

Carbon Composition and Source Influence on Microbial Community Function and Structure in the
Hyporheic Corridor

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Acknowledgements and dedication

Science does not move forward without collaboration. First and foremost, I would like to acknowledge my advisors, Matthew Ginder-Vogel and Eric Roden, as well as my thesis committee members Trina McMahon, Emily Stanley and Thea Whitman. I would like to acknowledge my collaborators at Pacific Northwest National Laboratory, John Zachara, James Fredrickson, James Stegen, David Kennedy, Evan Arntzen, Emily Graham, and Amy Goldman. I would also like to acknowledge collaborators from UW Madison: Bret Larget, Shaomei He, Jackie Mejia, Tina Hill, Sarah Bessey, Chris Worley, Nathaniel Fortney, Stephanie Napieralski, Anthony Allison, Elias Devoe and Brandon Converse. Thank you all for your commitment to moving scientific achievement forward.

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Abstract

The hyporheic zone is the area just below a river where constant mixing of groundwater and river water occurs. At this intersection between two sources of water, with different organic carbon and nutrient contents, complimentary pairing of electron donors and acceptors occurs, causing elevated rates of microbial metabolism. Due to these constant changes in electron acceptor and donor availability and because of microbial oxygen consumption dynamics, redox cycling process are constantly occurring and changing within the hyporheic zone. Iron oxides are theorized to play an important role in protecting sediment organic carbon from microbial metabolism through the formation of organo-metal aggregates. This thesis document outlines three related experiments investigating how different sources and forms of organic carbon found in freshwater river and groundwater systems effects hyporheic zone microbial metabolism rates. These studies revealed that deposition of autochthonous and allochthonous organic carbon to the riverbed sediment matrix (i.e. hyporheic zone) was correlated with release of labile dissolved organic carbon. Photosynthetic periphyton biomass (i.e. autochthonous epilithic biomass) was identified as a key source of organic carbon, which lead to highly elevated rates of aerobic respiration, anaerobic respiration, and methanogenesis. The presence of small pockets of anaerobic respiration and methanogenesis was observed within predominantly aerobic metabolizing sediment batch reactors; this shows that hyporheic zone sediment microbial community structure is highly heterogenous with patches of flexible metabolism occurring, depending on electron donor/acceptor availability. Iron bound organic carbon content was correlated with the presence of reactive iron oxide minerals, and these organo-metal aggregates were not biodegraded due to preferential metabolism of more labile pools of carbon such as fresh periphyton POM and DOC. The increase in microbial activity associated with fresh periphyton

biomass deposition shows that in freshwater river systems periphyton biomass is a major contributor to ecosystem respiration as well as gross primary productivity. Understanding how natural and anthropogenic inputs of organic carbon, nitrogen, phosphorous and sulfate cause changes in periphyton growth and hence changes in hyporheic zone microbial metabolism is an ongoing challenge to modeling the global carbon cycle.

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Chapter 1. Introduction

1.1 Thesis outline

This thesis document presents a collection of experiments investigating the influence of varying forms of organic carbon found in river systems on sediment microbial community metabolism rates. This work is part of a collaboration with Pacific Northwest National Lab (PNNL) SBR SFA division. The goal of the entire collaboration is to create a comprehensive model of all of the biogeochemical processes occurring at the PNNL Hanford Area 300 reach of the Columbia River. Our role in the project was to study the biogeochemical and ecological interactions involving organic carbon at the local (cm-m scale interactions) and fine (pore scale interactions) scales. The Hanford Area 300 cite is an ideal location for studying hyporheic zone interactions because of the Priest Holmes hydroelectrical dam, located just 10 km from the Handford Area 300 cite, which controls the river stage depending on hydroelectrical needs. Also, seasonal fluctuations in rain cause large increases in river stage causing river water intrusion far beyond the hyporheic zone over 100 meters into the adjacent groundwater zone. Due to all of these mixing events, river water exchange to the underlying hyporheic zone and even groundwater exchange to the hyporheic zone is common. Understanding how these mixing events lead to important changes in organic carbon sources and thus changes in microbial metabolism rates is the goal of this research.

