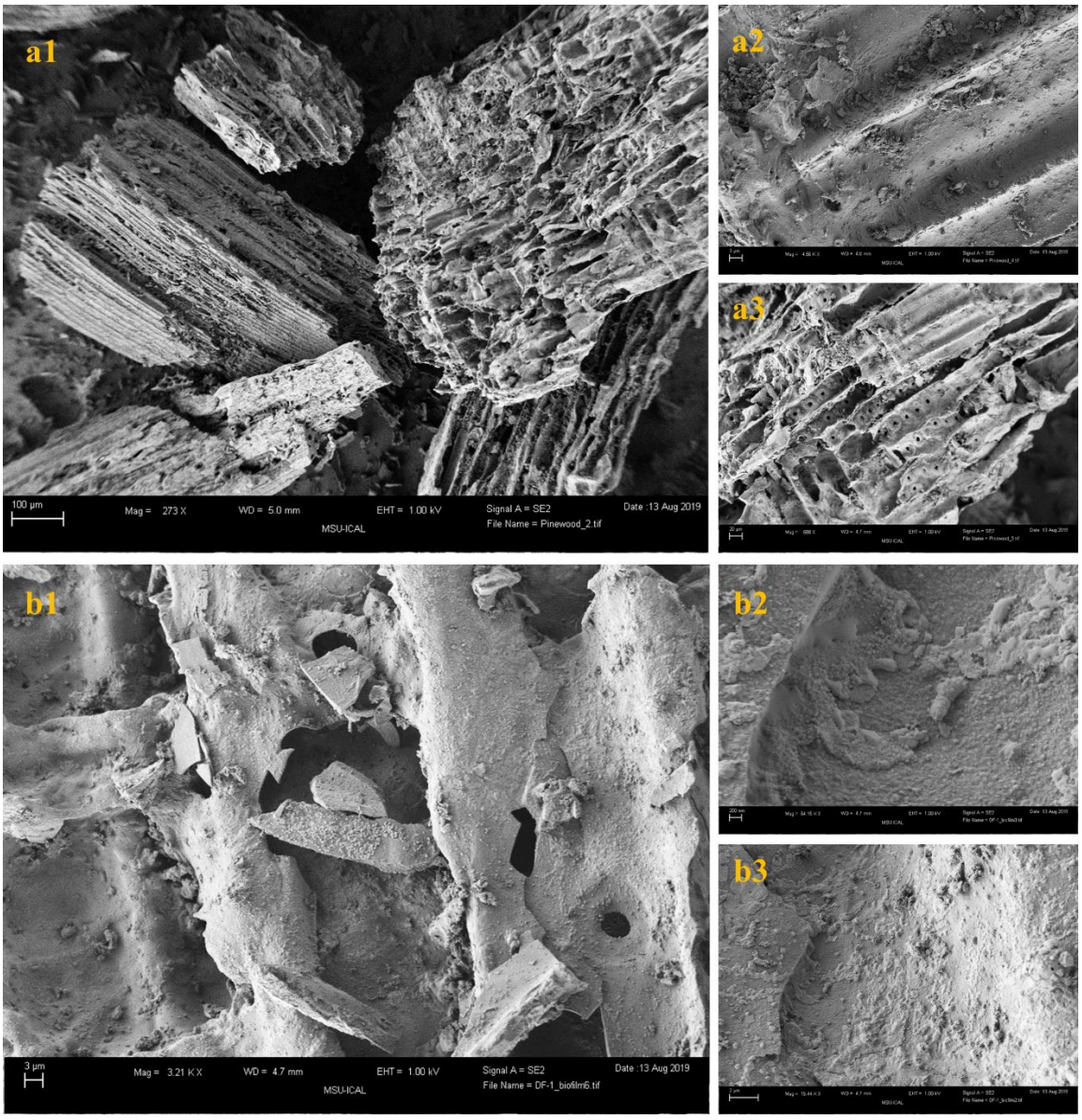

Figure 7.4 Scanning electron microscopy and energy-dispersive X-ray spectrometry mapping of pinewood biochar particles (a1-a3) and *D. chlorocoercia* DF-1 biofilm (b1-b3). Figure 7.4 a1-a3: a large number of hive-structures with the more complex microporous structures can be seen on the surface of pinewood biochar particles; Figure 7.4 b1-b3: the extracellular matrix deposition of the surface of pinewood biochar has been observed.

Total PCB concentration

The total concentration of PCBs was calculated for the individual samples (Figure 7.5). The biochar group had the largest reduction in the average total PCB concentration, which was 582.9 µg/g followed by biofilm group (221.5 µg/g). The samples with liquid bacterial inoculum showed a reduction of 149.3 µg/g. The total PCB concentration of the abiotic control was higher

after day 466 than the starting sample (19.2 µg/g), which likely was due to heterogeneity of the samples. Calculation of the average concentration of all samples at day 0 was used instead of the negative control in day 0 due to issues arising from autoclaving the sediment for the negative control. The differences between day 0 and day 466 were tested by repeated-measures ANOVA, and the P-value lower than 0.05, which means that the reduction of total PCB concentrations was significant for biochar group, liquid group and biofilm group (which was not the case).

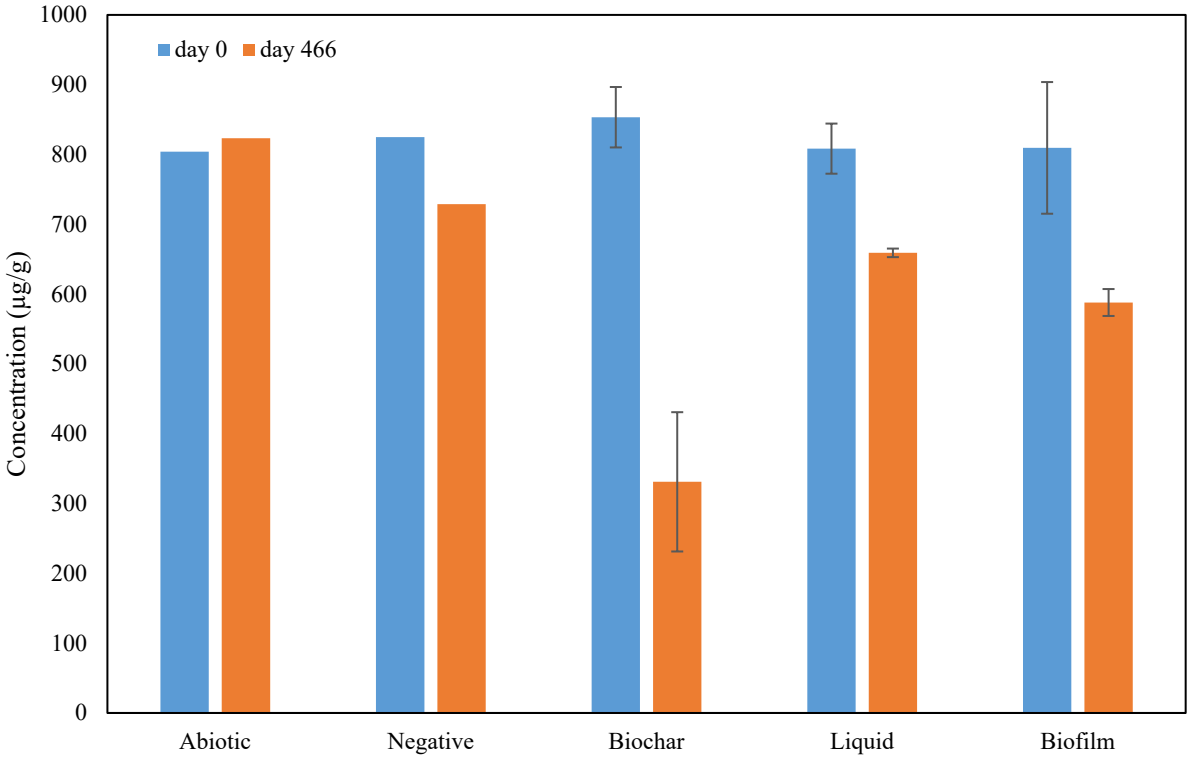

Figure 7.5. Total PCB concentrations of samples (Error bars show the standard deviation of the samples.

The difference of the PCB concentration changes among the biochar group, liquid group and biofilm group were tested by one-way ANOVA (P-value of 0.07), which higher than 0.05. This means that although the average values of PCB concentration reduction of biochar group from day 0 to day 466 was higher than liquid group and biofilm group, the concentration changes were not significant difference among groups. Multiple comparisons of every two groups also got all P-values higher than 0.05 (Table 7.3), which indicates the same results with one-way ANOVA test.

Table 7.3 Summary of comparisons by Holm's method on PCB concentration reduction.

P-value	Biochar	Biofilm
Biofilm	0.54	-
Liquid	0.54	0.54

Level of chlorination (Chlorines per biphenyl)

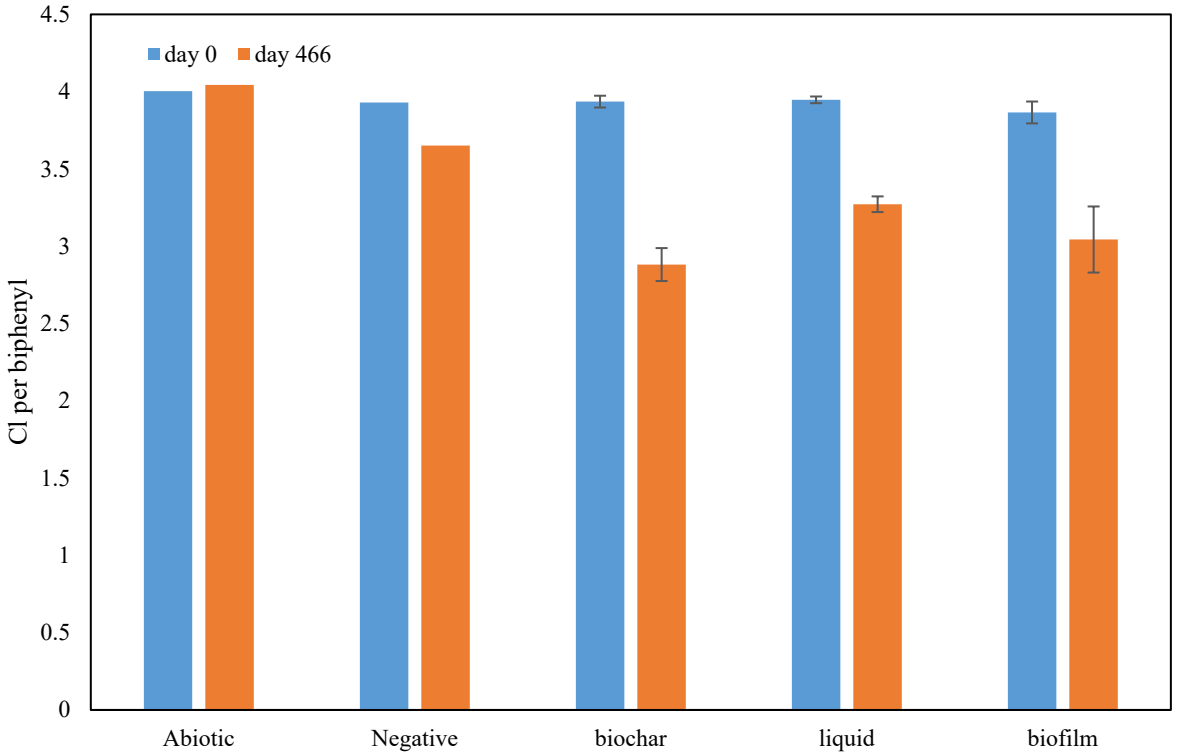

Figure 7.6 Cl per biphenyl of all treatment groups. (Error bars show the standard deviation of the samples. Cl per biphenyl of all samples at day 0 was used as Cl per biphenyl of negative control at day 0).

The Cl per biphenyl value of the negative control at day 0 was set as the average value of all samples at day 0. The average values of Cl per biphenyl of the biochar group, liquid group, and biofilm group samples were reduced from 3.94, 3.95, 3.87 at day 0 to 2.88, 3.27, and 3.04, respectively, at day 466 (Figure 7.6). The abiotic control had Cl per biphenyl value of 4.0 at day 0 and 4,1 at day 466, where the increase of Cl per biphenyl likely was caused by heterogeneity of the sample matrix. The differences between day 0 and day 466 were tested by repeated-measures ANOVA, and the P-value were lower than 0.05, which means that all treatment groups had significant lower Cl per biphenyl at day 466 than day 0.

One-way ANOVA was used to test the Cl per biphenyl changes among biochar group, liquid group, and biofilm group. The result shows that P-value was 0.07, which was higher than 0.05 and indicates there have no significant differences. Multiple comparisons between groups shows that every two groups also have no significant differences (table 7.4).

Table 7.4 Statistical summary of multiple comparisons by holm's method on Cl per biphenyl

P-value	Biochar	Biofilm
Biofilm	0.34	-
Liquid	0.21	0.36

Dechlorination Extent

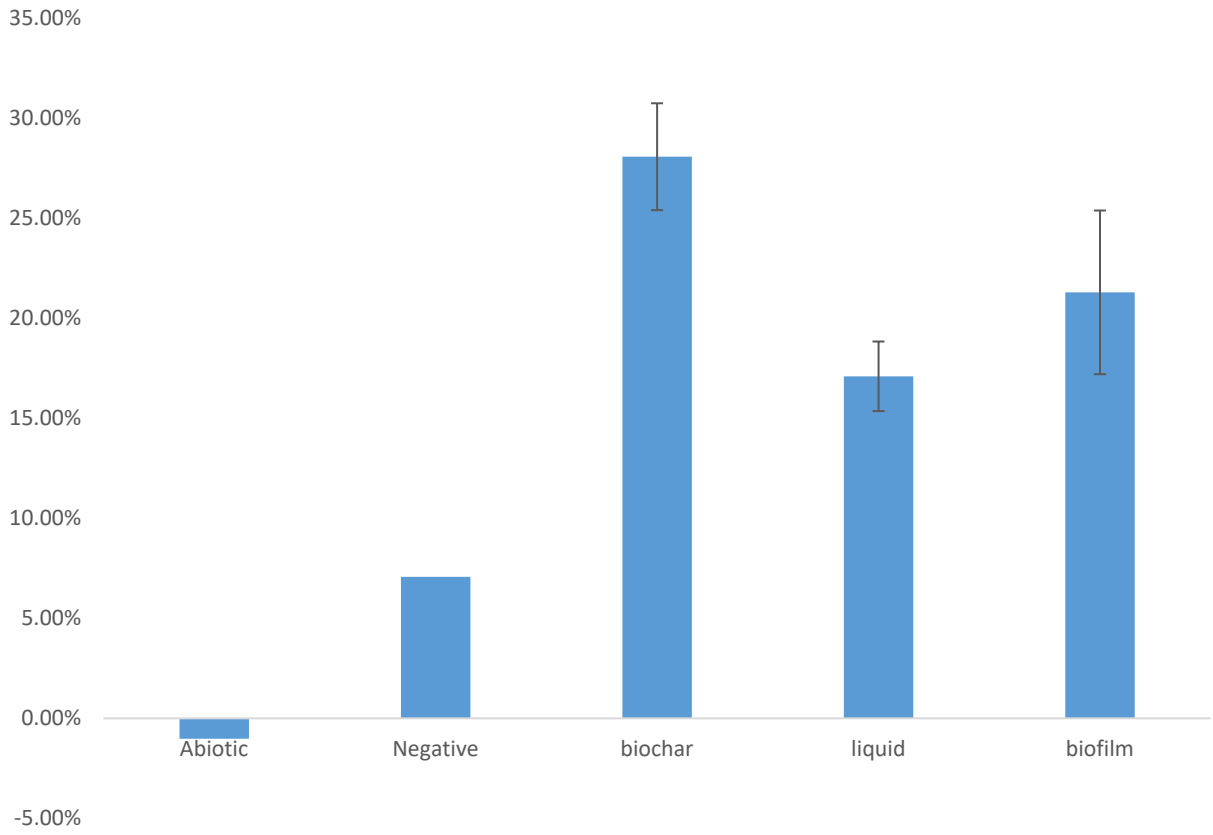

Figure 7.7 Dechlorination extent for all treatment groups. (Error bars show the standard deviation of the samples.)

The dechlorination extent was calculated for all samples using the Cl per biphenyl data (Figure 7.7). The dechlorination extents of the abiotic control was -1%, which likely was caused by the heterogeneity of the sample. The negative control showed a reduction of 7%. The three treatment groups had higher average values of dechlorination extents than the controls with average dechlorination extents of 26.8%, 17.1%, 21.3%, respectively.

One-way ANOVA was applied to assess the difference among the three treatment groups and get a P-value of 0.06, which was higher than 0.05, thus the three groups were not significantly different. As a result, the treatment groups showed no significant differences on dechlorination extent. Multiple comparisons by Holm's method showed the same result, P-values for all

contrast were higher than 0.05, which means that there have no significant differences between three treatment groups (Table 7.5).

Table 7.5 Statistical summary of multiple comparisons by Holm's method on dechlorination extents

P-value	Biochar	Biofilm
Biofilm	0.42	-
Liquid	0.16	0.42

Assessment of the microbial communities involved in dechlorination

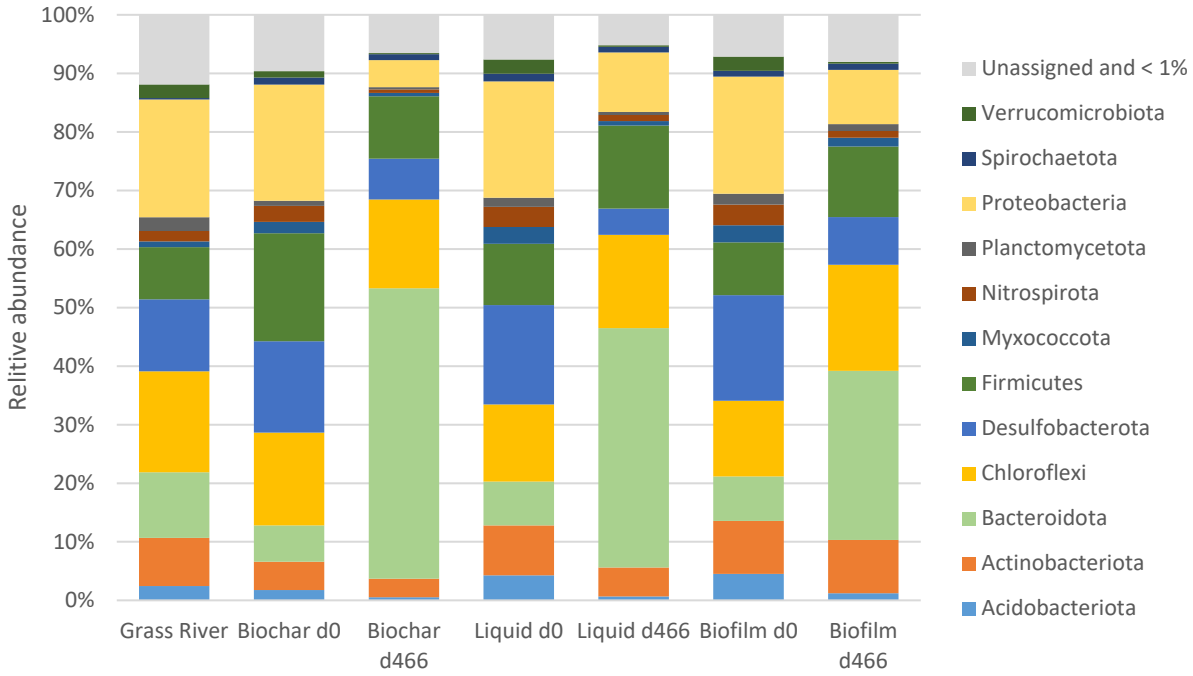

Figure 7.8 Relative abundances (>1%) of the microbial community on phylum level.

The microbial communities involved in dechlorination of PCBs was assessed using growth independent molecular methods to ensure that the full diversity was accounted for in the analysis. The microbial community (on the phylum level) of sediment samples changed from day 0 to day 466 (Figure 7.8). Before dechlorination at day 0, *Chloroflexi*, *Desulfobacterota*, *Firmicutes*, and *Proteobacteria* were the major members of the community. After dechlorination had taken place at day 466, the average abundance of *Bacteroidota* increased from 7.1% to 39.8% and became the most abundant phylum in all samples. *Chlorobiaceae* made the most contribution to the abundance of *Bacteroidota*. *Chlorobiaceae.* The anaerobic conditions of dechlorination provide a suitable living environment for *Chlorobiaceae*. Therefore, the increase of *Chlorobiaceae* on day 466 makes *Bacteroidota* the most abundant phylum in the community. The abundance of *Proteobacteria* decreased from 19.9% to 8.0%. Some members of *Proteobacteria* are aerobic bacteria that cannot survive in an anaerobic environment thus the abundance of *Proteobacteria* decreased throughout the experiment. The abundance of

Desulfobacterota was reduced from 16.9% to 6.5%. DF-1 belongs to the *Chloroflexi* phylum, and its abundance did not change for the biochar group or the liquid inoculum group during the experiment (15.9 to 15.2% and 13.2% to 15.9% respectively). The phylum *Chloroflexi* for Biofilm group increased from 12.9% to 18.1%. The phylum *Chloroflexi* for the biofilm group increased to 22.6% at day 466. The increasing relative abundance of *Chloroflexi* of the biofilm group was significantly higher than the biochar group (P-value < 0.05 by pairwise t-test). The results showed that bioaugmentation enriched the microbial community that subsequently enhanced dechlorination. Bioaugmentation by DF-1 biofilm made it possible for the dechlorinating bacterium DF-1 to survive in a new environment due to the mode of biofilm presence.

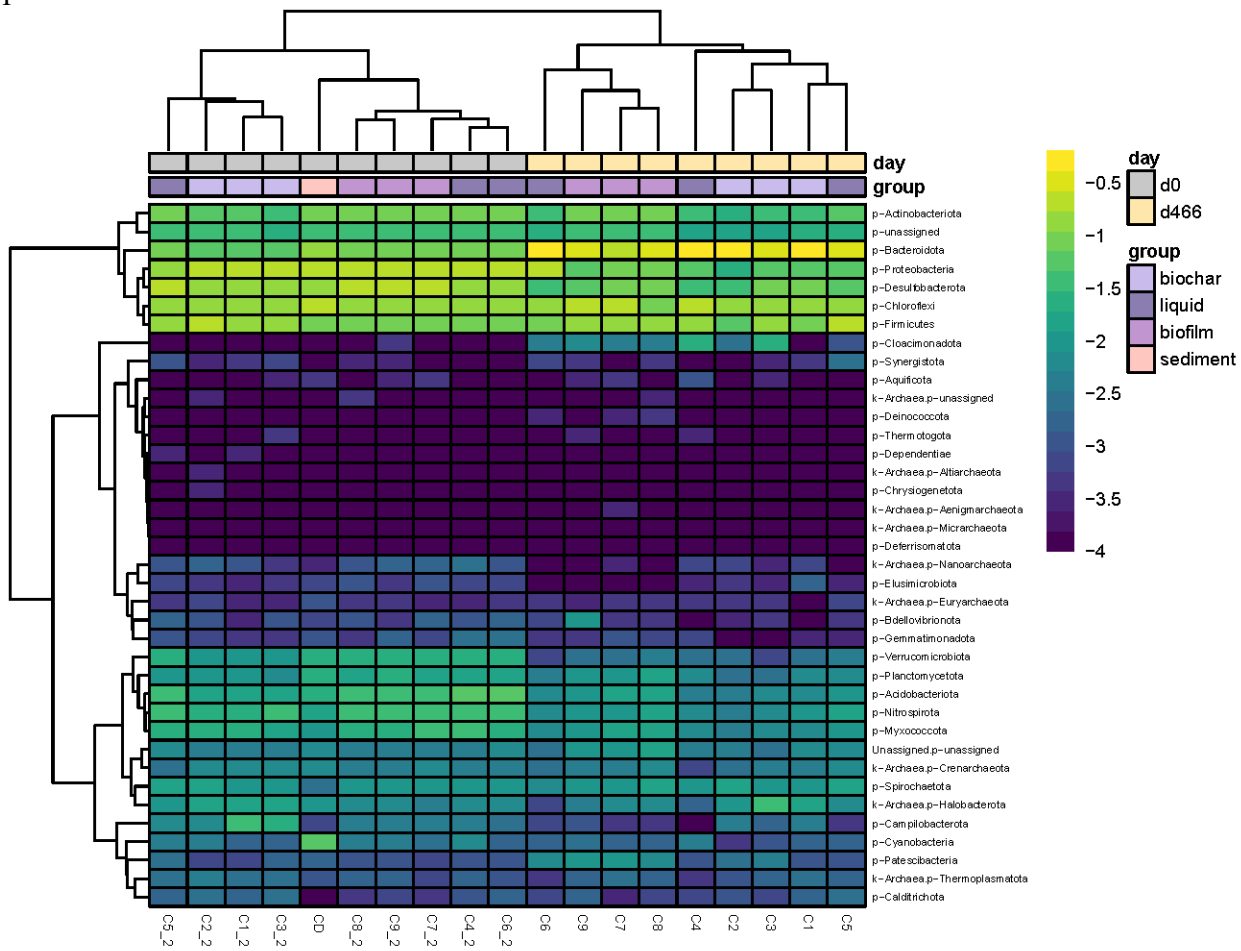

Figure 7.9 The dominant microbial phyla based on the relative abundance.

Further analysis of the microbial community of the sediment samples at day 0 and day 466, showed that they clustered together according to sampling time and the community of Grasse River sediments clustered with the samples at day 0 as expected, which means that the anaerobic dechlorination influenced the microbial community of sediments (Figure 7.9). The community of all groups except the liquid inoculum group clustered together before and after dechlorination. At day 0, two samples for the liquid inoculum group clustered with biofilm group, but at day 466, two samples from the liquid group clustered with the biochar group. This indicates that, although bioaugmentation by liquid DF-1 and biofilm DF-1 had similar influence on microbial community

at the beginning, bioaugmentation by DF-1 biofilm had a more stable influence on the sediment microbial community than liquid DF-1 inoculum. This may be because biofilms provide a more stable living environment for DF-1 that help them survive after being added to the river sediment. This is an important finding for the practical implementation of bioaugmentation of PCB impacted sediments.

Principal coordinates analysis of the microbial community composition showed similar results (Figure 7.10). Before dechlorination, all groups clustered together (day 0). At day 466, the three mesocosms from biofilm group still clustered together, while the biochar group showed a slightly larger variation. For the liquid group, three samples had relative lager differences than the other two groups, and the abiotic control had the largest difference.

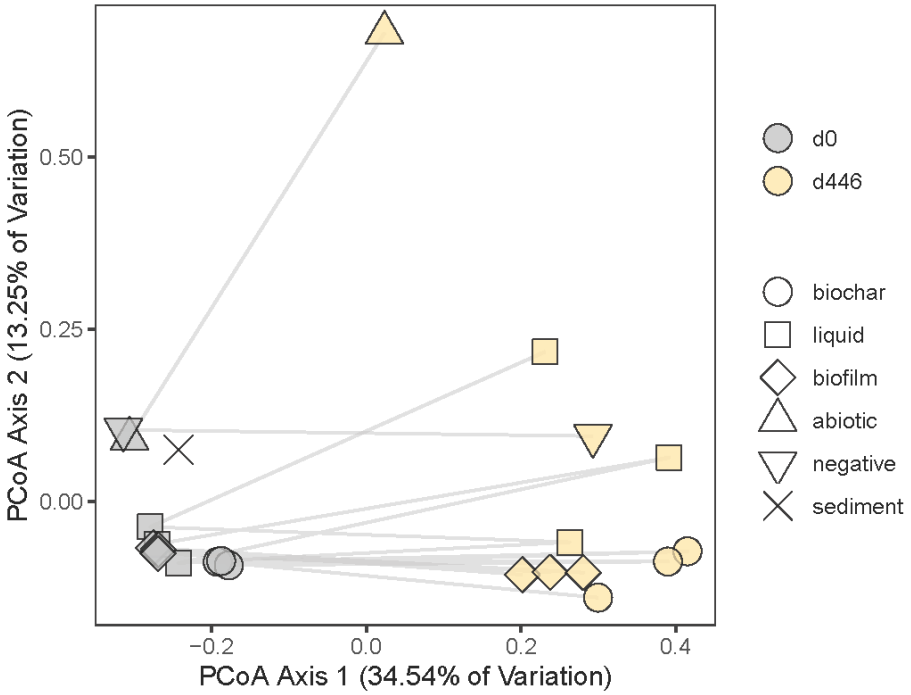

Figure 7.10 Principal coordinates analysis of microbial communities from different groups (Lines connect the same mesocosms at different time points).

The microbial communities of mesocosms before dechlorination were similar to microbial communities of sediments reported by other studies. Xiaomei et al. (2014) studied microbial community of PCB-contaminated river sediments, and the groups *Proteobacteria* (29.9–30.1 %), *Actinobacteria* (13.2–15.9 %), *Firmicutes* (11.6–12.9 %), and *Chloroflexi* (13.3%) were the major components of the present communities. Tong et al. (2017) studied the dechlorination capability of soil, and the major microbial phyla in the soil were *Firmicutes*, *Nitrospirae*, *Proteobacteria*, *Crenarchaeota*, *Chloroflexi*, *Bacteroidetes*, and *Acidobacteria*. In addition to DF-1 added to the sediment, some bacteria that originally existed in the river sediment have also been shown to have dechlorination ability. *Dehalococcoides mccartyi* (0.19% and 0.10% at day 0 and day 466 respectively) are members of the *Chloroflexi* phylum. They can use acetate as electron donor and carbon source, and dechlorinate PCBs as an electron acceptor (Laroe et al. 2014). *Desulfitobacterium* (<0.01% for day 0 and day 466) was identified and belongs to the

Firmicutes phylum and it is capable of PCE and TCE dechlorination (Sheu et al. 2016, Dugat-Bony et al. 2012). *Geobacter* (2.4% and 0.8% at day 0 and day 466 respective) in the *Desulfobacterota* phylum, is able to dechlorinate TCE (Adetutu et al. 2015). *Paraburkholderia* were also detected in the sediment samples and the species *Paraburkholderia xenovorans* LB400 is a strain that can aerobically degrade lowly chlorinated PCBs (Payne et al. 2013). The presence of these microorganisms listed above shows that the microbial community of Grasse River sediments has the ability for dechlorination. However, due to the low abundance of the native microorganisms, bioaugmentation can be used to enhance the dechlorination efficiency.

Discussion

According to the results, the total PCB concentration of biochar, liquid, and biofilm groups decreased significantly from day 0 to day 466. Negative control did not have dechlorination efficiency as high as biochar, liquid, and biofilm groups, and the dechlorination efficiency of the abiotic control was around 0%. The sediments used for abiotic control was autoclaved before the setup of the mesocosm, which means that microorganisms might have been eliminated from the sediments. As a result, dechlorination by microorganisms cannot take place in abiotic control. For negative control, neither biochar nor DF-1 were added into mesocosm. By the results of microbial community analysis, there were microorganisms with dechlorination ability already present in Grasse River sediments, such as *Dehalococcoides mccartyi*, *Desulfitobacterium,* and *Geobacter*. *Paraburkholderia* were also detected in the sediment samples. *Paraburkholderia xenovorans* LB400 has the ability to degrade PCBs aerobically (Payne, Fagervold et al. 2013). The dechlorination of PCBs in negative control may be caused by abiotic processes such as evaporation due to autoclaving the sediment. The relatively low dechlorination extent of negative control supports this observation. The results from this study, show that in order to effectively dechlorinate PCBs in river sediments, the addition of biochar or bioaugmentation is necessary. A study by Kjellerup et al, (2014) similarly showed that the presence of activated carbon without bioaugmentation enhanced dechlorination of Baltimore Harbor sediment.

There were no significant differences of dechlorination extents between the biochar group, liquid group, and biofilm group, which means that all three treatments can be used to improve the dechlorination efficiency in the river sediments. More than 20% of the Cl per biphenyl was reduced after 466 days. The dechlorination rate for the biochar, liquid, and biofilm groups were 2.35E-03 Cl per biphenyl/day, 2.08E-03 Cl per biphenyl/day, and 2.74E-03 Cl per biphenyl/day respectively.

The biochar added into the mesocosms can adsorb PCBs and provide an ideal environment for microorganisms to form biofilms. This advantage of close proximity to the electron acceptor may also help the original microorganisms in the sediments to improve the efficiency of dechlorination (i.e. priming) and thus impact the mesocosms with biochar to have the same relative dechlorination efficiency as the two bioaugmentation groups. However, the sediment that was bioaugmented with biofilms showed enhanced microbial community stability thus making this approach advantages for field applications of bioaugmentation.

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1. Chapter 8

2. Influence of Black Carbon Material on Organohalide respiration by *Dehalobium chlorocoercia*

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Highlights

- Enhanced kinetic effects on organohalide respiration were not observed in the presence of black carbon materials when the black carbon was first equilibrated with sediments followed by the addition of DF1
- Effects may be different when the organisms are applied as a coating on black carbon and is able to come in contact with sediment-bound PCBs first

Introduction

The most common strategies for remediation of PCB impacted sediments are focused on bulk removal or encapsulation by way of dredging or capping (Palermo et al., 2008). These remediation techniques are expensive and cause disruption and damage to the existing ecosystem as well as unintended releases (Patmont and Palermo, 2007). Better characterization of sediments and a better understanding of the role of carbon materials in the fate of hydrophobic contaminants resulted in new remediation strategies that target the sediment geochemistry. Naturally occurring black carbon materials, such as charcoal and soot, reduced aqueous concentrations in contaminated sediments due to their high sorptive capacity (Ghosh et al., 2001; Ghosh et al., 2003; Zimmerman et al., 2004). These initial finding have developed into a new field of *in-situ* remediation techniques that use black carbons, such as biochar and activated carbon, to treat contaminated sediments by reducing porewater concentrations in order to reduce bioavailability to macro-organisms (Sun and Ghosh, 2007; Beckingham and Ghosh, 2011; Ghosh et al., 2011).

Compounds sorbed to black carbons such as chars, soot, and activated carbon were previously believed to be chemically inert and therefore no longer available for transformation. This belief has recently been demonstrated to be incorrect resulting in a new field of study to determine the potential of black carbons to influence biotic and abiotic reactions. Initial work by Oh et al. (2002) observed enhanced reductions of DNT through the graphitic regions found on scrap iron. This observation was later determined to be through enhanced atomic hydrogen transfer through graphite (Ye and Chiu, 2006). Abiotic electron transfer through the conductive graphitic regions of black carbon has also been demonstrated to enhance the reduction of nitroaromatics, RDX, DNT, and DDT through π-π interactions with sorbed aromatic structures on the carbon surfaces (Oh and Chiu, 2009; Oh et al., 2013a; Xu et al., 2013; Ding and Xu, 2016).

The conductivity of the graphitic regions in black carbon was also found to enable extracellular microbial electron transfer. Direct interspecies electron transfer (DIET) has been observed between organisms *G. metallreducens* and *G. sulferreducens* in the presence of both activated carbon and biochar enabling the organisms to utilize an electron donor and acceptor that cannot be utilized directly by the individual species (Liu et al., 2013; Chen et al., 2014). Black carbon also contains redox active functional groups such as quinones and hydro-quinones capable of acting as both an electron acceptor and donor for redox reactions (Pignatello et al., 2017). These redox-active groups are accessible to microorganisms and can act both as an electron shuttle or to store electrons that can be utilized later, creating what has been called a "bio-battery" (Liu et al., 2013; Chen et al., 2014; Saquing et al., 2016; Zhao et al., 2016; Chen et al., 2018). The conductivity of the black carbon can vary significantly depending on the source material and pyrolysis temperatures. Yu et al. (2015) observed a linear correlation between the conductivity of the biochar material and the microbial dechlorination of pentachlorophenol (PCP) by *G. sulfurreducens*, however, only moderate increases in dechlorination rates were observed with pure graphite. Modeling their results, they found that three different reaction pathways were facilitated by the biochar. The pathways included direct cellular dechlorination, electron transport to sorbed PCP, and PCP reduction through electron transfer to redox active moieties (quinone groups) on the biochar surface revealing a very complicated redox system. Additional research on the influence of quinone groups on PCB dechlorination was reported by Chen et al. (2016) in which the addition of anthraquinone-2,6-disulfonic acid (AQDS) increased the rate of dechlorination of PCB 153 in a biologically active sediment mixture. Their results differ slightly from Zhang and Katayama (2012), who did not observe any increase in dechlorination with AQDS and only saw an effect with humic particles using different microbial communities. Recent laboratory and pilot scale studies have demonstrated the potential efficacy of in-situ microbial bioamendments to treat PCB contaminated sediments (Payne et al., 2011; Payne et al., 2013; Payne et al., 2017). Developing a good delivery medium for the microorganisms to contaminated sediments has been one of the difficulties in transitioning laboratory studies to the field despite their successes at the microcosm scale (Sowers and May, 2013). Specific challenges arise in passing an anaerobic microorganism through an aerobic water column without it washing away. Recent work by Payne et al. (2013) has demonstrated the potential to use granular activated carbon (GAC) as a delivery medium to bioamend weathered sediment with both anaerobic (DF1) and aerobic (LB400) bacteria (Payne et al., 2011; Payne et al., 2013; Payne et al., 2017). Kjellerup et al (2014) evaluated the effect of GAC on PCB dechlorination in sediments and found either no effect or a slight enhancement in the extent of dechlorination in a range of contaminated sediments. Currently, the effects of black carbon materials have not been reported in a sediment free system for any member of the family of *Chloroflexi.*

The key aim of the present study was to examine whether the activity of halorespiring bacteria DF-1 is enhanced by the presence of black carbon similar to observations in the species of *Geobacter*. PCB dechlorination rates were determined using methods developed in Chapter 3 in the presence of a humic acid surrogate (AQDS), graphite power, one biochar, and two activated carbons. Materials were selected to evaluate any potential stimulatory effect due to redox functional groups, conductivity, or sorptive capacity of the materials. These properties were selected as the dominant characteristics of black carbon materials that have been demonstrated to affect microbial kinetics in other bacteria but have not been reported for *Chloroflexi*.

Materials and Methods

Passive Sampler Preparation

Each passive sampler device consisted of a 0.10 g sheet of 2 mil polyethylene (PE) which was cut into 0.010 g pieces and attached to a heavy gauge stainless steel wire. The PE passive samplers were cleaned in a hexane:acetone (1:1) solution for 24 h then removed and allowed to dry.

Black Carbon Properties

Physical properties including surface area, porosity, and carbon content for the selected black carbons were reported by Gomeze-Eyles and Ghosh (2013). Partitioning coefficients for the carbon materials for PCB 61 was calculated at the start of each experiment. Partitioning coefficients were calculated based on passive sampling of the water concentration with PE and known initial concentration added to the microcosms. Microcosms were equilibrated on a rotary shaker for 14 d at 25º C prior to sampling. The equilibration period of 14 d was confirmed by abiotic controls in which no additional reductions of PCB 61 was observed in the aqueous phase.

Electron-accepting capacity (EAC) was measured by the Delaware Biotechnology Institute following the method published by Xin et al. (2018). Carbon samples for EAC measurement were tested in duplicate, ground, and sieved to <100 µm. Carbons were aerated in DI water to remove existing electrons, then dried, and residual O_2 was removed under vacuum followed by N_2 purging of the samples; then transferred to an anaerobic glove box. Samples were reduced with an excess of Ti(III) citrate at a pH of 6.4 The oxidation of Ti(III) citrate was monitored and used to calculate the electron-accepting capacity of the carbon samples.

Preparation of DF1

The anaerobic organohalorespiring bacterium "*Dehalobium chlorocoercia"* (DF1) was grown in co-culture with *Desulfovibrio* sp. in E-Cl medium containing 0.01 M sodium formate, and 0.2 mM perchloroethene (PCE) (Wu et al., 2002; May et al., 2008) in a 20L stainless steel bioreactor (BioFlo IV, New Brunswick Scientific). PCE was monitored throughout growth by GC-FID and replenished as required. DF1 was grown statically at 25 ºC in 14 L medium to a cell density of about $5x10^7$ cells mL^{-1}. DF1 cells were harvested anaerobically by gravity after the culture was sparged with N_2:CO_2 (80:20) to remove most of the residual PCE and dechlorination products.

DNA Extraction and Analyses

DNA was extracted from 0.25 ml liquid culture aliquots with a Power Soil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA) as previously described(Payne et al., 2011) and modified by using a QIACube robotic workstation (www.qiagen.com). Microorganisms were enumerated by real-time quantitative PCR (qPCR) using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) with primer pairs: (1)SKFPat9F/SKFPat9R specific for a putative reductive dehalogenase of DF1; or (2)348F/884R specific for the 16S rRNA gene of a deep branching putative dechlorinating clade within the Chloroflexi, using conditions described

previously (Payne et al., 2013). Amplification efficiencies of standards and samples were 99±10.0% with $R^2 = 0.98$.

Quantitative biofilm measurements

Black carbon materials were analyzed by our collaborators from the Kjellerup lab at the Center for Biofilm Engineering at Montana State University. A Leica TCS SP5 II confocal laser scanning microscope (Leica Microsystems, Model SP5 SMD) equipped with a 63x 0.9 NA long working distance (2.2 mm) water dipping objective (Leica Microsystems, Exton, PA, USA) was used to image the abiotic and biotic materials. CLSM 3-D images were compiled via Imaris x 64 software (version 8.4.0). Biofilm samples were placed in a petri-dish with sterile filtered water after they had been stained with SybrGreen I nucleic acid stain (30 minutes, in the dark), which was supplied as 10,000x concentrate in dimethylsulfoxide (Thermo Fisher Scientific, Waltham, MA) and diluted to a 40x working concentration in samples. Images of SybrGreen stained biofilms were obtained by using a fluorescein isothiocyanate (FITC) filter. Fluorescence and reflection images were captured at an excitation wavelength of 488 nm and SybrGreen emission wavelength collection was 500–595 nm. Images of SybrGreen stained microbial biofilms and reflection of the materials were overlaid in the Imaris software (Bitplane Scientific Software, South Windsor, CT). For each biofilm sample a total of 10 images were collected at randomly chosen fields of view. Images were analyzed using Imaris software and the area of biofilm coverage (i.e. biosurface area). CLSM imaging of the samples demonstrated the selective binding of the SybrGreen nucleic acid stain to the bacterial cells and not unspecific staining of the amendment materials.

DF1 Black Carbon Microcosms

Pinewood biochar (Biochar Co, CO), coconut GAC (OLC 50-120, Calgon Carbon, PA), and coal GAC (TOG 80x325, Calgon Carbon, PA) were selected to evaluate the effects of black carbon on dechlorination. In addition to the three black carbons, graphite powder (Alpha Aesar), 0.1 M AQDS (Alpha Aesar), and XAD-4 (Amberlite) resin were selected for their individual properties similar to GAC, namely, conductivity, redox activity, and sorptive capacity, respectively. Microcosms were prepared anaerobically in 125 mL wide mouth jars with PTFE lined caps consisting of 1x0.1gram PE passive sampler loaded with PCB 61, 90 mL of ECL media, and the evaluated material. Table 1 details the amount of each material added and the corresponding initial aqueous concentrations. Microcosms were prepared in triplicate and equilibrated for 14 d on a rotary shaker prior to the addition of DF1. Biologically active microcosms received 10 mL of DF1 culture at $1x10^7$ cells per mL for a targeted cell density of $1x10^6$ cells per mL and abiotic controls were dosed with autoclaved DF1 culture. Variations in the cell density from the fermenter stock are accounted for in the results (Table 1). PE samplers were removed, dried, and extracted with hexane on d 0, 1, 7, 14, 21, 28, and 42.

Sediment GAC Microcosm

PCB 61 (500 µg) (Ultra Scientific) dissolved in acetone was added to amber wide mouth jars and rolled by hand until the solvent evaporated. Rhode River sediment (250 g d.w.) and six 0.10 g PE passive samplers were added to each jar. Two jars received carbon amendments of 2.5 g (1% d.w.) and 12.5 g (5% d.w.) of GAC (TOG LF 80x325). Jars were then sealed with PTFE lined caps and plastic packaging tape and equilibrated by tumbling in a Bellco rotary shaker. The sediments were equilibrated for a duration of 14 d to minimize dechlorination by the native

population of dechlorinators. In initial trials with sediments equilibrated by rolling the sediment for 60 d to reach equilibrium, dechlorination products were formed by the native bacteria as discussed in Chapter 3. Sediment microcosms were prepared in triplicate anaerobically in 125 mL wide-mouth glass jars consisting of 30 g (d.w.) of equilibrated sediment and a 0.10 g PE passive sampler. Jars were maintained under anaerobic condition for 24 hours prior to the addition of DF1 inoculum. Microcosms received either autoclaved DF1 culture or live DF1 culture to account for native microbial dechlorination activity. Microcosm amendments consisted of 1% GAC (pre-equilibrated), 5% GAC (pre-equilibrated), 5% GAC with the DF1 inoculum added at d 0, and a control which received DF1 only. Each live bioamended microcosm received 20 mL of $7.7x10^7$ cells/mL (n=3, S.D.=$4.0x10^7$) to achieve a targeted cell density of $2.8x10^7$ cells/g (n=9, S.D.=$1.9x10^7$) wet sediment. Non-bioamended microcosms received 20 mL of autoclaved DF1 culture. Jars were maintained statically in an anaerobic atmosphere. Both PE sheets and sediment samples were removed at d 0, 17, 31, and 63 and analyzed for PCBs.

PCB Analysis

PE sheets were cleaned with DI water and wiped of any debris before being placed in 40 mL glass vials. Surrogate recoveries were based on 30 µL of PCB 65 at 500 µg/L added before the initial extraction. PE samplers were extracted with hexane for 24 hours on a rotary shaker 3 times. Carbon samples were lyophilized for 24 h until dry and extracted twice, first with hexane:acetone (1:1), then hexane using an Accelerated Solvent Extractor (Dionex) following EPA method 3545 as previously reported (Payne et al., 2011). Extracts were condensed to 1 mL under nitrogen then treated with copper to remove sulfur compounds (EPA method 3630b) and a modified silica gel clean-up (EPA 3630c) before analysis by GC-ECD following EPA method 8082a.

Estimation of Dechlorination Rate

The PCB dechlorination rate (k_b) in a passively dosed system that contains sorptive materials must account for the buffering capacity of the solid state(s) when measuring changes in the aqueous concentration. Based on the overall material balance in the system, the rate of dechlorination can be described by Eq. 1.

$$\frac{dC_w}{dt} * \left(\frac{V_w * + m_{PE} * K_{PE} + m_{AC} * K_{AC}}{V_w}\right) = C_w * k_b \qquad \text{Eq. 1}$$

Where, C_w is the concentration of the water, V_w is the volume of the water, m_{PE} is the mass of the polymer in the system, K_{PE} is the equilibrium partitioning coefficient for the polymer, m_{AC} is the mass of activated carbon, and K_{AC} is the equilibrium partitioning coefficient for the activated carbon. Based on the experimental construct, V_w, m_{PE}, K_{PE}, m_{AC}, and K_{AC} are all known constants and the equation can be further simplified by defining k_b':

$$k_b' = k_b * \left(\frac{V_w}{V_w * + m_{PE} * K_{PE} + m_{AC} * K_{AC}}\right) \qquad \text{Eq. 2}$$

Eq. 1 can be solved as a first order rate equation:

$$\frac{C_w}{C_{w\,o}} = e^{-k_b' t} \qquad \text{Eq. 3}$$

The buffering capacity (B) is represented by the inverse of the term in the parentheses of Eq 2. This term has also been called the bioavailability factor (B_f) by Zhang and Bouwer (1998) and demonstrated to be applicable in this experimental design by Lombard et al. (2014).

Assumptions made in this model are that the microbial population remains constant and that the mass transfer between the solid phases and water is faster than the microbial dechlorination rate.

2.1. Results and Discussion

Effects of Black Carbons

Three different black carbon materials consisting of a coal based GAC, coconut GAC, and pinewood biochar were tested to determine if any changes to the biological rate (k_b) could be observed with the different carbons. The carbons for this work were selected to include the most commonly used carbons for sediment remediation, and also based on the range of partitioning values previously measured by Gomez-Eyles and Ghosh (2013). The quantity of material tested and cell densities are reported in Table 1. The log K values for PCB 61 for the coal GAC, coconut GAC, and pinewood BC were measured to be 7.51, 6.75, and 6.05.

One difficulty in measuring changes to the aqueous PCB concentration is the strong sorption capacity of the carbons as reflected in the partitioning coefficients. In order to measure the rate of biodegradation indirectly with PE, the system needs to be at equilibrium among the different phases. Initial trials with adding GAC and DF1 simultaneously were unsuccessful due to the sorption by GAC dominating both the loss of PCB 61 and the formation of PCB 23. Additionally, the PCB 23 that was formed was not yet at equilibrium with the PE and GAC. Subsequent testing was conducted with pre-equilibrated black carbon to measure the loss of PCB 61 compared to autoclaved controls. As shown in Figure 1 the concentration of PCB 61 after pre-equilibrating the carbon does not change with respect to the initial concentration in the autoclaved control microcosms. There is no significant loss of PCB 61 in the autoclaved controls compared to the corresponding DF1 inoculated microcosms as shown in Figure 2. For each of the black carbon materials shown in Figure 1 we see no significant loss from the initial starting concentration shown by the dashed line, validating the assumption of equilibrium. Additionally, no dechlorination products were observed in any abiotic controls.