Chapter two contains an experiment titled: Dual role of humic substances as electron donor and shuttle for dissimilatory iron reduction. This chapter describes an experiment designed to test if humic acids and humins, previously thought recalcitrant organic matter, were susceptible to metabolic breakdown by anaerobic microorganisms and if this process could contribute to microbial iron cycling in sediments. This chapter has been published in the Journal of Environmental Science and Technology under the DOI: 10.1021/acs.est.7b06574.

Chapter 3 contains an experiment titled: Colonization Habitat Controls Biomass, Composition, and Metabolic Activity of Attached Microbial Communities in the Columbia River Hyporheic Corridor. This chapter describes an experiment designed to test how changes in DOC content due to river and groundwater mixing events affect river and groundwater sediment microbial community metabolism rates. This chapter has been published in the Journal of Applied and Environmental Microbiology under the DOI: 10.1128/AEM.00260-17.

Chapter 4 contains an experiment titled: Influence of Particulate Organic Matter Input on Riverbed Sediment Microbial Metabolism. This chapter describes an experiment to test the influence of autochthonous periphyton biomass deposition into the sediment matrix on aerobic and anaerobic metabolism rates. Also, of interest in this experiment is the if the presence of reactive iron oxide minerals promotes organic carbon-metal aggregate formation and if these aggregates are protected from microbial metabolism. This chapter will be submitted to a peer-reviewed journal in July of 2018. Chapter 5 contains a summary of the major findings and environmental implications of these three collective experiments.

**Chapter 2. Dual role of humic substances as electron donor and shuttle for dissimilatory
iron reduction**

2.1 Abstract

Dissimilatory iron-reducing bacteria (DIRB) are known to use humic substances (HS) as electron shuttles for dissimilatory iron reduction (DIR) by transferring electrons to HS-quinone moieties, which in turn rapidly reduce Fe(III) oxides. However, the potential for HS to serve as a source of organic carbon (OC) that can donate electrons for DIR is unknown. We studied whether humic acids (HA) and humins (HM) recovered from peat soil by sodium pyrophosphate extraction could serve as both electron shuttles and electron donors for DIR by freshwater sediment microorganisms. Both HA and HM served as electron shuttles in cultures amended with glucose. However, only HA served as an electron donor for DIR. Metagenomes from HA-containing cultures had an overrepresentation of genes involved in polysaccharide and to a lesser extent aromatic compound degradation, suggesting complex OC metabolism. Genomic searches for the Porin-Cytochrome Complex involved in DIR resulted in matches to *Ignavibacterium/Melioribacter*, DIRB capable of polymeric OC metabolism. These results indicate that such taxa may have played a role in both DIR and decomposition of complex OC. Our results suggest that decomposition of HS coupled to DIR and other anaerobic pathways could play an important role in global subsurface OC metabolism.

2.2 Introduction

Soil organic carbon (SOC) formation involves the biological, chemical and physical transformation of plant and microbial matter into organic products that vary in size at different stages of decomposition and is comprised of a heterogeneous mixture of cellulose, hemicellulose, lignin, lipids, tannins, proteins, sugar monomers and other organic products.(Schnitzer, 1978; Lorenz et al., 2007) Since all of these forms of organic carbon are in principle susceptible to microbial degradation, a combination of matrix protection, microbial ecology, enzyme kinetics and geochemical conditions control how SOC is formed and preserved.(Kleber, 2010) Materials produced through the degradation of these compounds have been commonly referred to as “humic substances” (HS), organic compounds that have undergone transformation such that they no longer resemble their original physicochemical state.(Schnitzer, 1978; MacCarthy et al., 1985) “Humic substances” have traditionally been recovered from soils via alkali extraction, which upon further processing leads to operationally defined humic acid (referred to here as HA), fulvic acid, and humin (referred to here as HM).(Rabenhorst, 1999) The term “alkali-extracts” has been recently introduced to explicitly acknowledge that these forms of organic carbon are the result of standardized extraction procedures and not necessarily representative of a native organic materials in soils or sediments.(Lehmann and Kleber, 2015)