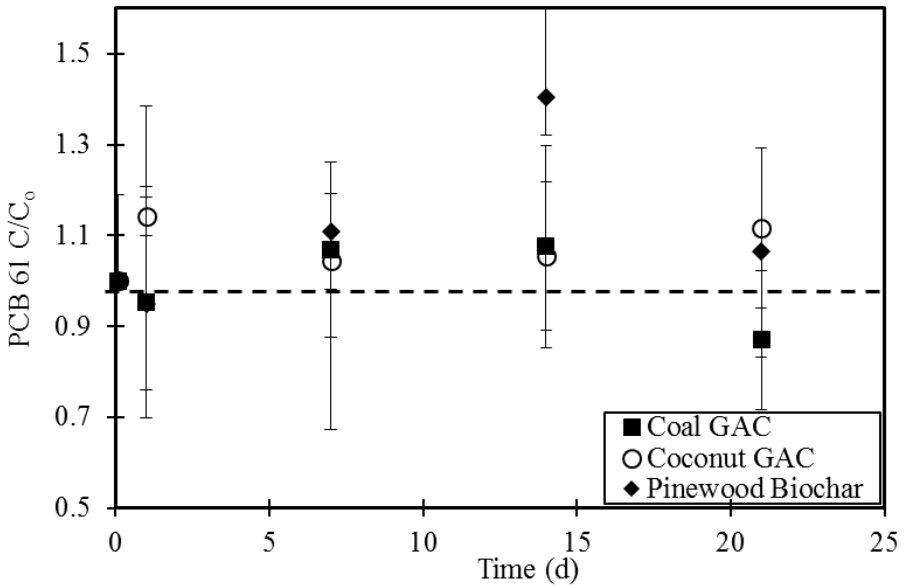

Figure 8.1 Changes in aqueous concentration (nM) of PCB 61 with autoclaved DF1 inoculum after a pre-equilibration time of 14 d. Results indicate the carbon materials were at equilibrium at the time of inoculation and demonstrate no abiotic loss of PCB 61

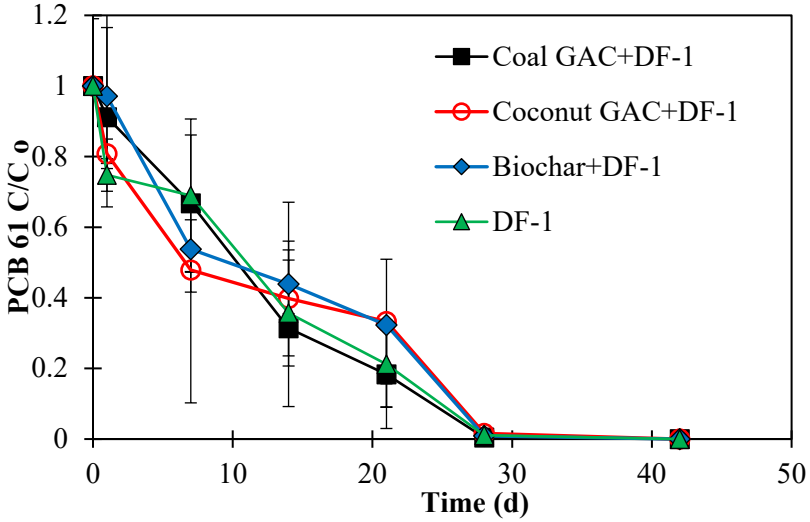

Figure 8.2: Dechlorination activity of DF1 in the presence of 3 black carbon materials. PCB 61 concentrations (nM) measured were normalized to the initial aqueous concentration of PCB 61$_o$ resulting in similar first order rates for each microcosm. Each data point is the mean of triplicate microcosms.

PCB dechlorination was observed immediately after 1 d in the presence of each black carbon material as shown in Figure 2 and Figure 3. The apparent aqueous rate (nM/d) for each microcosm was calculated based on the slope of the line of best fit (R^2 between 0.81-0.96) as shown in Figure 3 and Table 1. Normalizing the apparent aqueous rate to the initial aqueous concentration (nM) results in the apparent biological rate constant, k_b' (d^{-1}) reported in Table 1. A discussion on the assumption of first order kinetics versus zero order kinetics can be found Appendix III. No significant difference was observed between the calculated first order rate constant k_b' for the three carbon materials (k_b'= 0.021-0.026 d^{-1}) compared to the biological control (k_b'= 0.024 d^{-1}) which contained no black carbon material. The buffering capacity of the system as defined in Eq. 2 assumes that PCBs sorbed to the carbon are at equilibrium and will desorb as aqueous concentrations decreases. If PCBs sorbed to the carbon do not desorb and do not contribute to the buffering capacity of the system, then there will be no difference in the rate constant k_b between the different microcosms. Assuming that the carbon does contribute to the buffering capacity of the system then an increase in rate is observed in the coal GAC system due to the difference in buffering capacity when the carbon is included (Table 1, experiment 1). To evaluate if this difference is significant, a t-test (two-sample assuming unequal variances, α = 0.05) was performed and no significant difference in dechlorination rates between the coal GAC and biological control is observed (p= 0.31). Thus, while there is an apparent increase in dechlorination rate with activated carbon, due to the inherent uncertainty in the measurements involved, the change is not statistically significant.

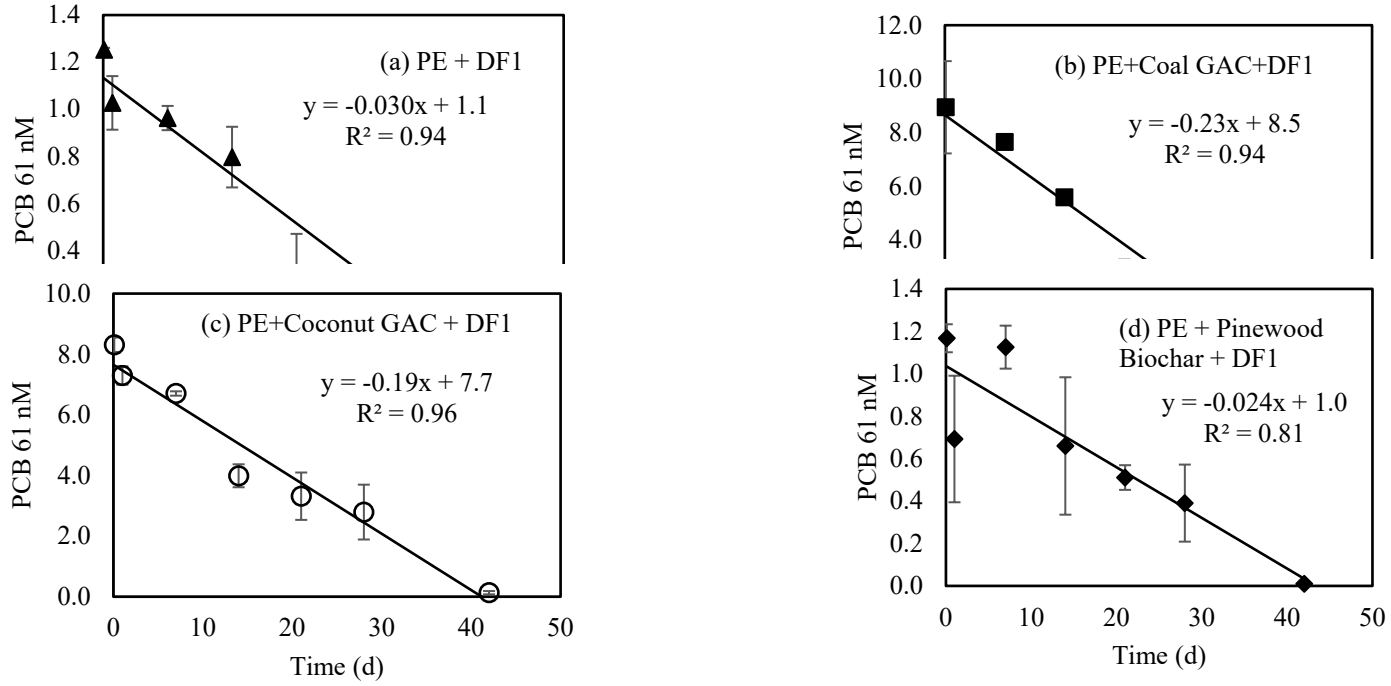

Figure 8.3 Apparent aqueous dechlorination rate (nM/d) of PCB 61 in microcosms containing (a) PE+DF1 only, (b) PE+coal GAC+DF1, (c) PE+coconut GAC+DF1, and (d) PE+pinewood biochar+DF1.

In order to determine if the mass of coal GAC used was too little to see an effect, the dose of GAC was increased to 0.5 g. Increasing the mass of GAC resulted in an increased buffering capacity of the microcosm from 5400 to 40000 as reported in Table 1, experiment 2. No dechlorination was observed in the aqueous phase by PE when 0.5 g GAC was added. Dechlorination activity was observed in the biotic control and other materials tested (XAD-4 resin) that were inoculated with the same DF1 culture confirming cell viability. The pH of the media was also tested to determine if a substantial shift in pH caused by the GAC was inhibiting the DF1, however, the media remained at the initial buffered pH of 6.8 when tested at the end of the experiment. The GAC was extracted at the completion of the experiment and PCB 23 was detected between 0.4 and 1.2 mole percent of the total PCBs extracted. If PCBs sorbed to the carbon surface were not bioavailable to DF1, it is expected that DF1 would dechlorinate the bioavailable fraction in the PE and aqueous states, but no significant reduction of PCB 61 or accumulation of PCB 23 was observed in the PE. Based on these observations, high doses of GAC is believed to be inhibitory since no significant quantities of PCB 23 were observed in the solid phase and no decrease in PCB 61 was observed in the PE. This inhibition may be due to the sorption of an essential nutrient from the media due to the high dose of GAC without the presence of an additional buffering material such as sediment.

Samples of the three different black carbon materials (coal GAC, coconut GAC, and pinewood biochar) were sent to the Center for Biofilm Engineering at Montana State University for confocal microscopy imaging. Biofilm growth was observed on each carbon surface after 49 d as shown in Figure 4. Before imaging the carbons, it was uncertain if planktonic cells added to the carbon microcosms would attach or interact with the carbon material. The presence of DF1, shown by the green stain, confirms that DF1 did attach and formed biofilm on all three carbon materials tested. Contact with the carbon surface would be necessary if the carbon materials enhanced an extracellular mechanism for transferring electrons similar to the mechanisms observed in *Geobacter* (Liu et al., 2013; Chen et al., 2014). Our results are consistent with similar rapid biofilm growth on GAC in sediment slurries tested with Arochlor 1260 reported by Mercier et al. (2013) where GAC was exposed to sediment slurries for 30 d.

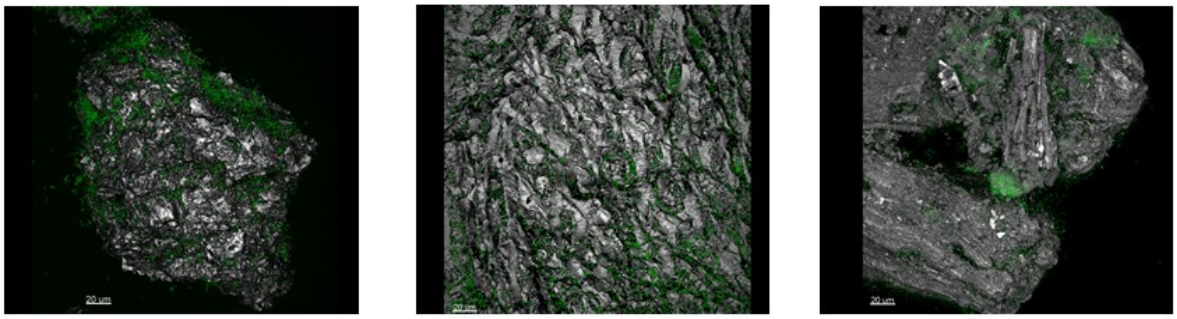

Figure 8.4 Biofilm growth (green stain) imaged after 49 d of contact with carbons pre-equilibrated with PCB 61. Left: Coal GAC. Center: Coconut GAC. Right: Pinewood biochar.

No dechlorination products were measured in any of the abiotic controls in either the PE or carbon extracts. Extracting the carbon materials in both the biologically active and abiotic microcosms consistently had low recoveries (5-20%) of the PCB sorbed to the carbon material after equilibration. High recoveries (80-95%) were maintained for surrogates added during the extraction process. The molar quantities of PCBs were conserved in microcosms that did not receive carbon materials making it unlikely that PCBs were lost from the system during the experiment. Without a complete mass balance that includes the sorbed PCBs on the carbon surface we cannot fully explain the extent of dechlorination of PCBs sorbed to the carbon material. However, the difficulty extracting the PCBs from the carbon using solvents at high temperatures and pressures in the ASE does not support the hypothesis that sorbed PCBs are bioavailable to DF1 through desorption to the aqueous phase.

Table 8.1 Dechlorination rates for the different materials tested with DF1. All materials were tested with an abiotic control containing autoclaved DF1 culture and a biological control that contained only DF1 and PE. Each material was tested in triplicate. Experiment number designating the same inoculum used is listed in column 1.

Exp.	Matrix	Cell Density (cell/mL)	Material Tested (g)	Rate (nM/d)	PCB 61$_{aq}$ initial (nM)	R^2	k_b' (d^{-1})	B	k_b (d^{-1})	k_b (d^{-1}) (1E6 cell/mL)
1	Coal GAC, PE	2.70E+06	0.01	0.23	8.9	0.94	0.026	5400	139	51
1	Coconut GAC, PE	2.30E+06	0.01	0.19	8.3	0.96	0.023	4000	91	40
1	Biochar, PE	1.80E+06	0.01	0.024	1.2	0.81	0.021	3700	76	42
1	PE	2.70E+06	---	0.03	1.3	0.93	0.024	3700	89	33
1	AQDS, PE	1.90E+06	---	0.037	1.0	0.92	0.035	3700	130	68
2	Coal GAC, PE	5.00E+06	0.5	0	20	---	---	40000	0	0
2	XAD, PE	5.60E+06	0.5	9.7	200	0.91	0.049	1500*	74	13
2	PE	5.60E+06	---	0.3	6.3	0.96	0.048	1500	71	13
3	Coal GAC, PE	2.00E+06	0.01	0.14	5.5	0.97	0.025	5400	140	70
3	Graphite, PE	2.20E+05	0.01	0.005	0.13	0.75	0.038	550	21	95
3	PE	1.60E+06	---	0.023	0.11	0.92	0.21	550	116	72

* Based only on PE, desorption from XAD-4 did not contribute to the buffering capacity of the system based on solids extractions.

Effects of Redox Active Groups on Dechlorination

As illustrated by the chemical structure shown in Figure 5, AQDS contains two quinone groups and two sulfonic acid groups, enabling the molecule to undergo multiple redox reactions. Similar to humic acids, black carbons contain quinone groups within the carbon structure that are formed during the pyrolysis process (Klüpfel et al., 2014). Electron accepting capacity (EAC) is a surrogate measurement to quantify the number of redox active groups, such as quinones in black carbons (Saquing et al., 2016; Xin et al., 2018). The black carbons tested, ranged in EAC from 3.08-3.62 mole/g (Table 2). The biological rate observed between the different black carbons tested correlates with the EAC for the materials as reported in Table 2. However, as previously discussed, there is no significant difference in the dechlorination rate between the presence of black carbon and the control microcosm.

To test the hypothesis that the redox active quinone and hydroquinone groups within black carbon materials will enhance dechlorination, 0.1 mM AQDS was evaluated as a surrogate for naturally occurring humic acids. The addition of AQDS resulted in an increase in the biological rate (k_b) from 33 d^{-1} in the control to 68 d^{-1} with AQDS normalized to a cell density of $1x10^6$ cells/mL (Table 1, experiment 1). The rate of individual replicates was also calculated to determine the statistical significance of the mean. A t-test (two-sample assuming unequal variances, $\alpha = 0.05$) showed no significant difference in dechlorination rates between the 0.1 mM AQDS and biological control (p=0.19). Work published by Chen et al (2016) found that the addition of 0.1 mM AQDS increased the dechlorination rate of PCB 153 in anaerobic sediments that contain a mixed culture of organisms. Electron shuttles such as AQDS or humic acids may play an important role in the redox cycle in mixed cultures in which various cell to cell transfers occur (Lovley et al., 1996). As a co-culture consisting of DF1 and desulfovibrio, electron shuttles may not be required to complete the redox cycle as observed in other mixed sediment cultures. The results of this work do not support the hypothesis that redox active groups will increase the rate of dechlorination by DF1 in a sediment free environment. The aqueous concentration of AQDS (0.1 M) exceeded the aqueous concentration of PCB 61 (1.0 nM) by orders of magnitude. If DF1 could utilize AQDS as an electron acceptor then it could have an inhibitory effect on the dechlorination rate by competing with PCB 61 as an electron acceptor. For AQDS to act as an electron shuttle, the reduced form of AQDS must be capable of reducing PCB 61. Future experimentation using chemically reduced AQDS and PCB 61 would be a more direct approach to determine if the presence of a quinone based electron shuttle enhances electron transport to PCBs as a terminal electron acceptor.

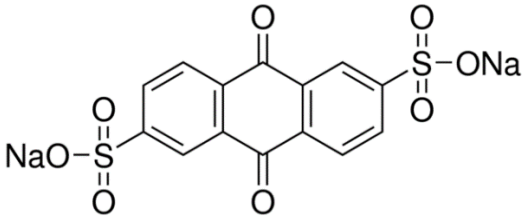

Figure 8.5 Chemical structure of 9,10-Anthraquinone-2,7-disulphonic acid (AQDS)

***Table 8.2**: Electron accepting capacity (EAC) of the black carbon materials evaluated.*

	Coal GAC	Coconut GAC	Pinewood BC	DF1 Control
EAC (mole/g)	3.62	3.16	3.08	----
k_b (d^{-1})	139	91	76	89

Effects of Conductivity on Dechlorination

Both the graphite powder microcosm and the biological control exhibited first-order rates of dechlorination as shown in Figure 6. Microcosms containing the graphite powder shown in grey reduced less than the microcosms that contained DF1 only shown in green. The qPCR analysis of the culture at d 7 found the graphite microcosms to be an order of magnitude lower in cell density than the biological control or the GAC. Both the control and the graphite microcosm were inoculated from the same stock culture with equal volumes of inoculum. The biological rate constant (normalized to $1x10^6$ cells/mL) in the graphite powder microcosm was 95 d^{-1} compared with the biological control k_b= 72 d^{-1} and coal GAC k_b=70 d^{-1} also tested (Table 1). Thus, normalizing the biological rate to a cell density of $1x10^6$ cells/mL results in no statistically significant difference in rate among the microcosms. The cause for the decrease in cell density could not be identified. However, neither the biological rate constant nor the adjusted rate resulted in a significant difference among the treatments. Graphite powder was selected to test if DF1 can utilize the conductive surface of black carbon materials to shuttle electrons and reduce PCB 61. Activated carbons and biochars contain graphitic regions that are capable of conducting electrons in redox reactions (Chen et al., 2014; Pignatello et al., 2017; Yuan et al., 2017). Graphite is an excellent conductor and has been used in a variety of applications to include serving as anodes in microbial fuel cells (Liu et al., 2010). These results however do not support the hypothesis that graphite increases the rate of dechlorination by providing a conductive surface; instead, graphite powder may be inhibitory to DF1 based on the observed decrease in cell density.

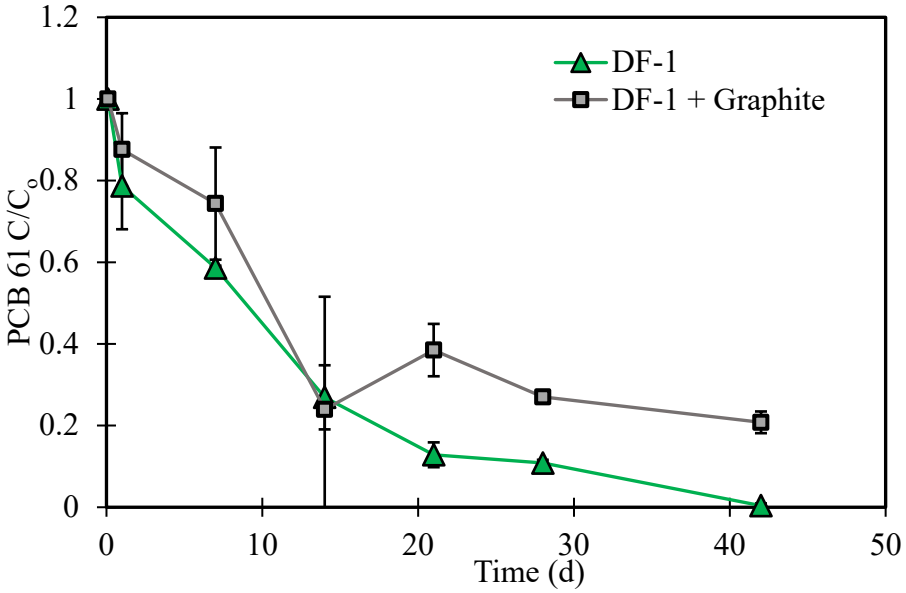

Figure 8.6 Dechlorination activity of DF1 in the presence and absence of graphite powder. PCB 61 concentrations (nM) measured were normalized to the initial aqueous concentration of PCB 61_o resulting in similar first order rates for each microcosm. Each data point is the mean of triplicate microcosms.

Effect of Sorption without Carbon

XAD-4 resin was tested to evaluate the possible effects of a highly sorptive material that is non-conductive and does not contain any redox active groups. XAD-4 resin is a styrene-divinylbenzene polymer with a strong affinity for hydrophobic organic compounds to include PCBs (Musty and Nickless, 1974; Carroll et al., 1994). The apparent rate of dechlorination (k_b') measured with PE when XAD-4 resin was added, was 74 d^{-1} compared with the biological control rate of 71 d^{-1}. If XAD-4 resin contributes to the buffering capacity of the system, then the biological rate (k_b) would be greater than the control due to the higher buffering capacity. The percent dechlorination in PE after 21 d was 95±5 % in XAD-4 resin microcosms and 89±4% in the biological control. The dechlorination observed in the XAD-4 resin solids was 15±2 % with no apparent decrease in PCB 61 concentration as shown in Figure 7. The XAD-4 resin appears to have accumulated some of the PCB 23 product from aqueous phase based on the molar quantities as shown in Figure 7. These results nullify the assumption in Eq. 1, in which all states are at equilibrium resulting in a buffering capacity from the solids. The low dechlorination observed in the XAD-4 resin solids is likely due to slower diffusion from the polymer beads compared to the PE sheets. XAD-4 resin is a macroporous polymer bead in which PCBs are absorbed to the surface within the pore structure. Diffusion out of the inner pores can be significantly retarded limiting the bioavailable fraction of PCBs absorbed to the surface. The addition of XAD-4 resin did not have an impact on the aqueous rate of dechlorination and did not contribute to the buffering capacity of the system within the exposure time. XAD-4 resin may be useful in future work to evaluate the effects of slow desorption on the biological rate to replicate the slow desorbing fraction of PCBs in weathered sediments.

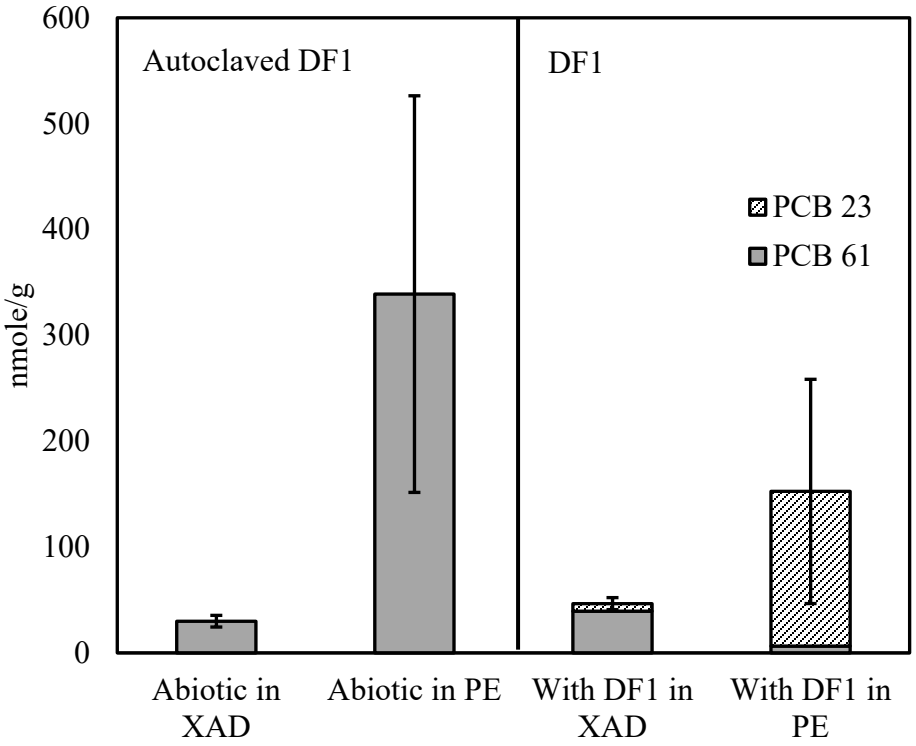

Figure 8.7 PCB concentration in nmole/g in the solid phases of PE and XAD extracted on d 21. Each microcosm contained 0.5 g XAD-4 resin and 0.2 g PE.

Effects of GAC Amendment on Dechlorination in Sediment

Static sediment microcosms were tested with pre-equilibrated GAC (1% and 5%) additions and one microcosm where 5% GAC was added with DF1 to evaluate the potential effects of GAC on the rate of dechlorination. Each microcosm received the same initial mass of PCB 61 unlike microcosms reported in Chapter 3 that used sediment slurries spiked with varying initial masses of PCB 61 but no activated carbon. The sediment pore-water concentrations ranged from 0.022-3.6 nM based on the amount of GAC added to the sediment (Table 3). The buffering capacity of the microcosms were calculated based on Eq. 2 with the addition of the mass of sediment and the partitioning coefficient for sediment as shown in Eq. 4. The addition of GAC increased the buffering capacity of the microcosms from 1.3×10^4 in unamended sediment to 1.2×10^5 and 1.6×10^6 in microcosms amended with 1% and 5% GAC respectively.

$$k_b' = k_b * \left(\frac{V_w}{V_w * + m_{PE} * K_{PE} + m_{AC} * K_{AC} + m_{sed} * K_{sed}}\right) \quad \text{Eq. 4}$$

The formation of PCB 23 was measured in all microcosms confirming dechlorination activity. The loss of PCB 61 in pre-equilibrated microcosms are shown in Figure 8 measured in both the PE samplers (▲) and sediment samples (■) normalized to the initial concentrations. Dashed lines represent the predicted dechlorination rates using the average biological rate (k_b=41 d^{-1} at 1×10^6 cells/mL) measured in Chapter 3, the buffering capacity of the sediment, and the cell density. A high cell density of 2.8×10^7 cells/g wet sediment was tested to shorten the experiment length and increase the potential interaction between DF1 and GAC. However, as discussed in Chapter 3, desorption from the sediments may retard the observed dechlorination rate if the microbial rate is

faster than the desorption rate. The dechlorination of PCB 61 in the sediment phase was slower than the predicted rate in each microcosm as shown by the dashed lines in Figure 8. Changes in the aqueous concentrations of PCB 61 (▲) were in closer agreement with the predicted rates (dashed lines) for both the unamended sediment and 1% GAC sediment. The aqueous rate of dechlorination in the 5% GAC sediment could not be calculated due to the low initial aqueous concentration of PCB 61 but was measureable in the sediment analysis. PCB 61 was dechlorinated to a greater extent in the PE (▲) than in the static sediment (■) as shown in Figure 8. The predicted biological rate (k_b=41) was close to the observed biological rate (k_b=12-21) after normalizing to a cell density of $1x10^6$ cells/g. As discussed in Chapter 3 of this work, normalizing to cell density by qPCR adds uncertainty due to the variability in the measurement. Predicting the biological rate (k_b) within a factor of 2-3 is within the margin of error for measuring cell densities in sediment.

Table 8.3 Results for biological rate in Rhode River sediments amended with DF1 and GAC. CF and k_b for 5% GAC D0 is not calculated because no assumption of equilibrium can be made.

Amendment	PCB 61aq$_o$ nM	k_b'	B	k_b	k_b ($1x10^6$ cells/mL)
DF1	3.6	3.0E-02	1.30E+04	390	14
1% GAC, DF1	0.29	2.9E-03	1.22E+05	354	13
5% GAC, DF1	0.022	4.3E-04	1.62E+06	697	25
5% GAC D0, DF1	3.3	1.2E-03	----------	---	--

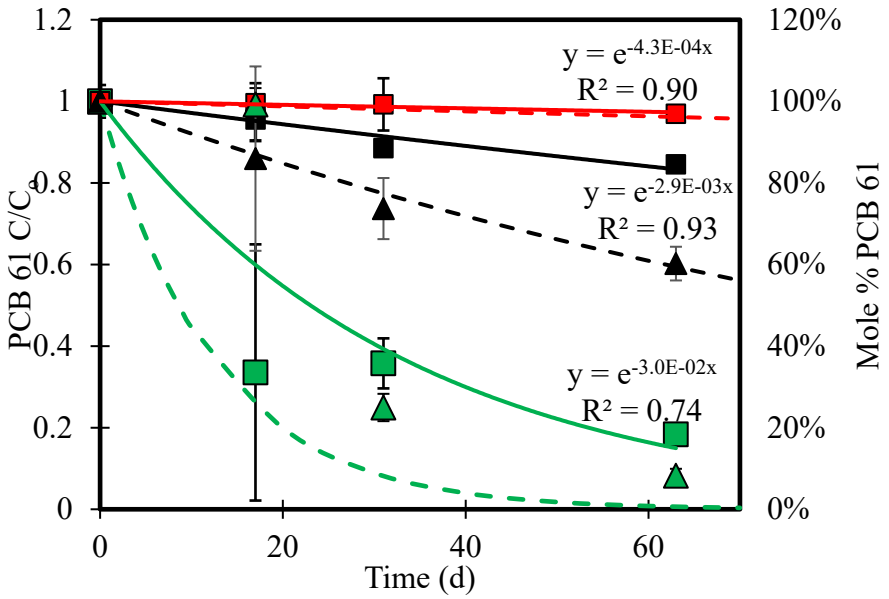

Figure 8.8 PCB 61 mole percent as measured in PE (▲) and sediment samples (■) in Rhode River sediment static microcosms consisting of bioamended control (green), bioamended 1% GAC (black), and bioamended 5% GAC (red). Dashed lines are predicted dechlorination rates based on cell density and biological rate measured in sediment free microcosms. Solid lines are exponential lines of best fit based on sediment values.

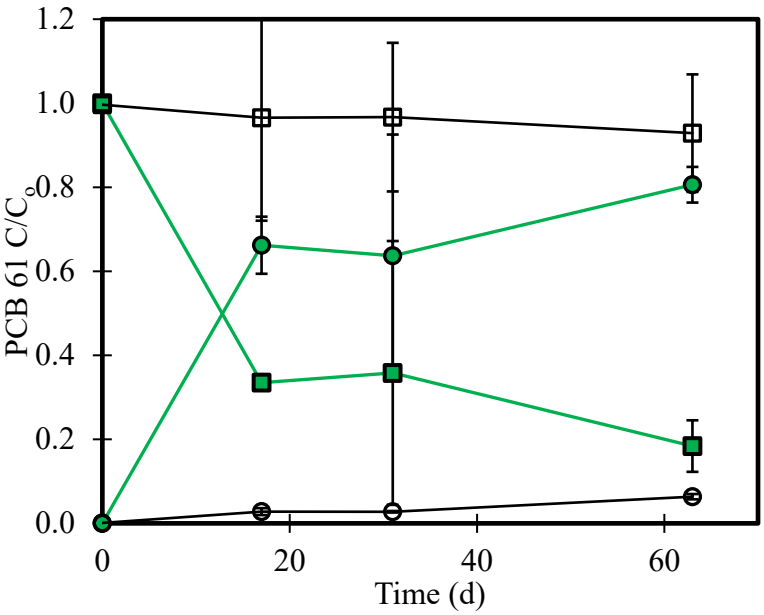

Figure 8.9 PCB 61 concentration (■) and PCB 23 (○) measured in sediments (nmole/g) normalized to the initial concentrations. Sediment microcosms containing 5% GAC and DF1 added at d 0 shown in black and DF1 only shown in green.

GAC was also added with DF1 at d 0 to equilibrated sediments to closely match the experimental design of Payne et al. (2011). Mesocosms conducted by Payne et al. (2011) used the same GAC (TOG 80x325) at 4.4% by dry weight to add DF1 to sediments. In the PCB 61 dosed sediments that received only DF1, an 80% reduction in PCB 61 was observed in the sediments as shown in Figure 9. Adding 5% GAC with DF1 reduced PCB 61 concentration in the sediment by 7% (Figure 9). Since the microcosms that received GAC at d 0 were not at equilibrium, the buffering capacity could not be calculated since the equation relies on the assumption that all states are in equilibrium. Without the buffering capacity the biological rate (k_b) could not be calculated from the observed rate (k_b'). Both microcosms started at comparable PCB 61 aqueous concentrations before GAC was added as reported in Table 3. The percent dechlorination observed in the sediments for the 5% GAC added at d 0 was greater than pre-equilibrated 5% GAC microcosms (3%) but less than the pre-equilibrated 1% GAC microcosm (15%) (Figure 8).

The freely dissolved aqueous concentration of PCB 61 reduced from 3.3±0.01 nM to 0.73 ±0.09 nM in 63 d when 5% GAC was added to static microcosms. Comparing the final aqueous concentration of the 5% GAC microcosm (0.73 nM) with the pre-equilibrated 5% GAC initial aqueous concentration (0.022 nM) (Table 3) indicates that the GAC (when added on d 0) did not reach equilibrium after 63 d in the static microcosms. The pre-equilibrated GAC reached equilibrium much faster due to the aggressive mixing prior to the addition of DF1. Studies conducted by Payne et al (2011) used weathered Baltimore Harbor sediments for their mesocosm studies with GAC but did not measure the aqueous concentrations. Diffusion from weathered sediments can be much slower than freshly dosed sediments used in this study. In the weathered sediments the GAC may take longer to reach equilibrium and enable DF1 to reduce the PCBs before they are sorbed to the carbon surface. Based on the results of this study we do not observe an enhanced dechlorination by DF1 in sediments with the addition of GAC. The observed rate of dechlorination in GAC amended sediments could be predicted within a factor of 3 based on the partitioning in equilibrated microcosm.

2.2. Implications

Past conceptual models of black carbon material as an inert sorptive material has been replaced by findings from new research into the biotic and abiotic mediated reactions that incorporate the multiple physiochemical properties of black carbon (Oh et al., 2012; Oh et al., 2013b; Chen et al., 2014; Klüpfel et al., 2014; Pignatello et al., 2017). In addition to the potential surface reactivity, activated carbon has been demonstrated to be a viable delivery media for amending contaminated sediments with PCB degrading bacteria (Payne et al., 2011; Payne et al., 2013; Payne et al., 2017). This research is the first analysis of the kinetic effects of black carbon material on the organohalide respiration rate of PCBs by DF1 in both sediment free microcosms and sediment microcosms. Past research on the potential impacts of GAC on organohalide respiration has been in weathered sediments containing PCB mixtures making the kinetic effects difficult to evaluate due to multiple dechlorination products and partitioning among the different phases. Enhanced dechlorination of PCBs in the presence of GAC has been reported, however, the enhancement is based on changes in the average number of chlorines per biphenyl ring not based on molar accumulation or depletion of specific PCBs (Payne et al., 2011; Kjellerup et al., 2014).

We tested the hypothesis that DF1 could use the conductive surface of black carbon to transfer electrons but found no enhanced dechlorination with graphite powder, biochar, or activated carbon. We evaluated if redox active groups in black carbon can act as an electron shuttle by adding AQDS to the microcosms and also measuring the electron accepting capacity of the carbon materials tested but did not observed any significant enhancement in the rate. There is no evidence of extracellular electron transfer with DF1 enabled by black carbon such as observed in other anaerobic bacteria found in the family of *Geobacter* (Chen et al., 2014; Yu et al., 2015; Saquing et al., 2016).

The results of this study found no significant stimulatory or enhanced kinetic effects on organohalide respiration in the presence of black carbon materials when the black carbon is first equilibrated with sediments followed by the addition of DF1. Black carbon amendments to contaminated sediments may enhance the rate of dechlorination in sediments when other microbial communities are present resulting in some benefit to DF1 that is not captured in this study. In addition, when the organisms are applied as a coating on black carbon and is able to come in contact with sediment PCBs first, the overall effect may be different as witnessed by Payne et al. (2011). The methods and results of this research provide the foundation for future research with other PCB degrading bacteria to include other members of the family of *Chloroflexi* to determine if black carbon materials enhance PCB dechlorination.

Supplemental Information

Zero Order Kinetics Discussion

First order kinetics were assumed in order to compare the rates measured in Chapter 3 with the rates measured in microcosms containing black carbon. As discussed, the aqueous rate (nM/d) for each microcosm was calculated based on the slope of the line of best fit as shown in Figure 3 and Table 1. The general linear pattern for each individual microcosm could also be fitted with a zero-order kinetics. If the degradation rate of PCB 61 by DF1 followed zero-order kinetics than the biological rate would be independent of the concentration of PCB 61. This would require the microbial kinetics to be significantly different than those observed in PE and sediment microcosms in which the apparent aqueous rate was dependent on the PCB 61_{aq} concentration. Zero-order kinetics would follow Eq. S4.1 in which C is reduced by a constant rate (k) with respect to time.

$$C_w = C_{w_o} - k_b * t \qquad \text{Eq. S4.1}$$

Applying the aqueous rate (nM/d) from the DF1+PE microcosm to observed degradation rate observed in the GAC microcosm does not explain the rate. However, using first order kinetics does predict the rate of degradation of PCB 61 as shown in Figure S1.

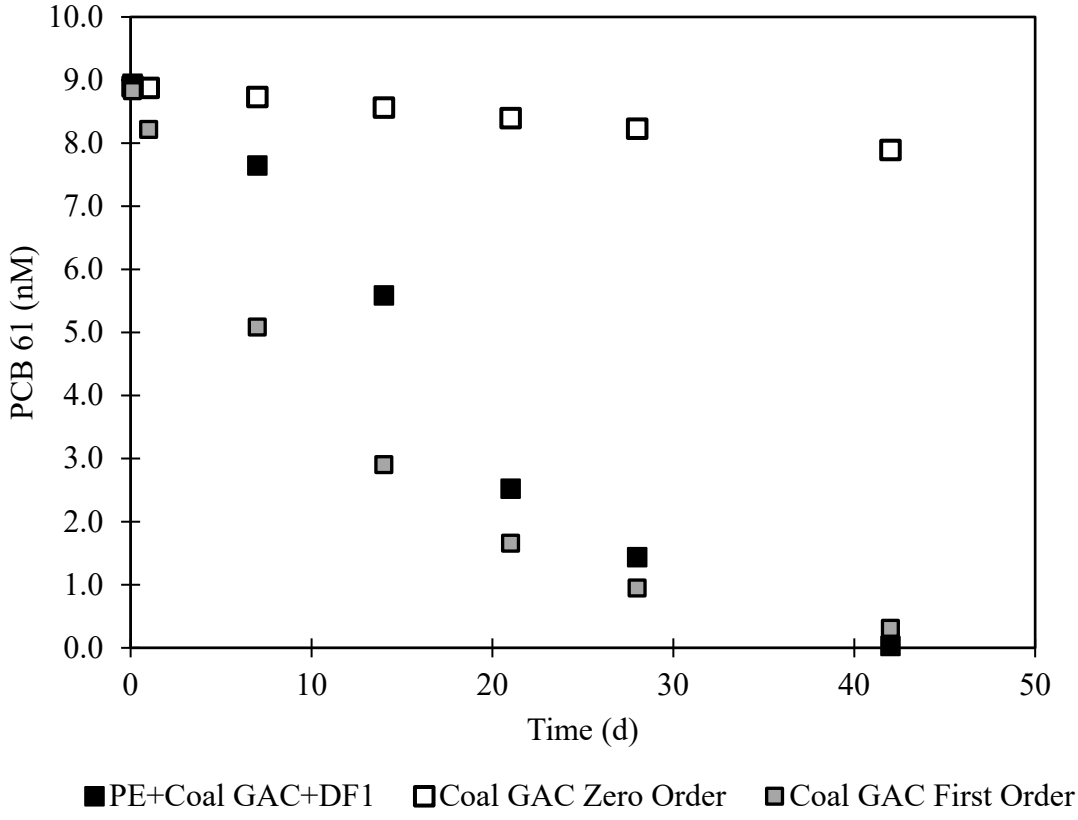

Figure 8.S1 Apparent aqueous dechlorination rate (nM/d) of PCB 61 in microcosms containing PE+coal GAC+DF1 (■). Apparent aqueous dechlorination rates predicted based on DF1+PE microcosms for first order kinetics (■) and zero order kinetics (□).

Graphite Testing
Initial trails were conducted using a custom-built sealed glass dialysis cell to replicate the experimental design of Oh et al. (2002). Due to the cost associated with building the prototype (Figure S2-S3) trials were conducted using DF1 and WBC-2 cultures to determine if full experiments should be conducted with multiple cells.

Cell Design
Dialysis cells were designed based on the experimental set-up by Oh et al. (2002). However, unlike the design by Oh et al. (2002) which utilized commercially available acrylic dialysis cells (Bel-Art Products, South Wayne, NJ) the dialysis cells were constructed with glass to reduce sorption by PCBs to the plastic surface. 0.13mm graphite foil (Alfa Aesar) was cut to fit between the two chambers and sealed with PTFE o-rings (therubberstore.com). The glass dialysis cell was constructed by the UMBC Glass Shop based on the sketch illustrated in Figure S4.2.

Initial Trials with DF1
The glass galvanic cell was autoclaved, then placed in an anaerobic hood for 24 h to equilibrate with the environment and release any residual O_2 prior to testing. Using the sampling ports, 0.10 g of PE was added to each side of the graphite cell consisting of one side with clean PE (no PCBs) and the other with PCB 61 preloaded. The PCB loaded side was then filled with 40 mL of ECL medium. The side of the cell with no PCB 61 was filled with DF1 stock culture ($5x10^7$ cells/mL). The cell was maintained under anaerobic conditions for 60 d then sacrificed and PE strips were removed. PE was analyzed following the methods previously described. No PCBs were detected in the cell containing DF1 culture only. No dechlorination products, PCB 23 or PCB 29, were observed in the PE containing PCB 61. Further tests with DF1 were not conducted based on these initial results and the inconclusive results with graphite powder.

Initial Trials with WBC-2
The glass galvanic cell was autoclaved then placed in an anaerobic hood for 24 h to equilibrate with the environment release any residual O_2 prior to testing. Using the sampling ports, 40 mL of low salt medium was added to one side of the cell with 125 µM PCE as the electron acceptor. WBC-2 culture was sparged under N_2 for 15 minutes to remove any residual PCE or daughter products. Headspace was sampled using GC-FID as described in Chapter 5 to confirm concentrations were below detection limits. WBC-2 culture was then added to the alternate side of the galvanic cell and 0.2 mL of 2M sodium lactate as the electron donor. The sampling ports were sealed with PTFE lined caps and the galvanic cell was maintained under anaerobic conditions. After 28 d the two sides of the galvanic cell were drained into separate 90 mL serum vials, capped, and the headspace as analyzed using GC-FID. No PCE or dechlorination products were detected in the WBC-2 culture. No dechlorination products were detected in medium with PCE only. Further testing with WBC-2 was not conducted based on these initial results.

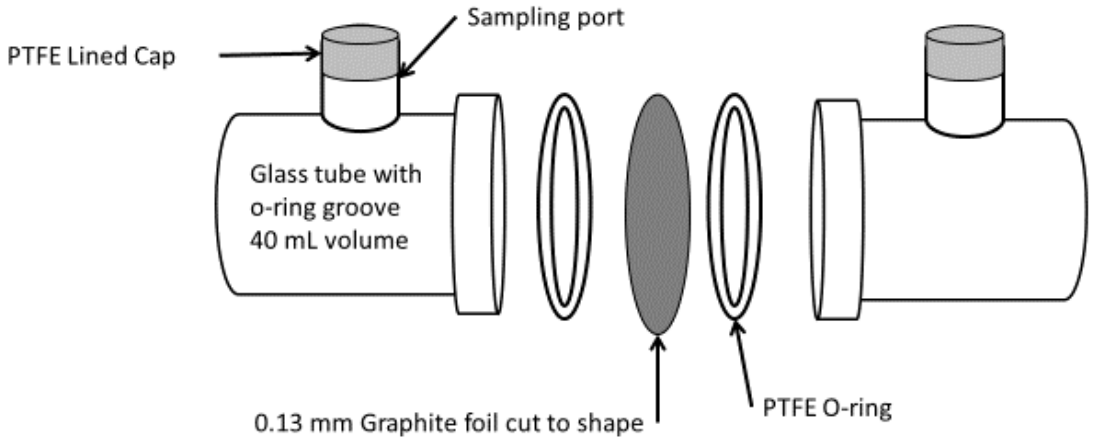

Figure 8.S2 Sketch of the glass dialysis cell.

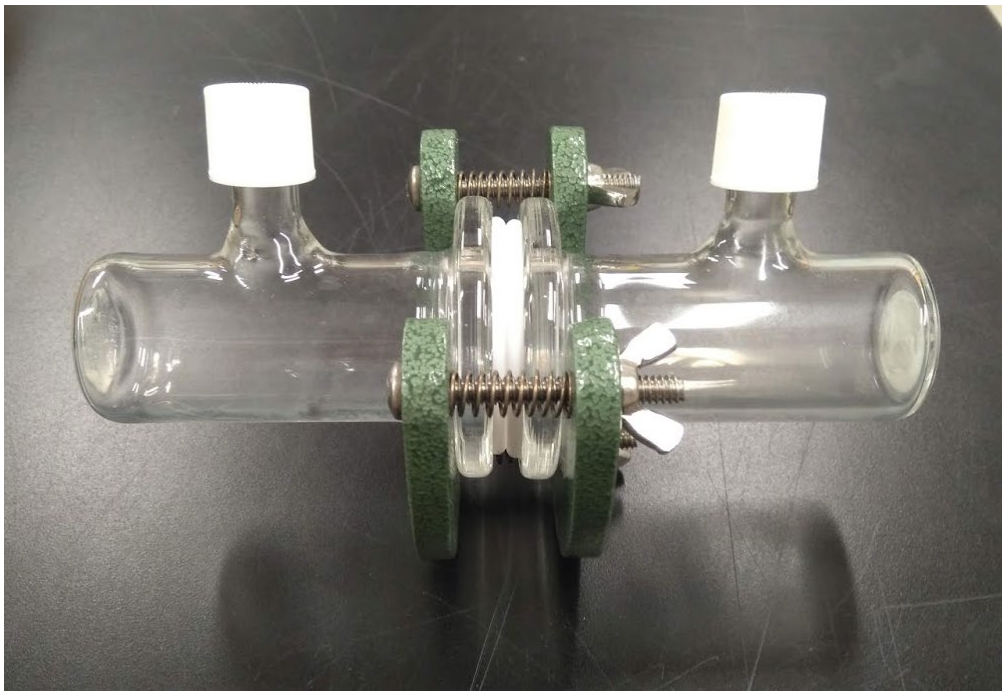

Figure 8.S3 Picture of the glass dialysis cell constructed by the UMBC Glass Shop.

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5. Chapter 9

Conclusions and Implications for Future Research/Implementation

Recent work with polychlorinated biphenyls (PCBs) contaminated sediments has used activated carbon (AC) to control in situ bioavailability of PCBs, with large reduction in the bioaccumulation of PCBs by aquatic organisms in field studies with sediment treated using 1-5% by weight granular activated carbon (GAC) (Ghosh et al., 2011). Sediment treated with GAC attained aqueous equilibrium PCB concentrations that were 85% and 92% lower than untreated sediment in one-month and six-month contact experiments, respectively (Zimmerman et al., 2004). It has been demonstrated that AC effectively outcompetes solid phases and benthic organisms for PCBs thus reducing PCB exposure to aquatic organisms.

Phase I of the SERDP project ER-2135 aimed at assessing how natural PCB dechlorination activity in sediments was affected by the addition of AC. Results showed that indigenous PCB dechlorinating bacteria were capable of PCB dechlorination even in the presence of a strong sorbent like AC. Although the results showed that there was a slight decrease in the overall concentration of parent congeners dechlorinated within the time frame studied, there was a significant increase in the extent of dechlorination in the presence of AC. The products of dechlorination were in the absence of AC tri-, tetra- and penta chlorinated congeners, whereas in the presence of AC the dechlorination products were mono- and di- chlorinated congeners. These dechlorination products generated in the presence of AC will likely make it possible for indigenous aerobic bacteria to complete the mineralization process in the presence of oxygen.

Bioaugmentation of PCBs in sediment and soils has been shown in several studies however, the dechlorination rates and the extent of PCB dechlorination obtained with bioaugmentation in weathered PCB contaminated sediments are often low. Therefore, to further enhance the degradation of weathered and low concentration PCBs in sediment, a combination of adsorbent sequestration and bioaugmentation in the form of biofilm covered sorptive particles is proposed. This approach benefits from bacteria being able to grow to a high density at the surface of sequestering sorbent. In addition, PCB dechlorinating bacteria in biofilms are surrounded by a hydrophobic layer enabling the bacterial cells to align in close proximity to adsorbent particles and thereby interact with hydrophobic PCBs that are adsorbed to particles (May et al. 2008). This biofilm community of microbes has greater cell density and activity than would be possible to obtain in free floating systems, where direct interaction between PCBs and the bacteria would occur less frequently. This interaction is required for optimal electron transfer and subsequent PCB dechlorination.

This biofilm-based approach is a significant improvement compared to previous bioaugmentation studies, where liquid cultures with free floating bacteria were injected or in other ways supplied to the sediment with reduced efficiency. An additional positive effect is that the dechlorinating bacteria will be fixed to the sorptive particles, so they will not be washed away or consumed by other microorganisms in the environment thereby increasing the potential for successful long term bioaugmentation. This concept was tested in the Phase I of project ER-2135, where biofilm-based inoculum on AC particles was applied as a delivery system in Aroclor 1248 contaminated sediment with high total PCB concentrations. The results showed that bioaugmentation with the biofilm inoculum enhanced the PCB dechlorination compared to

liquid inoculum and that the biofilm bacteria survived in the sediment environment and remained active throughout the 200-day experiment.

The intent of the work was to expand on the results with high PCB concentrations used in Phase I of project ER- 2135 by testing the application of dechlorinating bacterial biofilms on sorptive materials for treating sediments with lower total levels of weathered PCBs typically found in the environment. The **goal** for this research project is to obtain full degradation of PCBs in weathered sediment by focusing on the first and rate- limiting step of the initial anaerobic dechlorination and subsequently enable aerobic degradation by applying biofilm inoculum associated with AC as a delivery system. Many Department of Defense facilities face challenges from contaminated sediments, and existing remediation options are slow and expensive. This project assisted in the development of a novel approach to addressing PCB-contaminated sediments and management tools that will be applied in situ, resulting in more efficient and cost-effective reduction of risk at these sites.

An important aspect of the project was a more detailed assessment of the biofilm characteristics as the biofilm formed on activated carbon and other sorptive and carbonaceous materials. As a follow up to Phase 1, we showed that aerobic and anaerobic biofilms were established on GAC surfaces that subsequent could serve as a delivery system for contaminated sediment. Experiments where PCB contaminated Grasse River sediment was augmented with biofilm covered GAC showed increased Aroclor 1248 degradation. The results also showed that the augmentation benefitted other bacterial groups that were not directly linked to PCB degradation thus the delivery system could provide additional benefits compared to the objective.