The significance of SOC in the global carbon cycle is obvious as this reservoir contains more carbon than global vegetation and the atmosphere combined.(Intergovernmental Panel on Climate, 2014) In addition to serving as a large reservoir of organic carbon (OC), under anoxic conditions microorganisms can respire by transferring electrons to dissolved or solid phase quinone moieties in HS.(Lovley et al., 1996; Roden et al., 2010) Microbial reduction of

quinone moieties accelerates reduction of Fe(III) oxides via a process called electron shuttling, whereby microbially reduced quinones rapidly react to transfer electrons onto Fe(III) oxides.(Lovley et al., 1996; Coates et al., 1998; Lovley et al., 1998; Scott et al., 1998; Kappler et al., 2004; Jiang and Kappler, 2008; Roden et al., 2010) This process has received a great deal of attention owing to the broad impact of dissimilatory iron reduction (DIR) on the geochemistry of anoxic soils and sediments.(Lovley et al., 2004) The same pool of HS that contains quinone moieties also represents a large reservoir of OC that can potentially donate electrons to DIR itself. However, the metabolism of HS under Fe(III)-reducing conditions has not been previously explored. Rather, virtually all previous studies of DIR in the presence of HS have focused on the role of HS as electron shuttles in the presence of excess labile OC.(Lovley et al., 1996; Roden et al., 2010; Klüpfel et al., 2014) Coupling of HS degradation to DIR has the potential to create complexity in SOC degradation pathways. It has recently been shown that DIR can lead to the release of dissolved organic carbon (DOC) associated with Fe(III) oxide surfaces, which has important implications for Fe-C associations in soils and sediments.(Adhikari et al., 2017; Zhao et al., 2017)

It is well known that hydrolytic and fermentative microorganisms are responsible for breaking down complex OC into organic compounds that can then be utilized by dissimilatory iron reducing bacteria (DIRB).(Lovley and Phillips, 1988) However, the metabolic activity of DIRB such as *Geobacter* is more complex, as such taxa are able to utilize monoaromatic compounds for growth.(Lovley et al., 1989; Lovley and Lonergan, 1990; Rotaru et al., 2011) In addition, DIRB from the *Ignavibacteriales* (*Ignavibacterium*/*Melioribacter*) are capable of degrading complex polysaccharides.(Iino et al., 2010; Podosokorskaya et al., 2013)

Thus, the potential exists for DIRB to participate directly in the metabolism of HS, in addition to oxidizing the products of hydrolytic and fermentative metabolism.

Through a combination of geochemical and metagenomic sequencing data this study sought to disentangle electron shuttling and electron donating interactions involving HS and Fe(III)-reducing microbial communities from freshwater sediments. The potential for microbial communities to metabolize HS was determined by quantifying the presence of genes coding for carbohydrate active enzymes (CAZymes) such as glycoside hydrolases, polysaccharide lyases, glycosyl transferases, carbon binding modules, carbohydrate esterases, and genes involved in the degradation of aromatic carbon.(Lin et al., 2014) The presence of extracellular electron transfer (EET) systems that are involved in DIR was based on the normalized abundance of porin-cytochrome complex (PCC) and PilA genes first discovered in *Geobacter*.(Reguera et al., 2005; Shi et al., 2009; Reardon and Mueller, 2013; Liu et al., 2014) Investigating the reduction of Fe(III) oxides and the degradation of HS under controlled conditions provides insight into the microbial community structure and the metabolic pathways involved in coupled Fe-C cycling in soils and sediments.

2.3 Materials and methods

2.3.1 Humic substances

Two different HS obtained from an organic rich histosol from Amherst, Massachusetts were employed in our experiments: a humic acid (HA) fraction representing the combination of two extractions with 0.1M Na₄P₂O₇; and the final non-extractable humin (HM) fraction. The chemical properties of the isolated HS used in this study have been previously described.(Wang

et al., 2011; Yang et al., 2012) The $\text{Na}_4\text{P}_2\text{O}_7$ solution used to recover HA differs from the classical pH 13 sodium hydroxide extraction (IHSS, 2015), targeting materials associated with organo-metal complexes (Wagai et al., 2013) as opposed to bulk alkali-soluble SOC.