To further assess the mechanism behind the increased PCB dechlorinated from the Grasse River sediment, the importance of the material characteristics for selected sorptive and non-sorptive materials was evaluated. The results showed that *Paraburkholderia xenovorans* LB400 biofilms developed on each of the tested sorptive materials and that the biofilms did not display a preference for growth on any particular sorptive carrier material. For this analysis, we developed advanced microscopy tools that were subsequently used to determine the mechanism behind the increased Aroclor 1248 dechlorination in Grasse River sediment.

One of the main findings in this project was that sorption of PCBs to activated carbon the driving force behind the biofilm formation by *Dehalobium chlorocoercia* DF-1. DF1 formed a patchy biofilm ranging in cellular biomass thickness from 3.9 to 6.7 µm with an average of 4.6 ± 0.87 µm, while the biofilm coverage area varied from 5.5% (sand) to 20.2% (activated carbon) indicating a preference for highly sorptive carbonaceous materials. Quantification of DF1 bacteria in the biofilms showed abundances from 1.2 to 15.3 $x10^9$ bacteria per g material. These results show that biofilm-based inoculum for bioaugmentation of weathered PCBs in sediment can be an efficient approach.

An important objective in the project was to understand the fact that microbial dechlorination of PCBs has been observed in sediments over the last three decades, but translation to the field has been difficult due to a lack of clear understanding of the kinetic limitations. Therefore, a study using a passive dosing/sampling approach was applied to accurately measure the biological rate of dechlorination of 2,3,4,5-tetrachlorobiphenyl (PCB 61) to 2,3,5-trichlorobiphenyl (PCB 23)

by *Dehalobium chlorocoercia* DF-1. The results showed that the rate of PCB 61 dechlorination was linearly dependent on the freely dissolved concentration in both sediment and in sediment-free microcosms. This understanding of PCB microbial dechlorination kinetics can be used to predict PCB dechlorination activity in sediments within a factor of 2. This finding demonstrates that with a combination of accurate measurement of the aqueous phase dechlorination kinetics and an understanding of the site-specific partitioning characteristics, it is possible to predict PCB microbial dechlorination in sediments.

Another main objective of the project was to determine a practical and successful combination of growth conditions, bacterial strains and sorptive materials to for biofilm development that would be applied in subsequent mesocosm experiments. For this purpose, *Dehalobium chlorocoercia* DF1 was scaled up, grown and maintained using tandem 20 L bioreactors.

The observations and results from the overall project were applied in an assessment of biofilms made up by the organohalide-respiring bacteria *Dehalobium chlorocoercia* DF-1 formed on the surface of pinewood biochar. The biochar-biofilms were augmented into Aroclor 1248 contaminated and weathered sediment from the Grasse River, NY, USA. The goal was to evaluate the organohalide respiration of the PCB contaminated sediment in the absence/presence of the biofilm and free-floating bacterial inoculum. The results showed that dechlorination of weathered PCBs in Grasse River sediment occurred in the presence of biochar, liquid bacterial inoculum and biofilm formed on biochar with the highest dechlorination rate for the biofilm-biochar inoculum. This approach also showed that the microbial community stability (i.e. the biofilms were robust) was enhanced due to the fact that the dechlorinating bacteria were immobilized in biofilm at the biochar surface. This approach provides an efficient method for utilizing organohalide respiring bacteria for PCB bioaugmentation thereby increasing the potential for long-term and sustained organohalide respiration in sediment.

Summary

The results from this project have expanded the knowledge about mechanisms and rates for bioremediation of weathered PCB contaminated sediment. In addition, we have developed several new analytical approaches that will be applicable for treatment of other environmental compartments that are contaminated with PCBs and other persistent organic pollutants. These approaches include a predictive tool for PCB microbial dechlorination kinetics, quantitative methods for determining biofilm formation and presence on materials as well as scale up of microbial inoculum.

The project assessed the advantages (and limitations) for applying bioremediation and in particular biofilm-based bioremediation using sorptive materials such as activated carbon and biochar from different feed stocks. It was shown that sorption was a key mechanism for the enhanced biofilm formation on activated carbon and that the microbial communities formed on sorptive materials increased the stability of the overall microbial community in PCB contaminated sediment.

The developed approaches can be implemented for bioremediation of PCBs and other persistent organic pollutants beyond contaminated sediment including stormwater, ‘mixed waters’ such as rising groundwater mixed with stormwater or sediment and/or other surface related waters.

These environmental compartments are becoming increasingly important due to the impacts of climate change such as rising water tables and more unpredictable storms. Therefore, the presence of biofilms (robust microbial communities) together with sorptive materials that can adsorb persistent organic pollutants might become even more beneficial in an increasingly dynamic environment.

Appendix 1

The following pages include pdf files that show data from very large excel sheets that have been used to calculate the results in the reported chapters. These are difficult to read and we will be happy to share the electronic data upon request.

- Chapter 4
- Chapter 5
- Chapter 8

The data from these chapters have not been included since this would make up several hundreds of pages of pdf files with PCB congener specific data. We will be happy to share the electronic data upon request.

- Chapter 3
- Chapter 6
- Chapter 7

Appendix 2

January 29, 2018
ER-2135/BVK

WHITE PAPER #2: Progress update on project ER-2135

"Material characteristics responsible for enhanced dechlorination of PCBs on biofilm covered activated carbon particles"

1. Introduction

This White paper is submitted in response to the project ER-2135 and the decision point (go/no-go) regarding the material characteristics that are responsible for the enhanced dechlorination of PCBs due to the presence of biofilm covered activated carbon particles

Background: Enhanced dechlorination of Aroclor 1248 was observed in mesocosm studies with Grasse River sediment, when biofilm covered activated carbon particles were applied as a delivery system for bioaugmentation. The results were obtained in the SEED-project ER-2135 and a follow-on proposal was submitted. At the SAB Meeting on Dec. 18, 2013 further explanation was requested of the setup of laboratory studies with focus on understanding the fundamental processes that are responsible for the enhanced dechlorination in the presence of the biofilm based delivery system.

Objective: In the proposed laboratory studies, we will focus on determining the fundamental characteristics of the materials and the interaction with the biofilm that enhance the dechlorination process. The scope of this project does not allow us to go into detail with the fundamental mechanisms at the molecular/nanoscale level.

Key Research question: The results and conclusions in this White Paper related to Key Research Question #1 in the proposal: "What is the fundamental process for the biofilm delivery system and can porous substrates other than pure AC such as AC with Fe, mesoporous silica and/or biochar serve as biofilm support and also enhance the microbial dechlorination activity and aerobic degradation of PCBs?"

2. Evaluation of the fundamental process that are responsible for enhanced dechlorination

In the initial laboratory studies proposed in the ER-2135 full proposal, focus was on determining the fundamental characteristics of the materials and the interaction with the biofilm that could enhance the PCB dechlorination process. *The scope of this first part of the project (Research question #1) did not allow for detailed analyses of fundamental mechanisms at the molecular/nanoscale level as stated in the proposal.* To determine the characteristics of the enhanced dechlorination process with the biofilm based delivery system, the effect of different types of materials with biofilm growth on the dechlorination potential was evaluated. The different permutations allowed for identification of the material characteristics that were responsible for the enhanced PCB dechlorination process.

Bacterial cultures: For these experiments the dechlorinating bacterium DF-1 living present in co-culture with a native sulfate reducing bacterium (*Desulfovibrio sp.*) were applied. Due to a slow-down in the DF1 growth during parts of the initial project period (described in the White paper of November 2017), alternative dechlorinating cultures were applied as a backup. These included

the PCR-dechlorinating mixed culture WBC-2, PCB dechlorinating mixed culture CBDB1. In the proposal, it was also discussed that PCB dechlorinating cultures O-17 and SF1 could be tested. A study on the efficacy of DF1, DEH10, SF1 and o-17 for bioremediation of weathered Aroclor in sediment mesocosms showed that DF1 was more effective at reducing PCB levels than the other strains when added as a co-amendment with a PCB degrading strain (Payne et al, 2017). DF1 is the only strain that has been demonstrated to degrade weathered PCBs in a pilot field study (ER201215). We also obtained and tested WBC-2, a consortium that dechlorinates chlorinated ethenes (Lorah et al, 2007). However, it is not known whether this consortium will dechlorinate weathered Aroclors.

Evaluation of the material characteristics were based on the conceptual model (Figure 1) and the three hypotheses described and illustrated below.

Hypothesis 1: Surface area and porosity
Hypothesis: The increased number of active bacteria located in a biofilm on the solid surface will enhance the dechlorination process. Increased surface area of the solid surfaces will enhance the number of bacteria and thus the dechlorination process. To determine the influence of surface area/porosity, materials with a high surface area comparable to carbonaceous materials that are neither sorptive nor conductive.

Hypothesis 2: Electrical conductivity characteristics
Hypothesis: Electrical conductivity of activated carbon may allow for direct electron transfer from the dechlorinating bacteria to the external PCB molecules sorbed to the same carbon surface. Microbial electron transfer to extra-cellular electron acceptors such as Fe(III) via conductive nanowires has been suggested. Thus, increased rates of dechlorination can result due to the bacteria being abundant and close to a concentrated and electrically accessible PCB pool.

Hypothesis 3: Sorption characteristics and physical proximity of sorbed PCBs and biofilm
Hypothesis: The sorption capacity influences the physical proximity of the PCB that can be utilized by the PCB dechlorinating bacteria located in the biofilm on the material surface. Thus, the close proximity of a large number of active dechlorinating biofilm bacteria to a PCB-loaded strong sorbent surface will allow for enhanced removal/dechlorination of PCBs. To determine the importance of the sorption capacity, a range of activated carbons and biochars with two orders of magnitude difference in sorption capacity will be tested as potential solid substrates for biofilm growth.

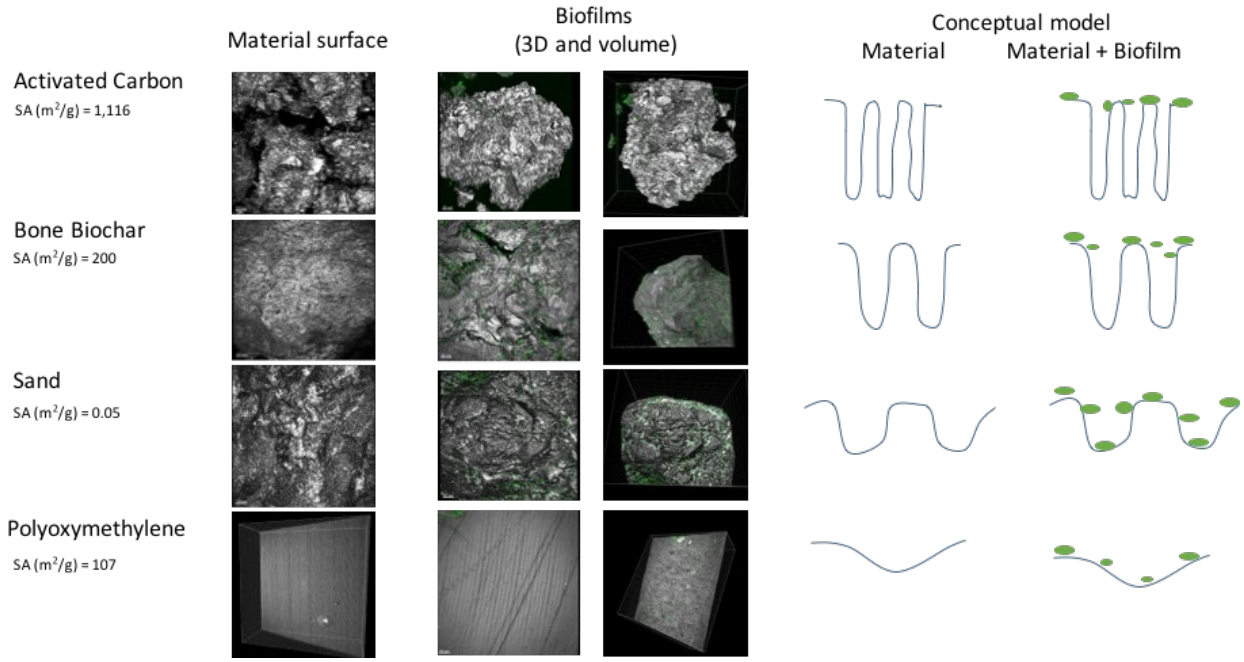


Figure 1*. Conceptual model illustrating the anticipated behavior of the tested materials covered in the presence of DF1 dechlorinating biofilms.*

3. Characterization of selected materials

The materials listed in Table 1 were all included in the primary evaluation of the material characteristics and their influence on biofilm formation. After the initial screening the number of material to be tested was reduced. Materials that contained/did not contain the characteristics tested in the outlined hypothesis were included.

Table 1. *Characteristics of materials used as amendments in microcosms experiments*

Material	Total surface area (m^2/g)	Sorption capacity Log(K_d)	Conductivity % Carbon	Avg. Pore Width (nm)	Commerciall y Available
Bone Biochar	200	N/A	11.0	6.25	Bonechar Carvao Ativado Do Brasil Ltd.
Coal AC (TOG)	1,116	(PCB-61) 8.70	80.9	1.48	Calgon TOG LF 80x325
Sand	0.05	N/A	0.0	$2x10^4$	Thermo Fisher Scientific
POM	107	(PCB-44) 5.65	N/A	N/A	Sigma-Aldrich

The results from the experiments are described in separate sections due to the difference in experimental approach. The conclusions from each approach will be discussed in Section 7.

4. Initial screening of material characteristics and importance for aerobic biofilm formation

From previous work in the Sowers' Laboratory, it was that different types of activated carbon can interact with bacteria in detrimental ways causing inhibition of the activity or killing the cells after long exposure. These studies were not performed in a quantitative way. Therefore, an array of materials has been tested for their potential to forming biofilms. The methods for quantification of biofilm included the following parameters: numbers of biofilm bacteria (plating and quantitative PCR) and surface coverage. The bacterial species *Burkholderia xenovorans* strain LB400, which is an aerobic PCB degrader was to evaluate any potential limitations.

Methods: Serum bottles containing a suspension of 300 ml of M9 media and 300 mg of each of the materials tested. Biphenyl was used as carbon source (5 mM) dissolved in acetone. One ml of the bacterial culture *Burkholderia xenovorans* strain LB400 (afterwards LB400) was used as seed microorganism. The cell concentration of the seed stock solution was approximately 10 cells per bottle. Each material was tested in triplicates. Samples were taken daily during the first part of the experiment (exponential growth) and then intermittently (every 1 or 2 days) until reaching stationary phase (plateau). Samples of materials (solids) and liquids were collected and analyzed at each time point. The solids were sonicated for 5 min in cell free buffer to resuspend the attached cells. The sonicated supernatant was stored at -20°C for further analysis. Biofilm characterization techniques included DNA extraction, and q-PCR with specific 16S rDNA primers. Active biomass was measured using a plating technique for Coloni Forming Unites (CFU).

Results: The results showed that *B. xenovorans* biofilms formed on all materials after 1 week. The results from the test of materials can be seen in Figure 2. No adverse effects in biofilm growth were observed by using any of the tested materials. In all the materials, an increasing trend on biofilm density (reported in cells/g of material) was observed. Figure 3 shows a side by side comparison of the biofilm development on some of biochars and activated carbons tested in this study. Although there was a clear increasing trend of biofilm growth for all materials, the difference among materials can be identified on a day by day basis.

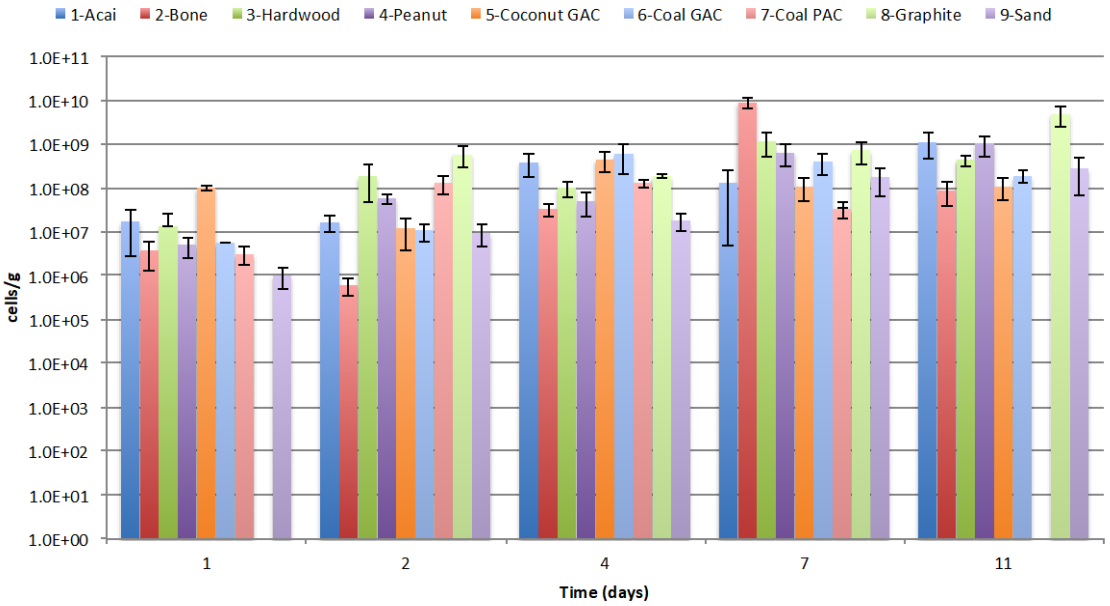


Figure 2. *Biofilm formation of B. xenovorans biofilms on materials after 10 days. The numbers were determined as CFU per g of material (Colony forming units on agar plates).*

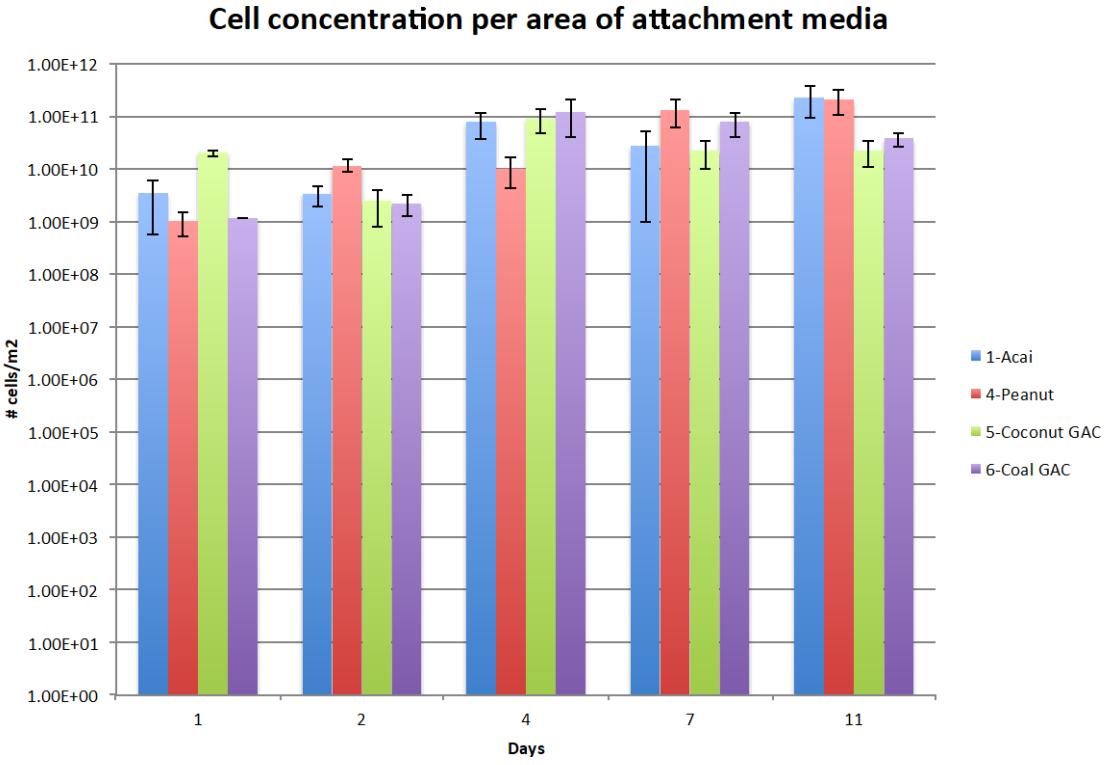


Figure 3. *Biofilm growth comparison between biochars (acai and peanut) and activated carbons (coconut and GAC).*

In the following, two different approaches were applied to independently test the outlined hypotheses: 1) The importance of the material characteristics were evaluated based on a microbiological approach. 2) The importance was evaluated based on a chemical approach. The results from each of these approaches will be described in separate sections and subsequently compared.

5. Impact of material characteristics on enhanced dechlorination based on biofilm formation

We investigated if surface area and porosity (*Hypothesis 1*), electrical conductivity (*Hypothesis 2*), and/or sorption capacity (*Hypothesis 3*) impact DF1 biofilm formation in addition to dechlorination in a microcosm experiment over 159 days. The following materials were tested: Polyoxymethylene (POM), Sand, Bone Biochar, and Coal Activated Carbon. Dechlorination activity was monitored using gas chromatography coupled to an electron capture detector (GC-ECD) and biofilm formation and cell abundance were monitored using confocal laser scanning microscopy (CLSM) and quantitative polymerase chain reaction (q-PCR), respectively. Methodology and results of the microcosm experiment are presented below.

Materials and Methods

Growth of bacterial cultures: Culture conditions were tested for *Dehalobium chlorocoercia* DF1 on selected materials: Coal Activated Carbon (TOG LF, 80 x 325), Bone Biochar, Sand and Polyoxymethylene (*POM)*. Physical-chemical characteristics of these materials are presented in Table 1 (Celzard, et al. 2002, Chun et al. 2013, Endo et al. 2017, Klüpfel et al. 2014, Pignatello et al. 2017, Saquing et al. 2016). For each material, three microcosm replicates along with a negative control were assembled and kept in an anaerobic chamber (5% H_2, 15% CO_2, 80% N_2). The replicates consisted of 0.1 gram of material (AC, BC etc.), 0.1 gram of autoclaved sediment (i.e. sterile), 1 mL of DF-1 co-culture (approximately $1x10^5$ of cells per ml), 9 mL of E-Cl medium, 1 mL of autoclaved *Desulfovibrio* extract, 10 mM degassed sodium formate (electron donor) and 10 ppm PCB-61 dissolved in acetone. Two controls were assembled in a similar fashion without materials, where one contained sediment and the other did not. Negative controls were assembled for each material treatment without the addition of culture. Samples were collected at days 22, 133, and 159 for confocal laser scanning microscopy analysis, DNA analysis and chemical analysis.

Quantitative biofilm measurements: A Leica confocal laser scanning microscope (CLSM) was used to image the abiotic material controls and the materials covered with biofilm. CLSM was based on collecting stacks of in-focus images through a thick sample, which were compiled via software to construct a 3-dimensional image. Images of SYBR Green stained microbes used a fluorescein isothiocyanate (FITC) filter Reflection of the material was acquired using reflected light and an Argon ion laser. Images of SYBR Green stained microbial biofilms, autofluorescence, and reflection of the materials, were overlaid in Imaris x 64 software (version 7.7.2) to produce the images shown in Figure 4. The Imaris software was also used to calculate the percent area of biofilm coverage (i.e., Biosurface area), where n = 10 images per sample for each time-point, on the different materials.

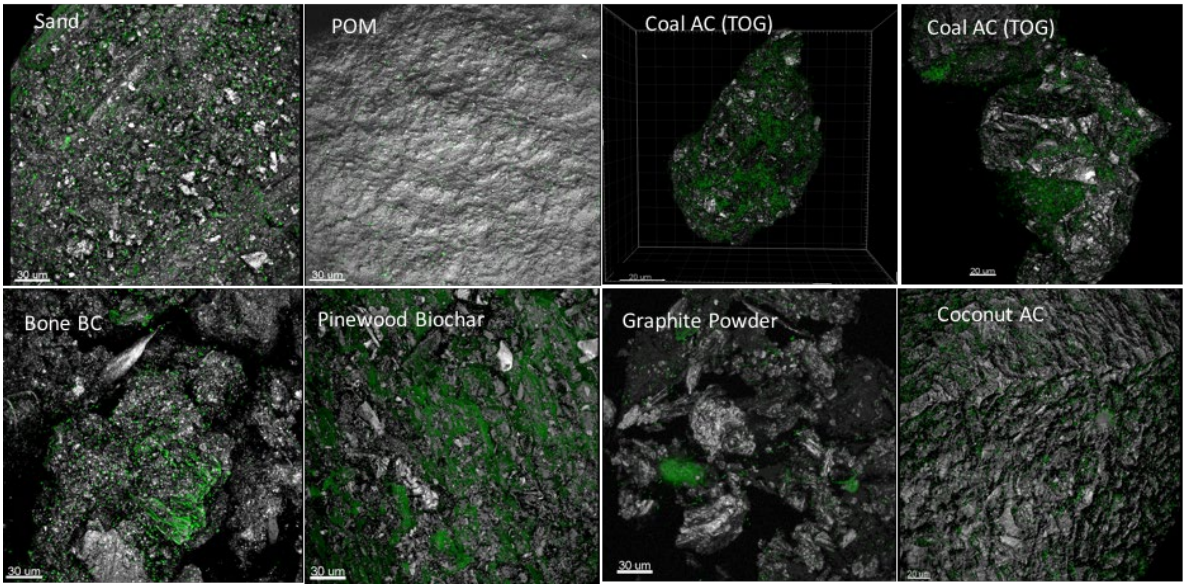


Figure 4*. CLSM images of DF1 biofilm on the surface of sand, POM, coal activated carbon, coconut activated carbon, bone biochar, pinewood biochar, and graphite powder.*

Enumeration of bacteria with q-PCR: Enumeration of DF1 cells present in biofilm and liquid samples were performed using quantitative PCR. Liquid samples were extracted using InstaGene kit (Bio-Rad Laboratories, Hercules, CA, USA) and solid samples were extracted using DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). A 10 order-of-magnitude dilution series of plasmid DNA containing the 16S rRNA gene was used as a standard for DF1 quantification. The q-PCR quantitations were run in triplicate. Total reaction volume was 25 µL, which included 4.5 µL Ultra-Pure Water (Gentrox, Claremont, CA, USA), 6 µL of template, 12.5 µL of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 1 µL each of the appropriate forward (dehcl – 348F: 5' GAG GCA GCA GCA AGG AA 3') and reverse primers (dehcl – 884R: 5' GGC GGG ACA CTT AAA GCG 3'). The thermocycler program was as follows: 2 min at 95 °C, followed by 40 cycles of (95 °C for 45 s, 55.7 °C for 45 s, 72 °C for 30 s). The 40 cycles were followed by an extension step of 72 C for 30 s after which melt curves generated by increasing temperature from 60°C to 95°C in 0.5°C per cycle increments confirmed that a single PCR product was synthesized in all reactions. Each increment was for 5 s.

Analysis of dechlorination in cultures: Chemical analysis was performed to evaluate DF1 dechlorination activity in the same microcosms, where surface coverage was evaluated via microscopy and q-PCR. Samples we collected (0.5 mL liquid) and transferred to a clean glass vial. Next, 3 mL of hexane are added as well as a mass recovery standard (tetrachloro-m-xylene). The samples were vortexed for 5 minutes and allowed to sit for 10 minutes for further separation of the organic and aqueous layers. The organic layer was transferred to a clean amber vial and the process was repeated twice. The sample was then concentrated via nitrogen evaporation and transferred to a clean GC vial. PCB-61 and PCB-23 were quantified against a five-point calibration curve using the internal standard (PCB-30) method of quantification. All samples were analyzed for PCBs on an Agilent 7890B gas chromatograph (GC) equipped with an Electron Capture Detector (ECD) using DB-5 (5% diphenyl dimethyl polysiloxane) capillary column (60m × 0.25mm i.d. × 0.25µm film thickness).

Results

Biofilm growth and the relationship with selected surface characteristics was examined via Confocal Laser Scanning Microscopy (CLSM), quantitative PCR (Q-PCR) and chemically via PCB dechlorination experiments. PCB-61 dechlorinated in all microcosms containing materials and DF1 culture. No activity was detected in negative controls. The results from the CLSM analysis showed the percent area coverage (afterwards: "Biosurface area") increased during the experiment (Figure 7). Determining biofilm coverage based on area of the biofilm allows for the monitoring of biofilm formation and structure of the biofilm on surface of the materials tested (i.e., Coal AC, Bone BC, Sand, and POM). Doing so will aid in quality assessment of the inoculum during the experiment as well as in future scale-up processes.

Table 2. *Results from quantitative microscopy using Confocal Laser Scanning Microscopy.*

Material	**Biosurface Area Coverage**	
	Average (%)	**Standard Error (%)**
Bone Biochar	14.7	5.8
Coal AC (TOG)	20.2	4.1
Sand	5.5	1.4
POM	10.2	2.5

Statistical analyses (ANOVA) were performed confocal data to examine trends between groups of materials over time as well as between samples. The analyses revealed a statistically significant difference in Biosurface area for the materials as the biofilms were formed thus showing significant biofilm formation over time for all materials tested (between 22 days and 133 days and between 22 days and 159 days). Furthermore, a t-Test showed a significant difference between Bone BC and Coal AC samples at day 133 day.

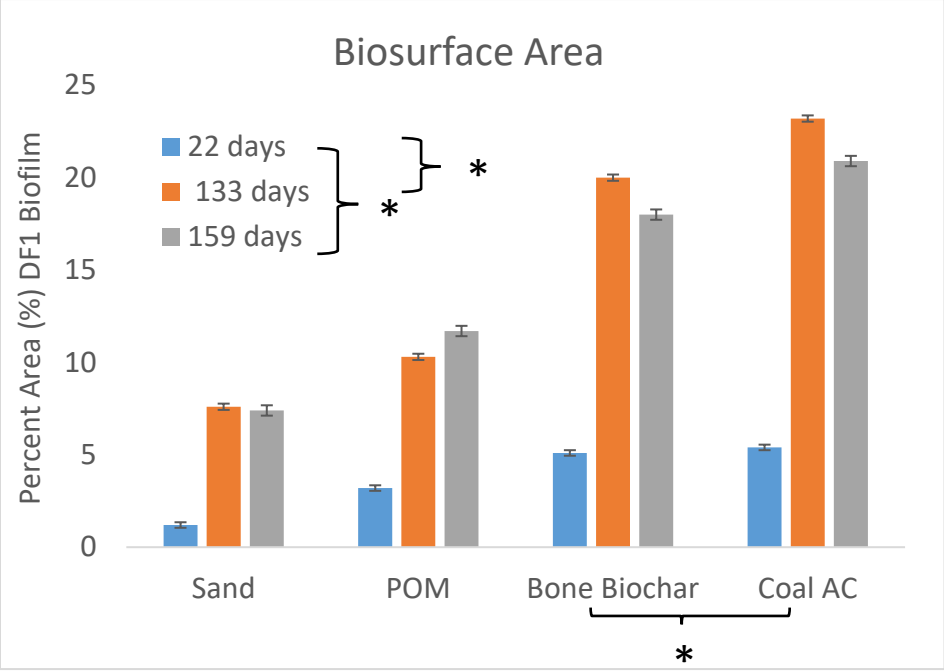
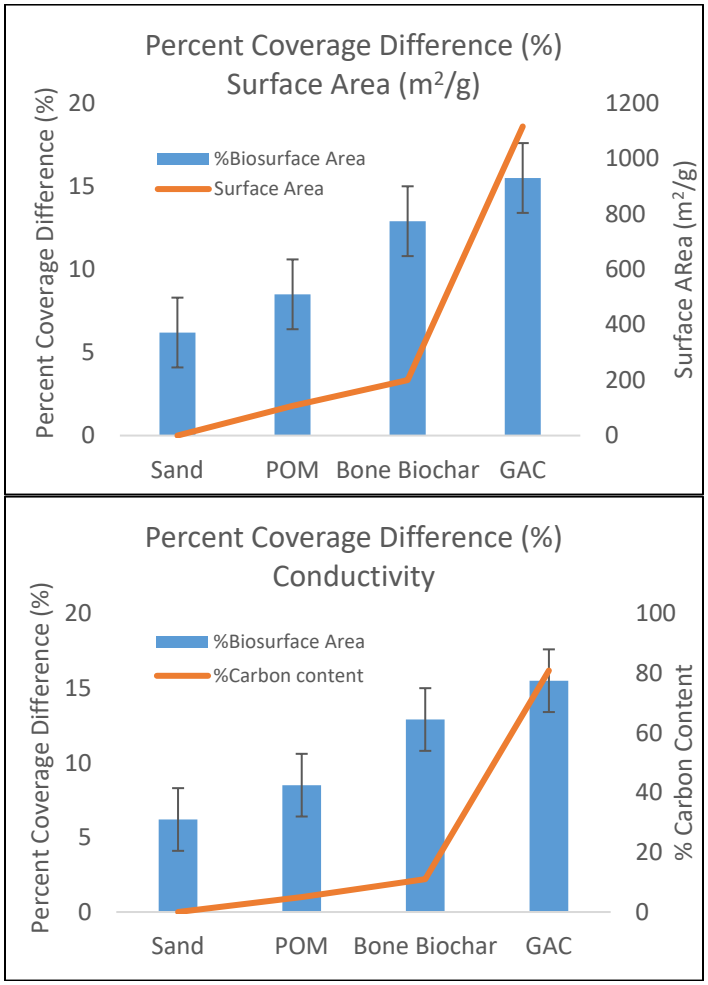


Figure 5. *Biosurface area (%Area coverage) of DF1 biofilm on various materials over time. An asterisk (*) denote a significant difference between groups over time ($p < 0.05$). Error bars indicate standard deviation ($n = 10$ images). Statistically significant difference in Biosurface area was observed for all materials over time. This means that the biofilm actively grew on the materials.*

To further investigate the Biosurface area, the data was sorted according to material characteristics (Figure 6, a-c). The data was assessed in such a manner to observe trends resulting from increasing total surface area, electrical conductivity and sorption capacity of the materials tested to investigate the effect of the material characteristics on biofilm formation and subsequently on dechlorination activity.



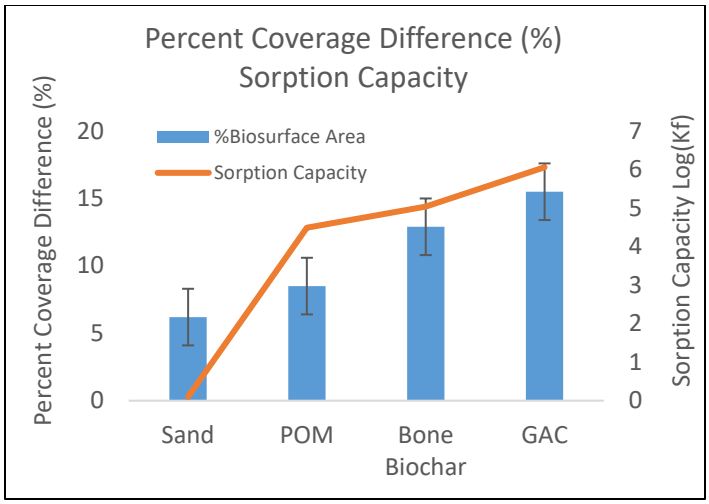


Figure 6, a-c*. The percent coverage difference over the duration of the experiment of DF1 biofilms on various materials. The plots are organized by increasing "quantitative value" of material characteristics. Error bars indicate standard error. The data showed that the Biosurface area (i.e. area covered with DF1 biofilm) increased more with increasing sorption capacity than surface area and electrical conductivity over time.*

Enumeration of bacteria on surfaces: The results of the qPCR data for the tested materials are shown in Figure 7. In all experiments a significantly larger fraction of the total number of cells in the microcosm was associated with the materials than the liquid (<1%) for all the microcosms (liquid data not shown). Therefore, the observed dechlorination activity can be assigned to DF1 bacteria present in biofilms. There was an increasing trend on the materials over time for all materials except for sand, where a minor decrease was observed between the last two time points. The q-PCR data from the solids did not support the Biosurface area coverage results, since the number of bacteria did not increase linearly as the biofilm coverage increased. The reason for this was likely that biofilm is made up both by bacterial cells and extracellular polymeric substance (EPS), which is the "slime" that keeps the bacteria together. During the quantitative analysis, it was also observed that some of the materials caused slime to be formed to a larger extent than others. Quantification of the EPS coverage of the materials was evaluated and reported in the quarterly report for October 2016, which supports the findings with DF1 biofilms. However, there was an increasing trend for sorptive materials and materials with higher total surface area suggesting the formation of more expansive biofilms on materials with larger available surface area and more sorptive characteristics. This is in agreement with the independently obtained microscopy results and support the notion that materials with more available surface area and sorptive characteristics support DF1 biofilm formation.

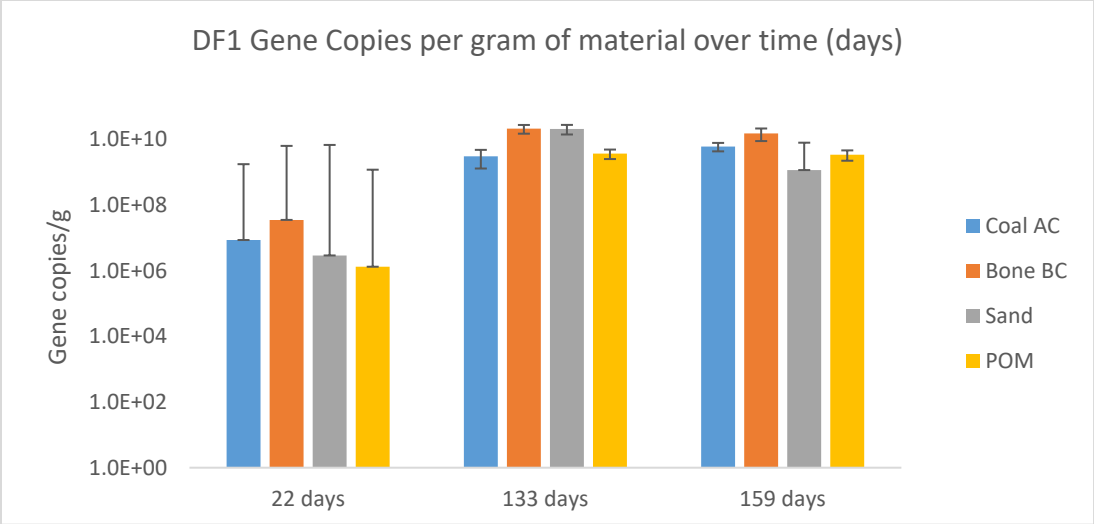


***Figure** 7. Enumeration of DF1 cells attached to material surfaces using qPCR. Error bars represent standard error.*

Dechlorination analysis: PCB-61 was dechlorinated by DF1 biofilms in all microcosms containing materials and by cells in the liquid phase for the control with DF1 culture. No activity was detected in negative controls. The mole percent (%mole) of daughter products as a sum of parent and daughter products was calculated over the observations period. After 22 days, Coal AC, bone BC, sand and POM microcosms dechlorinated to maximum of 27%, 22%, 79% and 47%, respectively. After 53 days the microcosms amended with sorptive materials showed a decrease in dechlorination, whereas those amended with non-sorptive materials showed continued dechlorination. These results may be attributed the parent and daughter products were re-absorbed into sorptive materials.

Overall conclusion

Materials with larger surface area allow for more area for cells to attach to and biofilm to form thus resulting in more extensive biofilm coverage on the materials. Materials with sorptive characteristics *pull* the PCB toward the material, where the driving force of sorption can be *ad*sorption of *ab*sorption depending on the material (both for BC and AC, and adsorption for POM). Over time, not only will the PCB equilibrate with the carbon source but the biofilm will also form. Once the material has reached equilibrium, there will be steady forward and reverse transfer of the electron acceptor (PCB). The combination of the equilibrated carbon and formation of a biofilm (cells and EPS) will aid in a steady transfer of an electron acceptor for the microbe and the biofilm matrix will assist in providing a secure region with a carbon source and electron donor.

6. Impact of material characteristic on enhanced dechlorination based on chemical approach

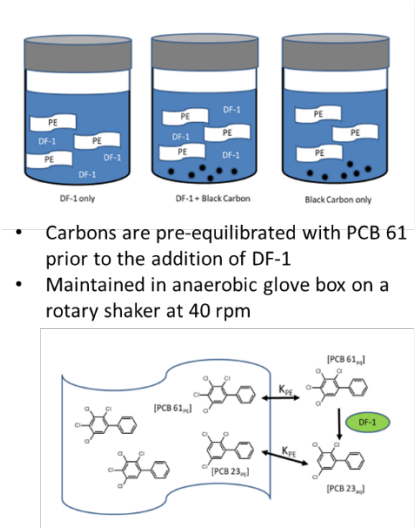
The physiochemical properties of carbonaceous materials have been demonstrated to enable various biological and abiotic redox reactions. These properties can vary greatly based on the source material, pyrolysis temperatures, and activation methods (Pignatello et al. 2017, Celzard et al. 2002, Klüpfel et al. 2014). Subsequently, which property of AC or biochar enhances redox

reactions for microorganisms can vary greatly depending on the organism and the electron acceptor (Saquing et al. 2016, Chen et al. 2014, Chen et al. 2018). The following experiments were conducted to determine which of the physiochemical properties of AC enhance dechlorination of PCB 61 by DF-1.

Sorption characteristics and physical proximity of sorbed PCBs and biofilm

Materials and Methods

Two activated carbons and one biochar with different sorption capacities (K_d) were tested in order to evaluate the effects of sorption on the rate of dechlorination of PCB 61 by DF-1. Dechlorination rates were measured using a passive dosing-passive sampling approach using polyethylene sheets that were pre-loaded with PCB 61 and equilibrated with the system prior to the addition of the DF-1 inoculum. The experimental design as illustrated in Figure 8 closely follows the method reported by Lombard et al (2014). An initial study confirmed that the rate of dechlorination in the absence of carbons was dependent on the initial aqueous concentration and the rate kinetics matched those reported by Lombard et al (2014). The accumulation of the dechlorination product, PCB 23, was observed in all materials tested. Accurate measurement of PCB 23 kinetics is confounded by the kinetics of sorption by the different carbonaceous materials, therefore the rate of dechlorination was measured based on the loss of PCB 61 and comparison between control and bioamended systems. Each material tested included an abiotic control where autoclaved DF-1 culture was added.



Materials tested:

PCB 61:
Coal GAC (TOG LF 80x325)
Coconut GAC (OLC 30x50)
Pinewood biochar
AQDS (0.1 mM)
Graphite Powder

PCE:
XAD
Coal GAC
Clay
Graphite Powder

Figure 8. *The PCB biodegradation kinetics was measured using a passive dosing/passive sampling approach as described in Lombard et al. 2014. PCB 61 was dosed in PE and pre-equilibrated with media and a range of materials. The loss of PCB 61 and appearance of the dichlorination product PCB 23 were tracked in the PE over time. In the PCE experiments, the headspace was sampled to measure PCE and the dichlorination products.*

Results

As illustrated in Figure 9 top panel, there is no loss of PCB 61 over 21 days in the absence of DF-1. Thus, the presence of the carbons alone does not lead to abiotic removal of PCB 61 either through further adsorption or through any abiotic reaction. Also, the abiotic experimental results indicate the carbon, water, and PE phases were all at a steady state equilibrium condition

throughout the experiment. The four plots at the bottom of Figure 9 indicate loss of PCB 61 when DF-1 is present in the mesocosms. Each mesocosm received the same amount of DF-1 inoculum. In each case there is a marked loss of PCB 61 with time as measured based on extraction of a PE sheet from the mesocosm at each time interval. The data plotted in Figure 9 are concentrations of PCB 61 measured in PE normalized to initial concentration in PE. While the slopes of the normalized concentrations look similar, each mesocosm had a wide range in total PCBs which needs to be accounted for in a material balance to determine the true rate of transformation.

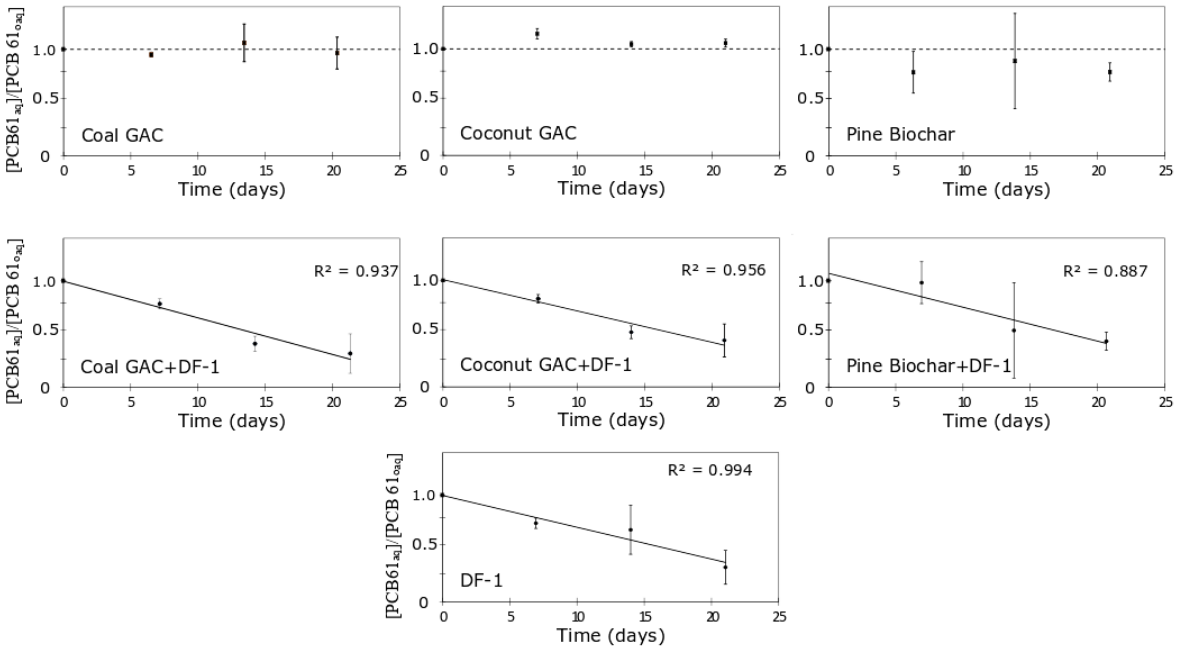


Figure 9. *Top panel: No loss of PCB61 from mesocosms with carbons and no DF-1 demonstrates: i) no abiotic loss of PCB61, and 2) steady state equilibrium conditions between carbon, water, and PE. Bottom four plots show PCB 61 loss kinetics in PE when DF-1 is present in the mesocosm.*

A linear first order aqueous rate was observed in all materials tested. The PCB dechlorination rate (k_b) in a passively dosed system that contains strong sorptive materials must account for the contribution and buffering capacity to the changes in the aqueous concentration. Based on the overall material balance in the system, the rate of dechlorination can be described by equation 1.

$$\frac{dC_w}{dt} * \left(\frac{V_w * + m_{PE} * K_{PE} + m_C * K_d}{V_w}\right) = C_w * k_b \qquad (1)$$

Where, V_w is the volume of the water, m_{PE} is the mass of the PE in the system, K_{PE} is the equilibrium partitioning coefficient, m_C is the mass of carbonaceous material, and K_d is the equilibrium partitioning coefficient for the carbonaceous material. The buffering capacity represented by the term in the parentheses of equation 1 or the inverse as in the bracketed term in

equation 2 (often called the bioavailability factor; Zhang and Bouwer, 1998) is composed of known values for each system. Therefore, the apparent biodegradation rate (k_b') can be expressed as the true biodegradation rate (k_b) multiplied by the bioavailability factor as shown in equation 2. Substituting k_b' for k_b simplifies the first order rate equation (4) and can be estimated based on the slope of the normalized concentration plots in Figure 9. Table 3 presents the apparent and true aqueous phase biodegradation rates along with the respective partition constants for each solid tested. As shown in Table 3, and Figure 10, the estimated net bi odegradation rate was different in the presence of the various carbon sorbents. The k_b value with DF-1 only (no sorbents) was similar to those reported by Lombard et al. (2014) and increased by about an order of magnitude in the presence of coal GAC. The value of k_b demonstrated proportionality with the partition constant in carbon as illustrated in Figure 10. Thus, the true rate of dichlorination is increased in the presence of activated carbon.

$$k_b' = k_b * \left(\frac{V_w}{V_w * + m_{PE} * K_{PE} + m_{AC} * K_{AC}}\right) \quad (2)$$

$$\frac{dC_w}{dt} = k_b' * C_w \quad (3)$$

$$\frac{C_w}{C_{w\,o}} = e^{-k_b' t} \quad (4)$$

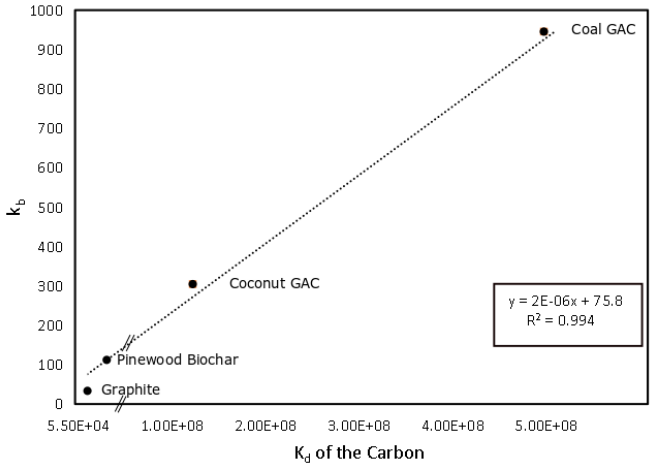


Figure 10. *True biological rate constant k_b in [PCB61$_{tot}$]/[PCB 61$_{o\ tot}$] day^{-1} with respect to K_d.*

Table 3*: Biological rate constants observed and calculated for different carbonaceous materials.*

Material	**k_b'** ([PCB61$_{aq}$]/[PCB 61$_{o\ aq}$] day^{-1})	**k_b** ([PCB61$_{tot}$]/[PCB 61$_{o\ tot}$] day^{-1})	**K_d** (L/kg)
DF-1 Only	0.0284	105	
Coal GAC	0.0329	945	5.01E+08
Coconut GAC	0.0305	304	1.26E+08
Pinewood Biochar	0.0302	112	6.31E+05

Electrical conductivity characteristics

Materials and Methods

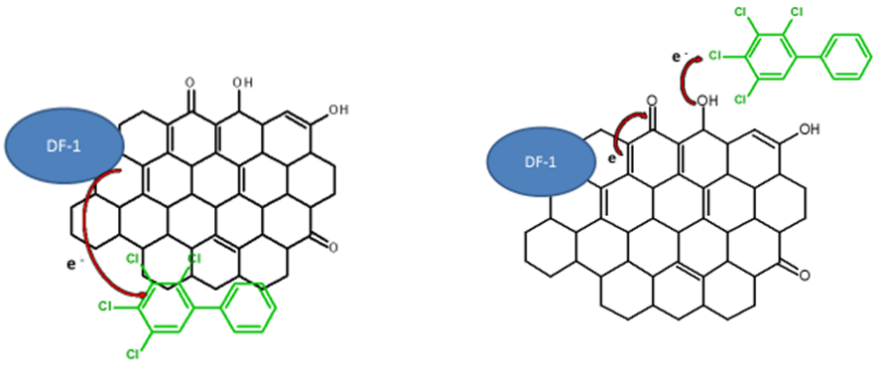
Graphite powder and anthraquinone-2,6-disulfonate (AQDS) were tested in addition to the black carbon materials following the experimental design illustrated in Figure 8. High purity graphite powder (Alfa Aesar) was selected since it does not contain any functional groups compared with biochars or activated carbons.