2.3.2 DIR experiments

DIR experiments were conducted in anoxic 10 mM PIPES (1,4-piperazinediethanesulfonic acid) buffered medium containing 100 μM NH_4Cl and 10 μM KH_2PO_4 . Duplicate reactors were amended with 100 mmol L^{-1} of reagent-grade hematite (Fe_2O_3 , Fisher Scientific) as the Fe(III) oxide mineral phase and 180 mg L^{-1} of either HA or HM as a source of SOC, with or without the presence of 100 μM glucose as an auxiliary electron donor to test the potential for electron shuttling. Note that the HA and HM were added in particulate form to mimic solid-phase SOC. Sterile control reactors containing only 100 μM NH_4Cl , 10 μM KH_2PO_4 and HA/HM showed DOC values below 0.3 ppm after 72 days. Hematite was chosen as the Fe(III) oxide phase for the sake of consistency with recent studies of biochar-accelerated Fe(III) oxide reduction. (Xu et al., 2016) Previous studies have demonstrated its reducibility by freshwater DIRB (Roden, 2003), and unlike ferrihydrite and other Fe(III) oxides hematite does not undergo reductive phase transformations, which have a confounding effect on oxide reducibility. (Roden and Urrutia, 2002) The inoculum used for the experiment was taken from a freshwater pond in Middleton, WI. DIR was determined by monitoring the accumulation of aqueous and 0.5M HCl-extractable Fe(II) using the ferrozine assay. (Stookey, 1970)

2.3.3 Metagenome sequencing and analysis

Shotgun Illumina sequencing was performed on six metagenomes. On average, 10 Gbp of raw sequences were obtained for each metagenome. Metagenome assembly was performed using the CLC Genomics workbench (version 6.02; CLC bio, Inc., Cambridge, MA, USA). The raw reads were merged to obtain paired-end reads; quality trimmed and filtered by length; and assembled using a *k*-mer of 63 with scaffolding (Table S1). After assembly, the average fold coverage of each contig was estimated and all contigs were uploaded to the Integrated Microbial Genomes with Microbiomes (IMG/MER) database (<http://img.jgi.doe.gov/mer>) for gene prediction and functional annotation.(Markowitz et al., 2014) Genome reconstruction (i.e. binning) from metagenomes was carried out by a coverage-composition algorithm using MaxBin.(Wu et al., 2014) The completeness and potential contamination of the bins was assessed using CheckM(Parks et al., 2015a) and the resulting high quality bins of interest were further evaluated.

2.3.4 Microbial community analysis

Conserved phylogenetic markers and 16S rRNA genes from metagenomes were used to estimate microbial community composition. Phylogenetic marker genes from Phylosift,(Darling et al., 2014) that are present in >99% bacteria and archaea, were chosen for this analysis. These genes are represented by 32 COGs (Cluster of Orthologous Groups) listed in Supplementary Table S2. Genes annotated by these 32 COGs were extracted, and BLASTP was conducted against the Ref_Seq protein database with an e-value of $1e^{-5}$, with the top 20 hits being retained. MEGAN was used for taxonomic classification of BLASTP results using the lowest common ancestor algorithm.(Huson et al., 2011) As these phylogenetic marker genes have different lengths due to

different gene completeness and different COG families that they represent, the abundance of each phylogenetic marker gene was estimated using its coverage depth weighted by its recovered length and then normalized by its expected full length (i.e. the consensus length of each COG listed in Table S2) according to He et al.(He et al., 2015) The relative abundance of each phylum within the total community was calculated using the normalized abundance of all phylogenetic marker genes.