Electron Acceptance Capacity Measurement. A sample of the two activated carbons and one biochar were analyzed for electron acceptance capacity (EAC) to determine if there is a correlation to the dechlorination rate. The carbon samples were ground and sieved to <100 um in 0.5 g duplicates. Existing electrons were removed from the samples by aerating DI water then the residual O_2 was removed by vacuum and N_2 purging. The samples were further degassed in an anaerobic glove box and reduced with excess Ti(III) citrate at a pH of 6.4. The electron accepting capacity was then calculated based on the oxidation of Ti(III) over time.

PCE Rate Kinetics: 100mL of high cell density DF-1 cell culture (10^7 cells per mL) was added to 160 mL serum vials containing either XAD-2 resin, coal GAC, graphite powder, or clay. 5mg of PCE was added to each culture. PCE and the dechlorination products TCE and DCE were measured at 48 hour intervals by headspace analysis. Clay was selected as non-sorptive, non-conductive control. A control for each material received autoclaved DF-1 culture to serve as an abiotic control.

Results

Biochars and activate carbons are both conductive and contain quinone groups that are capable of acting as both an electron acceptor and electron donor in different redox conditions. Species of *Geobacter* have been demonstrated to use the conductive surfaces to enhance direct interspecies electron transfer (Liu et al. 2013, Chen et al. 2014) and enable redox reactions on the carbon surface (Chai et al 2012, Yu et al. 2015, Chen et al 2018). Additionally, 0.1 mM AQDS has been previously reported by Chen et al. (2016) to increase dechlorination of PCB 153 by acting as an electron shuttle by receiving intracellular electrons then donating those electrons to an external electron acceptor (Chen et al. 2016). AQDS is a surrogate for humic acids which are ubiquitous to sediments and necessary for some anaerobic processes (Lovley et al 1996) but has not been demonstrated to be necessary for DF-1 in halorespiration. The effect of electrical conductivity on dechlorination can be evaluated based on two possible mechanisms. First, that the graphitic regions on carbonaceous materials enable direct electron transfer to sorbed electron acceptors. Second, that redox active sites, such as quinone groups or humic acids, act as an electron shuttle for anaerobic dechlorination. A representation of these two possible mechanisms is illustrated in Figure 11.



Mechanism 1: Direct electron transfer to sorbed electron acceptors

Mechanism 2: Electron shuttle through redox active sites

Figure 11. *Two possible mechanisms for electrical conductivity. Mechanism 1 (left) is based on extracellular electron transfer to a sorbed electron acceptor. Mechanism 2 (right) is based on an electron shuttle model where redox active sites facilitate the electron transfer to the electron acceptor.*

As shown in Figure 12, the addition of graphite powder did not result in an enhancement of the dechlorination rate (k_b). Graphite provides a conductive surface for electron transfer like activated carbons, but does not have a strong sorption capacity for PCBs. Electrical conductivities for graphite, pinewood biochar, coconut GAC, and coal GAC have been reported by Celzard et al (2002) (Table 4). If direct electron transport through the conductive surface was responsible for the increase in rate observed in the presence of GAC and biochar then a similar result would be expected with graphite powder. *However, since no increase in the biological degradation rate was observed in the presence of graphite (Figure 6) and there is no correlation between electrical conductivity and k_b reported in Table 4, the hypothesis that conductivity alone enhances dechlorination is not supported.*

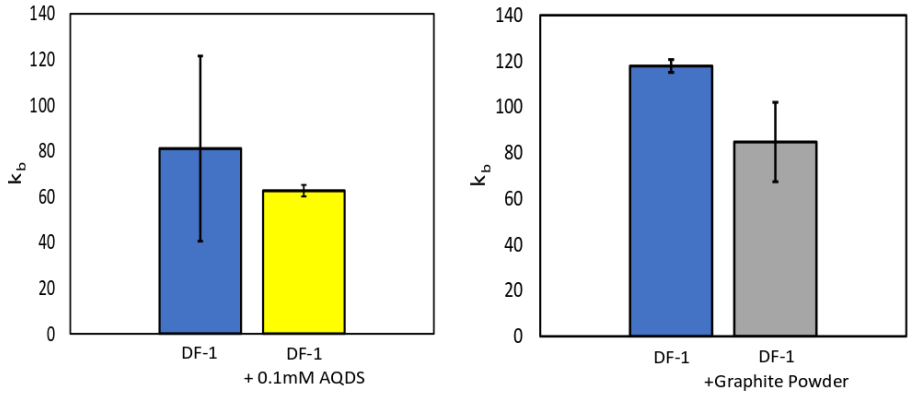


Figure 12. *Biological rate comparison between mesocosms consisting of DF-1 culture alone and the addition of graphite powder (grey) or 0.1 nM AQDS (yellow). The results are based on two separate experiments where a control receiving the same cell inoculum (blue) was set up to account for any variation in the culture.*

Table 4**:** *Electrical conductivity and redox properties of the carbon materials*

Material	k_b [$PCB61_{tot}$]/[$PCB\ 61_{o\ tot}$] day^{-1}	Conductivity σ (S cm-1)	EAC (mmol e^-/g)	% Carbon
Coal GAC	946	50.41	3.62	80.9
Coconut GAC	304	34.84	3.16	90.8
Pinewood Biochar	112	0.418	3.08	22.1
Graphite Powder	34	106.5		99.0

A second characteristic of activated carbons and biochars is the ability to act as an electron acceptor and donator through the presence of redox active groups as depicted in mechanism 2 of Figure 11. The redox active groups of activated carbons and biochars are primarily associated with quinone and phenol groups within the carbon structure (Klüpfel et al. 2014). *Geobacter* species have been reported to be capable of utilizing the electron accepting capacity (EAC) of biochar to enhance different biological redox reactions (Yu et al. 2015, Saquing et al. 2016, Chen et al. 2018).(Yu, Yuan et al. 2015, Saquing, Yu et al. 2016, Chen, Wang et al. 2018) The EAC for the three-carbon species were coal GAC 3.62 mmol e^-/g, coconut GAC 3.16 mmol e^-/g, and pinewood biochar 3.08 mmol e^-/g (Table 3). These results correlate with the true biological rate constant (k_b, R^2=0.993). The correlation between the true biological rate constant and the EAC is reported in Figure 13. *These results indicate potential interaction between DF-1 and redox active sites as depicted in mechanism 2 of Figure 11 however, the EAC values for the materials also correlate with the K_d of the carbons making it difficult to separate the potential effects of sorption and EAC.*

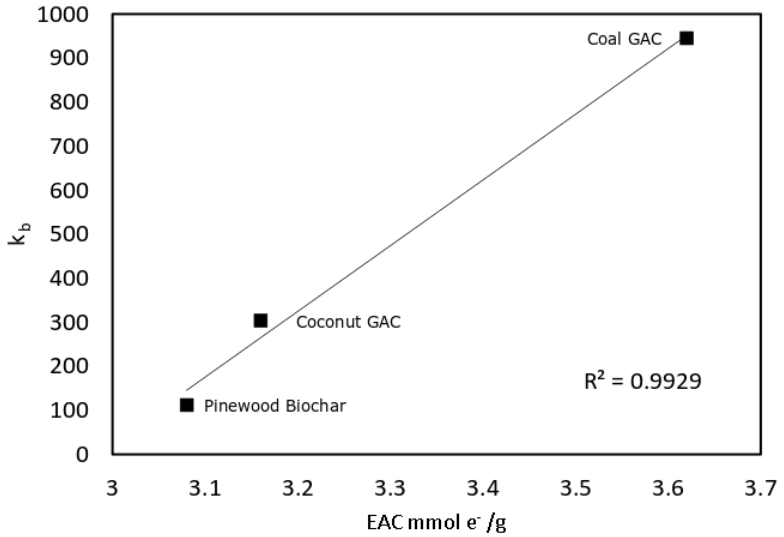


***Figure 13**: Electron accepting capacity (EAC) for three carbon species versus the true biological rate constant (k_b) in [$PCB61_{tot}$]/[$PCB\ 61_{o\ tot}$] day^{-1}.*

To evaluate if sorption in addition to a conductive surface is necessary for enhanced dechl1orination, additional studies were performed comparing coal GAC with a polymeric adsorbent resin, XAD-2. XAD was evaluated against GAC using polychlorinated ethylene (PCE) to screen if XAD should be evaluated with PCB 61. DF-1 dechlorinates both PCBs and PCE, but the dechlorination kinetics are much faster for PCE and PCE equilibrates much quicker with the sorbents. The mass of PCE in each mesocosm is plotted showing the sorbed (black) and freely dissolved (blue) masses in Figure 14. For clay and graphite, there is very little transformation of PCE during the study duration. However, for XAD and GAC large reductions of total PCE are observed compared to the abiotic controls. Thus, initial results support the hypothesis that sorption alone is the material characteristic that enhances rates of dechlorination. Currently XAD is being evaluated against coal GAC for PCB 61 dechlorination following the same experimental method performed on the different carbonaceous materials. Estimated completion for this experiment is February 2018.

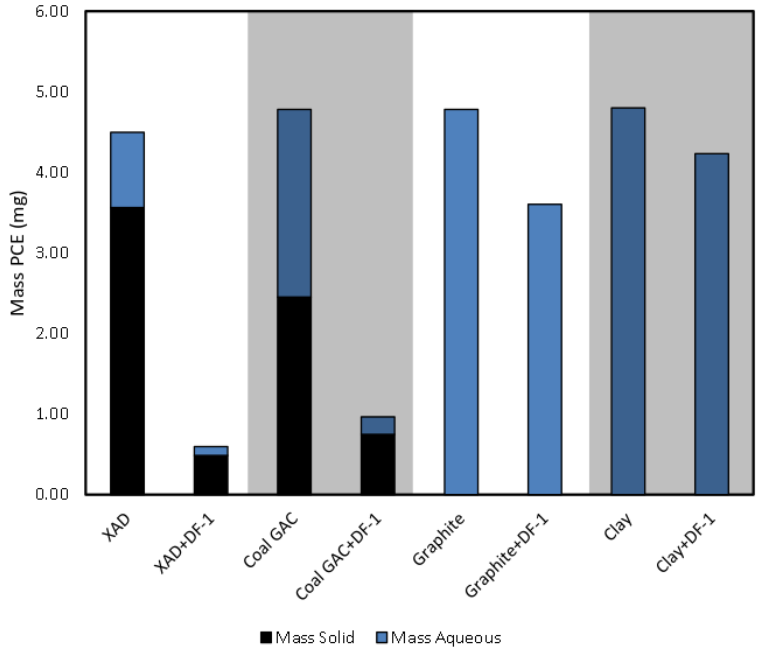


Figure 14. *Mass of PCE after 7 days. Each material was tested with autoclaved DF-1 culture and live DF-1 culture and plotted side by side as shown in the shaded panels. Mass distribution of PCE in the solid (sorbed) and aqueous phase is calculated based on the observed partitioning in the abiotic system at day 7. Each system received the same spike of 5 mg PCE.*

Surface area and porosity

Results

The activation process for black carbon material increases the surface area by orders of magnitude over non-activated material such as coke and biochar. The effect of activation is evident when comparing the surface area between the two GACs and pinewood biochar in Table 5. To evaluate the possible effects of surface area and porosity on the dechlorination rate, the biological rate constants were plotted against surface area to see if a correlation could be found. *No correlation was observed between the surface area and the rate of dechlorination of PBC 61 in the materials tested* (see Figure 15). Coal GAC enhanced dechlorination the most out of the materials tested but did not have the greatest surface area when compared with coconut GAC.

However, it is important to note that coconut shell GAC is known to have more micropores compared to coal based GAC. DF-1 (~300 nm) is likely size excluded from the micropore (1-2 nm) surface area in GAC.

At the completion of the experiment, a subsample of each material was analyzed by confocal microscopy in to assess the formation and coverage of biofilm by DF-1. The cell coverage did not correlate with the surface area with each carbon receiving the same inoculum of cells with comparable mass of each carbon. *These results do not support the hypothesis that surface area and porosity enhance the number of bacteria on the surface resulting in enhanced dechlorination*. Biofilm growth and how it relates to the surface characteristics of these materials is discussed in greater detail in Section 4.2 of this document.

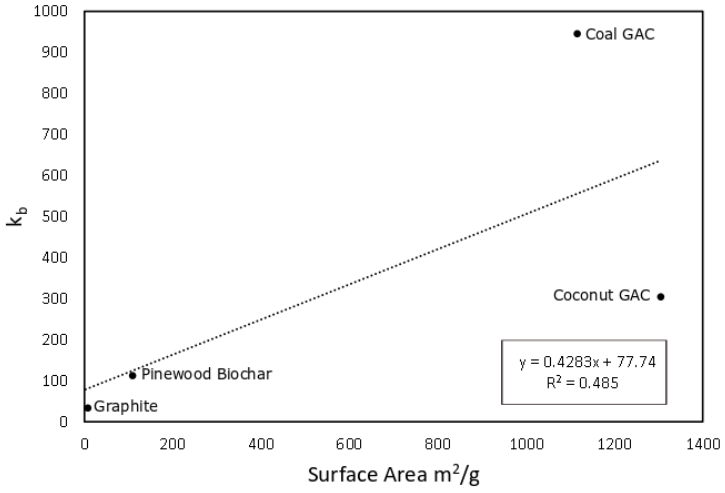


Figure 15. *True biological rate k_b in $[PCB61_{tot}]/[PCB\ 61_{o\ tot}]$ day^{-1} with respect to surface area of the carbonaceous materials.*

Table 5*. Measured biodegradation rates, surface area, pore diameter, and cell coverage of four carbonaceous materials tested.*

Material	k_b $[PCB61_{tot}]/[PCB\ 61_{o\ tot}]$ day^{-1}	SA (m^2/g)	Average Pore Diameter (nm)	Cell Coverage
Coal AC	946	1116	1.48	79%
Coconut AC	304	1305	1.45	30%
Pine Biochar	112	109	1.56	89%
Graphite	34	2.79	N/A	N/A

7. Conclusion

The results from the two independent approaches showed that the sorption capacity of the tested materials played the most important role in enhancing PCB dechlorination on the presence of DF1 biofilms.

In a continuation of the overall project (ER-2135), the approach will be to employ the biofilm based delivery system utilizing with a focus on materials with a high sorption capacity as attachment and growth surfaces for biofilm formation by PCB dechlorinating bacteria. The

system benefits from bacteria being able to be grown to a high density at the surface of sequestering adsorbent material. PCB-dechlorinating bacteria in biofilms are surrounded by a hydrophobic layer that enables the bacterial cells to align in close proximity to the highly sorptive particles and thereby interact with the adsorbed hydrophobic PCBs. This biofilm community of microbes has a much larger cell density and activity than would be possible to obtain in free floating systems, where the chances are rare for direct interaction between PCBs and the bacteria. This interaction is required for electron transfer and subsequent PCB dechlorination. The dechlorinating bacteria will also be fixed to the surface of the particles so they will not be washed away or consumed by other microorganisms in the environment, increasing the potential for successful long-term bioaugmentation. This two-phased approach will provide an efficient and cost-effective method for delivering microorganisms for bioaugmentation.

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March 12, 2018
ER-2135/BVK

WHITE PAPER #3: Progress update on project ER-2135

Status on current activities
This White paper (#3) describes the progress made on project ER-2135 in addition to the presentation at the IPR on February 7, 2018. In addition, a proposal for a path forward has been outlined together with a revised time line.

The progress will be described for the individual tasks for this project. Due to several delays during the project period (move to another university for PI Kjellerup, delays due to growth of DF1 (Sowers)), the deadlines for the tasks have been updated several times. The tasks involved in ER-2135 are shown in Table 1.

Table 1*. Tasks involved in project ER-2135 as they are present in SEMS2.*

Task #	Task	Completed?
3	Fundamental characteristics (Initial Lab Study)	Yes. White Paper 2 submitted January 2018
4	Isotherm studies	Yes. See this White Paper (#3).
5	Inoculum decision	Yes. White Paper 1. Submitted November 2017.
6	DOD site visits, sediment collection and characterization	Yes. See this White Paper (#3). Collected sediment from DOD sites will be used.
7	Setup of new analytical methods – Microbiology	Yes. See this White Paper (#3).
8	Setup of new analytical methods - Chemical and other	Yes. See this White Paper (#3).
9	Culture setup	Yes. See this White Paper (#3).
10	Not present in SEMS	
11	Biofilm inoculum (Anaerobic vs material)	In progress. Outlined in White Paper #3
12	Biofilm inoculum (Aerobic vs material)	Yes. See this White Paper (#3).
13	Removal methods for PCE on sorbents	In progress. Outlined in White Paper #3
14	Scale-up method develop: FBR approach	In progress. Outlined in White Paper #3
15	Scale-up method develop: MBBR approach	On hold. Depends on results from Task 14.
16	Standard inoculum assessment methods	In progress. Outlined in White Paper #3
17	Cost estimate	In progress. Outlined in White Paper #3
18	Decision on inoculum scale up method	On hold. Depends on results from Task 14, 15.
19	Scale-up mesocosm experiment	In progress. Outlined in White Paper #3
20	Mesocosm studies - Set up	In progress. Outlined in White Paper #3
21	Mesocosm studies - microbiological analysis	In progress. Outlined in White Paper #3

22	Mesocosm studies - chemical and other analysis	In progress. Outlined in White Paper #3
23	Reporting	Taking place

Task 3: Fundamental characteristics (Initial Lab Study)
This task was completed and a White Paper (#2) was submitted in January 2018 where the results were presented. These were also presented at the February 2018 IPR. Two manuscripts for peer-reviewed journals are in preparation based on work performed for this Task (see list of manuscripts).
Status: Task completed.

Task 4: Isotherm studies
Several isotherm studies were performed to characterize the sorption characteristics of the sorbent materials used for this project and specific parent compounds and degradation products in the dichlorination reactions studied. These included isotherms for PCB 61 and PCB 23 for the sorbent materials coal GAC, coconut GAC, and pinewood biochar. PCB isotherms were measured using a passive sampling approach as described in Gomez-Eyles et al. (2013). Results of these isotherms were presented in White Paper #2.

Additionally, isotherms were measured for coal GAC for tetrachloroethylene (PCE), trichloroethylene (TCE), dichloroethylene (DCE), vinyl chloride (VC), and Ethylene and graphite powder for PCE and TCE. Freundlich isotherm parameters were calculated for each of these isotherms and used to interpret based on a partitioning model, the experimental observations from kinetic studies with WBC-2 and DF-1 cultures.
Status: Task completed.

Task 5: Inoculum decision
This task was completed and a White Paper (#1) was submitted in November 2017, where the results were presented. One manuscript has been submitted to a peer-reviewed journal based on work performed for this Task and another is in preparation (see list of manuscripts). The manuscripts describe the opportunities for aerobic and anaerobic biodegradation in wastewater treatment plants, where the original proposal outlined a potential for obtaining enrichment cultures.
Status: Task completed.

Task 6: DOD Site visits, sediment collection and characterization
The team has been able to leverage site visits from other DOD projects to collect sediment samples that can be applied in this project. We have sediment collected from 3 PCB contaminated sites: Abrahams Creek at MCBQ, VA, Shiawassee River, MI and a sewage treatment pond in Altavista, VA. The sediment from all three sites has been characterized for PCB congeners and we have adequate material available in storage anaerobically at 4C for the proposed mesocosm studies. In addition, samples from Canal Creek, Aberdeen Proving Grounds, where Ghosh and collaborators collected sediment samples for a long-term performance

monitoring project (ER-201580) is available as well. The sediment has been characterized for PCB congeners, TOC, black carbon, and partitioning characteristics and will be applied for the mesocosm testing in Tasks 19-22.
Status: Task completed.

Task 7: Setup of new analytical methods – Microbiology
New microbiological methods have been evaluated and implemented in the Kjellerup and Sowers Laboratory groups. These include: 1) Quantitative PCR (Q-PCR) for enumeration of bacterial groups in sediments, enrichment and laboratory cultures using several primer sets based on the 16S RRNA gene as well as functional genes; 2) Biofilm imaging and quantitative analyses using Confocal Laser Scanning Microscopy (CLSM); 3) Scanning electron Microscopy (SEM) and elementary analysis EDX for biofilm imaging and surface characterization, respectively; 4) Identification of bacterial populations using Illumina sequencing. Standard Operating Procedures have been developed for these methods and for several of them Video SOPs are also available.
Status: Task completed.

Task 8: Setup of new analytical methods – Chemical analyses and other
New chemical analyses have been evaluated and implemented in the Kjellerup Laboratory. These include: 1) Microwave extraction of PCBs from sediment and mesocosm cultures; 2) Quantification of low level PCBs in weathered sediment samples using GC-MS; 3) QA/QC of analyzed samples including analyses of recoveries using surrogate compounds (non-PCBs). Standard Operating Procedures have been developed for these methods and for several of them Video SOPs are also available.
Status: Task completed.

Task 9: Culture setup *(Experimental methods for Tasks 9, 13, 14 and 20 are in supplemental material)*
In late 2016 we discovered that our stock culture of *Dehalobium chlorocoercia* DF1 would not recover when transferred following normal procedures. The next nine months were spent recovering the culture with a variety of amendments (described White Paper #1, Nov. 2017) to revive growth. These included addition of activated carbon, vitamin B12 +/- 5,6-Dimethylbenzimidazole, sediment, extract from older re-isolated *Desulfovibrio*, and concentrated *Desulfovibrio* extract. We also transferred several older cultures in the lab, which ultimately led to complete recovery. We confirmed the authenticity of the culture by comparing growth rates and dehalogenation products from PCE, PCB61 and Aroclor 1260. We now maintain a critical cell mass in multiple cultures with regular transfers. We are currently testing long-term preservation methods including: 1) freeze-drying using several freezing media (e.g., ATCC standard medium, dried milk, etc.); 2) freezing in anaerobic glycerol; 3) storage of high density culture at 4^{O}C. The preservation experiments are on-going. When DF1 was recovered, scale-up was started to allow for the following experiments.

A) **Development of optimal scale-up protocols.** The PCB dehalorespiring species *Dehalobium chlorocoercia* DF1 was cultures in two types of bioreactors: 1) New Brunswick BioFlo IV 20

L. stainless steel bioreactors, 2) 15 L glass bioreactor constructed from a modified glass carboy.

Results: Both style of bioreactors performed similarly. We developed a method for continuous growth with periodic purging of the PCE dehalogenation product and re-amendment with PCE. This method has enabled us to maintain active DF1 and maximum density for over 9 months. *The bioreactors have served as a consistent source of active, high density inoculum for all three laboratories to conduct the experiments described in this project.* Both bioreactors have maintained continuous growth of DF1 at similar rates and densities over a period of 290 days (stainless steel) and 200 days (glass). The glass carboy is less expensive to construct since it has fewer controls enabling us to perform more replicate experiments in the later stages of the project.

B) Improving yield of DF1 during scale up. Since DF-1 grows to limited density ($\leq 1x10^8$ cells per ml) experiments were conducted to improve the yields and possible the rates of growth. We had previously shown that continuous mixing inhibited growth of the microorganisms. Three additional bioreactors configurations were tested: 1) medium was recirculated from the bottom to the headspace to determine if recirculating PCE accumulated in the bottom of the vessel would improve growth (**Supplemental Figure 1**); 2) the gas phase in the headspace was recirculated through the bottom of the DNAPL layer to mix non-solubilized PCE into the medium and create gentle mixing (**Supplemental Figure 2**): 3) culture was mixed discontinuously for 30 minutes every 24 hr (**Supplemental Figure 3**).

Results: the first two approaches did not adversely affect growth, but neither did they improve the cell yields or rate of growth. However, the third approach inhibited growth. A forth approach is currently being tested to improve yields. Based on earlier experiments that showed AC stimulates the rate and extent of PCB dehalogenation (Water Research 2014), we added 0.1% sterile AC powder to a 15 L glass bioreactor with DF1. Conclusions cannot be drawn yet, because this experiment is in the preliminary stages. However, we are observing dehalogenating activity and we will monitor both the rates and extent of dechlorination as well as the cell titer over time to determine if this is an effective approach for improving yields.

Alternative dechlorinating cultures were also cultured during the period, where *Dehalobium chlorocoercia* DF1 was inactive (White Paper #1). While the team decided to move on with the PCB dehalorespiring species *Dehalobium chlorocoercia* DF1, valuable information was obtained that will be published in a peer-reviewed journal. One publication is in preparation based on the alternative cultures.

Status: Task completed.

Task 10: Does not exist in SEMS2.

Task 11: Biofilm inoculum (Anaerobic vs material)

Biofilm formation and growth potential for the anaerobic PCB dechlorinating bacterium DF1 was evaluated for an array of materials (part of Task 3). The developed microbiological and chemical methods (Task 7-8) were applied and the results were shown in White Paper #2. The next step for this Task was to consistently grow the biofilm on the sorptive material, which we have also accomplished. Currently, we are working on establishing the combined aerobic-

anaerobic biofilm that would make it possible to deploy only one type of inoculum compared to separately deploying anaerobic and aerobic biofilm cultures. The combined approach would make deployment easier and more cost effective.
Status: Task in progress.

Task 12: Biofilm inoculum (Aerobic vs material)
Biofilm formation and growth potential for aerobic PCB degrading bacteria (*Burkholderia xenovorans* LB400) has been evaluated for more than 10 types of materials. The developed microbiological and chemical methods (Task 7-8) were applied and results were shown in White Paper #2. The biofilm can consistently be formed on sorptive materials to the level of deployment within 3-4 days.
Status: Task completed.

Task 13: Methods for removal of residual PCE on sorbents.
One potential concern with this project is the removal of residual PCE associated with the formed biofilm on sorptive material. Current methodology for growing DF1 for use in experiments is to sparge residual PCE and dehalogenation products from the culture with nitrogen prior to using the cells. This prevents "carryover" of residual PCE. However, since we propose forming biofilms on sorptive material, removal of residual substrate by sparging might not be effective and an alternative method could be needed. We already began forming biofilms on sorptive materials in one of our bioreactors and we are in the process of developing a method for removal of residual organochlorines using zero valent iron. The iron itself will serve as an additional nutrient for the bacteria and residual iron will also stimulate reductive dechlorination.
Results: Once the bioreactor with biofilm is fully developed, we will test the effectiveness of Fe^{O} for dechlorinating PCE and its microbial products. Method have already been developed using Accelerated Solvent Extraction and Microwave Extraction to efficiently extract dehalogenation products from the biofilm on AC. Tests will include different ratios of Fe^{O} to AC and incubation times. Both the degradation product concentrations and cell viability will be monitored to identify the optimal conditions for treating the biofilms.
Status: Task in progress.

Task 14: Scale-up method develop - FBR approach.
As described in Task 9, we have already initiated a fluidized bed bioreactor in a glass bioreactor. In addition to monitoring dechlorination rates and growth, the Kjellerup lab will use this material to assess biofilm formation during scale up and the long-term viability during potential storage. The goal is to assess the optimal condition for biofilm formation. As stated in Task 13 our ability to build multiple glass bioreactor will enable us to determine the optimal condition for biofilm formation.
Status: Task in progress.

Task 15: Scale-up method develop - MBBR approach
The moving Bed Biofilm Reactor was described as an alternative in the original proposal, since we did not know if the scale-up approaches in the Sowers Lab would be suitable for biofilm

growth as well as the suspended growth that these reactor types were designed for. As describe din Task 14, the biofilm scale-up has been started and the results look promising thus far. Therefore, the start of the activities in this Task has been paused until we know more about the results from the current scale-up approaches. If the scale-up approach from Task 14 show to be effective, we will not initiate Task 15.
Status: On hold.

Task 16: Standard inoculum assessment methods
During the development of new microbiological methods (Task 7) for the determination of the Fundamental Characteristics (Task 3), these methods were also standardized so they could be applied for assessment of the biofilm inoculum cultured in the scale-up process. Standard Operating Procedures have been developed, so biofilm inoculum from the scale-up approach can be assessed during the production stages to ensure a high quality and active biofilm inoculum for subsequent deployment.
Status: Task in progress.

Task 17: Cost estimate
Collection of information about the composition and characteristics of commercially available activated carbons and biochars is ongoing. Based on the results from White Paper #2, we know that **sorption** is the most important parameter for dechlorination activity. Therefore, we will evaluate the options of applying activated carbons/biochars based on alternative biomass sources instead of freshly produced activated carbons that are expensive. The goal is to identify a type of sorptive material based on a biomass "waste" product that has comparable sorption capacity to the newly synthesized materials used in White Paper #2. This would make the overall biofilm solution cheaper and with a smaller environmental footprint.

The calculations of the cost estimate have been started and are at this point including the costs based on the current scale up approach (Task 14). Here, we will be able to calculate the cost for production of a specific biofilm mass on the material. In Task 19-21, we will determine the mass of biofilm needed for treatment of weathered PCB contaminated sediment. We are also in contact with Drs. Simon Vainberg and Paul Hatzinger at APTIM Federal Services, LLC regarding significantly larger scale-up approaches for potential commercial applications. The cost based on commercial scale-up will be included as well.

In addition, we have started evaluating approaches for deployment of the biofilm covered particles, since the employment approach also will influence the cost for the overall biofilm remediation technology. We are evaluating the settling characteristics by forming biocement containing biofilms on top of the PCB dechlorinating biofilms. Our goal is to form a protective layer that will increase the survival of the PCB dechlorinating biofilms but not negatively affect the settling velocity for the biofilm through the water column. This work includes evaluation of the survival of these particles after deployment. The survival will be evaluated by measuring the biofilm mass and cell numbers on the particles and the dechlorination activity level before and after deployment. In the laboratory, we are currently setting up experiments, where specific shear forces that mimic the stress that the particles face in the environment (**Figure 1**) will be applied.

The protective biofilm that we have evaluated was made up by *Sporosarcina pasteurii*. It is a bacterium capable of producing calcium carbonate. In the experiments, it was determined

which materials a biofilm *S. pasterurii* was able to form on by measuring the increasing weight (due to biocement and biofilm formation). The materials tested included sand, pinewood biochar, bone biochar, activated carbon, and coconut biochar. Through application of developed microbiological tools, it was determined that materials that best supported *S. pasteruii* biofilm growth were pine wood, coconut and activated carbon. Upon testing the survival and settling characteristics in a 1 m cylinder (water column of 1 m), it was determined that the biofilms were intact after the passage of the water column and that ability of the materials to settle was not affected by the biofilm formation, but rather was a property of the material itself. New experiments have been initiated, where we are growing 3-4 types of aerobic PCB degrading bacteria in the same biofilm to increase the biofilm thickness and obtain increased PCB degradation potential from several bacterial pathways.

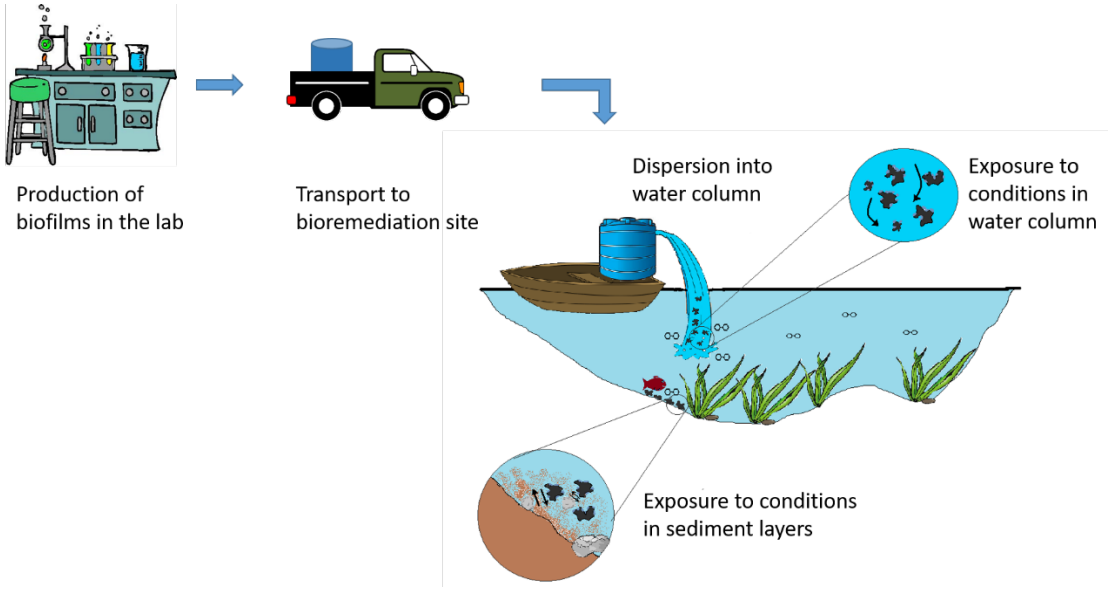

***Figure 1**. Conceptual model illustrating the potential shear forces that biofilm particles can face from production to deployment.*

Status: Task in progress.

Task 18: Decision on inoculum scale up method

As described for Task 15, we will not initiate the alternative approach for scale-up unless problems occur during the biofilm scale up using the FBR approach (Task 14) that would make this reasonable. As mentioned, a lot of experiences has been gained by the Sowers group on scaling up suspended bacterial cells with the FBR approach and these will be useful for the scale-up of the biofilm inoculum as well. Therefore, Task 18 is also on hold until results from Task 14 are available.

Status: On hold.

Task 19: Scale-up mesocosm experiment

The scale-up of inoculum will take place when we are ready to initiate the large mesocosm experiment (Task 20). The scale-up will be based on the decisions for the previous tasks that

involve type of sorptive material, type of biofilm growth (combined anaerobic-aerobic or separate), type of reactor for scale-up etc. These aspects have been outlined in Tasks 11-18.
Status: Task in progress.

Task 20: Mesocosm studies - Set up
We have started a smaller scale experiment with the goal of quantifying the amount of biofilm inoculum that will be needed for bioremediation of the weathered contaminated sediment. These smaller scale experiments will also be used to evaluate if cheaper materials with high adsorption potential can be used. This would reduce both the price and the environmental foot print for the solution. Based on these results, we will know how much inoculum to produce for the scaled up mesocosm experiment and whether we will use high quality activated carbon or if activated carbon/biochar based on alternative biomass sources can be applied.

Mesocosm tanks have been assembled and are ready for use. The mesocosm tanks were designed and used successfully for ER-201215. They consist of sealed TLC tanks and 12 channel peristaltic pump for recirculating water from the target site to simulate anaerobic and aerobic zones similar to those found *in situ.* The system is ready for inoculation once the biofilms are available.
Status: Task in progress.

Task 21: Mesocosm studies - microbiological analysis
Activities for this task have been started for the ongoing smaller scale experiment and will continue when the TLC tank experiment will be started. These methods have been developed in earlier tasks, so no further development will be required.
Status: Task in progress.

Task 22: Mesocosm studies – chemical and other analysis
Activities for this task have been started for the ongoing smaller scale experiment and will continue when the TLC tank experiment will be started. These methods have been developed in earlier tasks, so no further development will be required.
Status: Task in progress.

Task 23: Reporting
Status: Ongoing

Proposed Path Forward

Based on the description of activities within the Tasks above, we have estimated how far along we are with the separate Tasks (**Table 2**). This estimate is based on anticipated work load and the time that it takes for anaerobic experiments to reveal results. This has also lead to a revised time schedule shown in the Gantt diagram (**Figure 2**). The revision of the project plan reflects that other projects have leveraged results that have benefitted the progress of this project. This include the ESTCP project in Drs. Sowers and Ghosh Labs as well as projects on PCBs from Maryland State Highway in Dr. Kjellerup's lab.

For the Path Forward, we propose a completion of the Tasks that currently are ongoing. In this way, we will be able to deliver a scientifically sound and well documented product by the completion of the project. The proposed work will include the Tasks in **Table 2**.

Table 2*. Proposed Tasks involved in a Path Forward for project ER-2135. These are all "In Progress".*

Task #	Task	Status of Task (estimate of completion)
11	Biofilm inoculum (Anaerobic vs material)	60% completed
13	Removal methods for PCE on sorbents	40% completed
14	Scale-up method develop: FBR approach	60% completed
15	Scale-up method develop: MBBR approach	On hold
16	Standard inoculum assessment methods	60% completed
17	Cost estimate	35% completed
18	Decision on inoculum scale up method	On Hold
19	Scale-up mesocosm experiment	35% completed
20	Mesocosm studies - Set up	15% completed
21	Mesocosm studies - microbiological analysis	20% completed
22	Mesocosm studies - chemical and other analysis	20% completed
23	Reporting	Ongoing

The proposed time table for ongoing Tasks in Table 2 are described in the Gantt diagram below (**Figure 2**).

Year	**2018**				**2019**			
Quarter no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Tasks								
11. Biofilm inoculum development	x	x						
13. Removal of PCE	x	x	x					
14. Inoculum scale-up: FBR	x	x	x					
15. Inoculum scale-up: MBBR								
16. Inoculum assessment method		x	x					

17. Cost estimate	x	x			x			
18. Decision on scale-up			x					
19. Scale-up mesocosm experiment			x	x				
20.-22. Mesocosm studies	x		x	x	x	X		
23. Final Report							x	

Figure 2*. Gantt diagram for the remaining activities.*

Proposed revision to Task 15

We are also proposing to substitute Task 15 (Scale-up method develop - MBBR approach) if Task 14 shows results as expected. The new Task would be: "Evaluation of deployment aspects". This revision is based on the comments from the SAB on February 7, 2018. Here, several questions dealt with deployment and engineering aspects with regards to deployment and the logistics on how to bring this solution into the field in a faster manner. Even though the SAB (3013) discouraged us from dealing with these delivery and deployment aspects, we fell that we are so far along with this solution that these aspects could be covered.

In a revised Task 15, we plan to evaluate the deployment mechanisms and the impact on the biofilm-activated carbon inoculum and the survival over depth of the water column, shear forces and other physical, chemical and biological impacts that these biofilm particles would experience during the transportation phase through deployment in the sediment. These aspects are shown at the conceptual model below (**Figure 1**).

Dissemination of research results (Transition Plan)

The project group has been very productive in dissemination research results and experiences based on the completed Tasks in this project.

- 38 presentations have been delivered at meetings and conferences (Appendix A).
- 7 peer-reviewed manuscripts have been published (added to SEMS2) and 6 are currently in preparation with expected submission during spring/summer 2018 (Appendix B).

Several students have been involved in the performed research:

- *Stephen Grabowski*, Baltimore Polytechnic Institute, HSDG with Honors May 2017. Currently enrolled in Aerospace Engineering, Perdue University, expected graduation May 2021.
- *Kirstie Coombs*, Bioengineering Undergraduate at UMD. Currently Laboratory Manager at Dr. Fischer's Laboratory at UMD.
- *Shireen Khayat*, Bioengineering Undergraduate at UMD.
- *Bradley Hogan*, Civil and Environmental Undergraduate at UMD.
- *Raymond Jing*, Ph.D. student at UMD.
- *Trevor Needham*, Ph.D. student at UMBC.

Furthermore, several other activities involving Technology Transfer have also been performed during the project period (Appendix A).

Appendix 3: Publications and presentations

Publications citing SERDP funding:

Capozzi, S.L., Bodenreider, C., Prieto, A., Payne, R.B., Sowers, K.R. and Kjellerup, B.V., 2019. Colonization and growth of dehalorespiring biofilms on carbonaceous sorptive amendments. *Biofouling*, *35*(1), pp.50-58.

Capozzi, S.L., Jing, R., Rodenburg, L.A. and Kjellerup, B.V., 2019. Positive Matrix Factorization analysis shows dechlorination of polychlorinated biphenyls during domestic wastewater collection and treatment. *Chemosphere*, *216*, pp.289-296.

Edwards, S.J. and Kjellerup, B.V., 2013. Applications of biofilms in bioremediation and biotransformation of persistent organic pollutants, pharmaceuticals/personal care products, and heavy metals. *Applied Microbiology and Biotechnology*, *97*(23), pp.9909-9921.

Jing, R., Fusi, S. and Kjellerup, B.V., 2018. Remediation of polychlorinated biphenyls (PCBs) in contaminated soils and sediment: state of knowledge and perspectives. *Frontiers in Environmental Science*, *6*, p.79.

Jing, R. and Kjellerup, B.V., 2020. Predicting the potential for organohalide respiration in wastewater: Comparison of intestinal and wastewater microbiomes. *Science of the Total Environment*, *705*, p.135833.

Kaya, D., Sowers, K.R., Demirtepe, H., Stiell, B., Baker, J.E., Imamoglu, I. and Kjellerup, B.V., 2019. Assessment of PCB contamination, the potential for in situ microbial dechlorination and natural attenuation in an urban watershed at the East Coast of the United States. *Science of the Total Environment*, *683*, pp.154-165.

Kjellerup, B.V., Naff, C., Edwards, S.J., Ghosh, U., Baker, J.E. and Sowers, K.R., 2014. Effects of activated carbon on reductive dechlorination of PCBs by organohalide respiring bacteria indigenous to sediments. *Water Research*, *52*, pp.1-10.

Needham, T.P., Payne, R.B., Sowers, K.R. and Ghosh, U., 2019. Kinetics of PCB microbial dechlorination explained by freely dissolved concentration in sediment microcosms. *Environmental Science & Technology*, *53*(13), pp.7432-7441.

Presentations at meetings citing SERDP funding:

Jing, R.; Capozzi, S.L., Prieto, A., Sowers, K., Ghosh, U., Payne R.; Kjellerup, B.V. Application of Biofilm-Based Inoculum Delivery System for Organohalide Respiration of Polychlorinated Biphenyls in Sediments. Poster presentation at SERDP ESTCP 2019 Symposium in Washington D.C., December 3 – 5, 2019.

Kaya, D., Kjellerup, B.V., Sowers, K. 2019. Bioremediation Assessment for Baltimore Harbor Sediments Contaminated with PCBs. Poster presentation at SERDP ESTCP 2019 Symposium in Washington D.C., December 3 – 5, 2019.

Kaya, D., Capozzi, S.L. and Kjellerup, B.V. Efficacy of Biofilm Covered Activated Carbon Particles on the Enhancement of PCB Dechlorination. Poster presentation at the American Society for Microbiology, San Francisco, CA, June 20 – 24, 2019.

Kaya, D., Kjellerup, B.V., Sowers, K.R. Bioremediation of Baltimore Harbor Sediments Contaminated with PCBs. Poster presentation at the American Society for Microbiology, San Francisco, CA, June 20 – 24, 2019.

Kaya, D., Kjellerup, B.V., Sowers, K.R. Microbial Reductive Dechlorination of PCBs in Sludge. Poster presentation at the American Society for Microbiology, San Francisco, CA, June 20 – 24, 2019.

Jing, R. Capozzi, S.L. Kjellerup, B. V. The Potentials for Bioremediation of Polychlorinated Biphenyls in Effluents from a Large Advanced Wastewater Treatment Plant. Poster presentation at the Fifth International Symposium on Bioremediation and Sustainable Environmental Technologies in Baltimore, MD, April 15 – 18, 2019.

Kaya, D., Kjellerup, B.V., Sowers, K.R. Contamination of Baltimore Harbor Sediment with PCBs Has Increased over the Last Century: Is Bioremediation Possible? Treatment of Urban Toxic Contaminants in Stormwater. Poster presentation at the Fifth International Symposium on Bioremediation and Sustainable Environmental Technologies in Baltimore, MD, April 15 – 18, 2019.

Kaya, D., Kjellerup, B.V. Biofilm Covered Activated Carbon Particles Enhances Degradation of PCBs in Sediments. Poster Presentation at the Postdoctoral Research Symposium. Sep. 13, 2019, University of Maryland, College Park, MD.

Capozzi, S.L., Rodenburg, L.A., Jing, R., Kjellerup, B.V. Positive matrix factorization analysis shows dechlorination of polychlorinated biphenyls during domestic wastewater collection and treatment. Geosyntec Quarterly Department Seminar Presentation, Maryland Brach, January 24, 2019.

Capozzi, S.L. Bodenreider, C., Prieto, A; Payne, R.B., Sower, K.R.; Needham, T., Ghosh, U., Kjellerup, B.V. Colonization and growth of PCB dehalorespiring biofilms on carbonaceous sorptive amendments. Poster presentation at SERDP ESTCP 2018 Symposium in Washington D.C., November 27 – 29, 2018.

Capozzi, S.L., Jing, R., Rodenburg, L.A., Kjellerup, B.V. Forensic analysis of polychlorinated biphenyls in wastewater in the Mid-Atlantic region of the USA. Oral presentation at the SETAC North America 39th Annual Meeting in Sacramento, CA, November 4 – 8, 2018.

Capozzi, S.L., Kjellerup, B.V. Colonization and growth of organohalide respiring biofilms on carbonaceous amendments. Poster presentation at the SETAC North America 39th Annual Meeting in Sacramento, CA, November 4 – 8, 2018.

Capozzi, S.L., Jing, R., Rodenburg, L.A.; Kjellerup, B.V. Positive Matrix Factorization analysis shows dechlorination of polychlorinated biphenyls during domestic wastewater collection

and treatment. Oral presentation at the 10th International PCB Workshop, Krakow, Poland, August 26-31, 2018.

Capozzi, S.L. and Kjellerup, B.V. Colonization and growth of PCB respiring biofilms on carbonaceous amendments. Poster presentation at the 10th International PCB Workshop, Krakow, Poland, August 26-31, 2018.

Capozzi, S.L.; Jing, R., Rodenburg, L.A., Kjellerup, B.V. Oral presentation at the SETAC Young Environmental Scientists Meeting in Madison, Wisconsin, March 25 – 29, 2018.

Capozzi, S.L., Ghandehari, S.S., Bodenreider, C., Jing, R.; Kjellerup, B.V. Enhanced bioremediation of polychlorinated biphenyl in sediment: Application of biofilm-activated carbon aggregates as delivery vehicle. Poster presentation at Strategic Environmental Research and Development Program & Environmental Security Technology Certification Program 2017 Symposium in Washington D.C., November 28-20, 2017.

Capozzi, S.L., Jing, R., Rodenburg, L.A., Wilson, E.K, Kjellerup, B.V. Source apportionment of polychlorinated biphenyls in District of Columbia wastewater. Poster presentation at SERDP ESTCP 2017 Symposium in Washington D.C., November 28-20, 2017.

Capozzi, S.L., Jing, R., Rodenburg, L.A., Wilson, E.K, Kjellerup, B.V. Source apportionment of polychlorinated biphenyls in District of Columbia wastewater. Oral presentation at the SETAC North America 38th Annual Meeting in Minneapolis, MN, November 12-16th 2017.

Capozzi, S.L., Ghandehari, S.S., Bodenreider, C., Jing, R., Kjellerup, B.V. Enhanced bioremediation of polychlorinated biphenyl (PCBs) in sediment: Application of biofilm-activated carbon aggregates as delivery vehicle. Oral presentation at the SETAC North America 38th Annual Meeting in Minneapolis, MN, November 12-16th 2017.

Capozzi, S.L., Edwards, S.J., Kjellerup, B.V. Impact of Biofilm Based Bioremediation Technologies on Public Health. Oral presentation at the 5th European Congress on Microbial Biofilms, Amsterdam, September 2017.

Capozzi, S.L., Jing, R., Rodenburg, L.A., Wilson, E.K, Kjellerup, B.V. Source apportionment of polychlorinated biphenyls in District of Columbia wastewater. Oral presentation at the 254th American Chemical Society Fall National Meeting & Exposition, Washington, DC, August 20-24, 2017.

Capozzi, S.L., Ghandehari, S.S., Bodenreider, C; Jing, R., Kjellerup, BV. Biofilm covered activated carbon particles: Application as a microbial inoculum delivery system. Poster presentation at the 254th American Chemical Society Fall National Meeting & Exposition, Washington, D.C., August 20-24, 2017.

Jing, R., Capozzi, S.L., Wilson, E.K., Kjellerup, B.V. Polychlorinated biphenyls in effluent discharged from a wastewater treatment plant. Oral presentation at the 254th American Chemical Society Fall National Meeting & Exposition, Washington, DC, August 20-24, 2017.

Jing, R., and Kjellerup, B. K. Mesocosm study for enrichment of natural PCB-dechlorinating bacteria in wastewater samples using activated carbon particles for enhanced dechlorination of Aroclor 1248. Poster Presentation at the 254th American Chemical

Cao, S., Chan, A., Capozzi, S.L., Davis, A.P., Kjellerup, B.V. Occurrence of polychlorinated biphenyls (PCBs) in storm water sediments and their dechlorination by soil biofilms. Oral presentation at the 254th American Chemical Society Fall National Meeting & Exposition, Washington, DC, August 20-24, 2017.

Ghandehari, S.S.,Capozzi, S.L.; Bodenreider, C., Flores, M., Kjellerup, B.V. Bioremediation of PCE-contaminated groundwater using mixed organohalide-respiring biofilms. S. Poster presentation at the 254th American Chemical Society Fall National Meeting & Exposition, Washington, DC, August 20-24, 2017.

Capozzi, S.L. and Kjellerup, B.V. Biofilm covered activated carbon particles: Application as a microbial inoculum delivery system. Oral presentation at the Baltimore Region Toxics Workshop at the USGS Office at University of Maryland Baltimore County, August 2017.

Capozzi, S.L., Jing, R., Rodenburg, L.A., Wilson, E.K, Kjellerup, B.V. Source apportionment of polychlorinated biphenyls in District of Columbia wastewater. Poster presentation at the 2017 Chesapeake Potomac Regional Chapter of the Society of Environmental Toxicology and Chemistry, Annapolis, MD, April 24, 2017.

Jing, R., Draghi, C., Edwards, S., Andrade, N., Kjellerup, B.V. Biological transformations and toxicity of PCBs in wastewater treatment. Poster presentation at the 252nd American Chemical Society. Philadelphia, PA. August 21-25, 2016.

Jing, R., Wilson, E., Fusi, S., Chan, A., Kjellerup, B.V. Analysis of polychlorinated biphenyls in effluent discharged from a wastewater treatment plant during dry and wet weather periods. Poster presentation at the 252nd American Chemical Society. Philadelphia, PA. August 21-25, 2016.