2.3.5 Identification of OC degrading systems

The metagenomes were compared based on functional units of Carbohydrate Active enZymes (CAZymes) or KEGG Orthology (KO). CAZymes are divided into five classes: glycoside hydrolases (GHs); glycosyl transferases (GTs); polysaccharide lyases (PLs); and auxiliary activities (AA) enzymes. The database also includes modules such as the cohesin module, dockerin, the S-layer homology (SLH) module and carbon binding modules (CBMs). CAZyme genes were determined by uploading the annotated metagenomes to a database for automated CAZyme annotation (dbCAN) and using a $1e^{-6}$ cutoff E-value and a 30% cutoff coverage value.(Yin et al., 2012) When redundancies were detected, classification was determined based on the lowest E-value or highest coverage value. KEGG-KO assignments were performed during IMG/MER gene annotation. We focused on 140 KOs belonging to 22 KEGG modules within the Aromatics Degradation (AD) category.(Kanehisa and Goto, 2000; Kanehisa et al., 2013; Kanehisa et al., 2016; Kanehisa et al., 2017) Comparison across all six metagenomes was possible by multiplying the scaffold coverage (i.e., scaffold read depth) by a normalization factor, which was computed by dividing 8,420,617,307 bases by the number of bases of raw reads that matched after the assembly of each sample (Table S1). The abundance of genes

reported from here on represents normalized gene copy numbers. To determine the taxonomic distribution of genes involved in OC degradation, all reads were subjected to BLASTP searches against the NCBI nr database with an E-value cutoff of $1e^{-5}$. MEGAN was used for taxonomic classification of BLASTP results using the lowest common ancestor algorithm.(Huson et al., 2011)

2.3.6 Identification of PCC and PilA genes

PCC gene systems involved in DIRB are composed of one beta-barrel protein and adjacent multi-heme cytochromes(Shi et al., 2009; Liu et al., 2014; Shi et al., 2014). These systems were identified by a hidden Markov model (HMM)(Eddy, 2009) based on 29 PCC porin gene homologs(Shi et al., 2014) and created using the hmmbuild function in HMMER 3.0.(Finn et al., 2011) Confirmation that the identified porin gene contained a beta-barrel protein structure was preformed using PRED-TMBB.(Bagos et al., 2004a, 2004b) Adjacent cytochrome genes and their respective heme-binding sites were searched manually in IMG.(Markowitz et al., 2011) The cellular location (periplasmic, outer membrane or extracellular) of all genes was determined by Cello2go.(Yu et al., 2014) PilA genes(Reguera et al., 2005; Reguera et al., 2007; Reardon and Mueller, 2013) involved in DIRB were identified by IMG protein BLAST using 15 known *Geobacter* specific PilA gene sequences and a maximum E-value of 10^{-5} . The cellular location of the PilA genes was confirmed by Cello2go(Yu et al., 2014) and gene sequence alignment was performed using PROMALS3D multiple sequence and structure alignment server.(Pie et al., 2008) Taxonomic assignments of all identified EET gene systems was carried out by BLASTP as described in the previous section.

2.4 Results and discussion

2.4.1 DIR in the presence and absence of humic substances

The initial rate and long-term extent of hematite reduction were both enhanced by the presence of HA (Figure 2.1A). In the presence of HA the addition of 0.1 mM glucose stimulated a further increase in DIR reduction; however, the rate and extent of Fe(III) oxide reduction was greater in the presence of HA-only compared to glucose only. These results clearly indicate that HA served as a source of electrons for DIRB. In addition, the higher initial rate of DIR in the presence of HA compared to the glucose only reactors is suggestive of an electron shuttling effect analogous to previous studies with solid-phase humic substances.(Roden et al., 2010) The total amount of Fe(II) produced in the HA+glucose reactors was equivalent, within experimental error, to the sum of Fe(II) production in the HA-only and glucose-only treatments. The apparent inability of glucose to promote HA metabolism cannot be attributed to an electron acceptor limitation of OC metabolism, because the long-term extent of hematite reduction was 2-fold lower than in previous studies where natural freshwater bacteria were provided with greater amounts of labile OC.(Roden, 2003) This observation suggests that DIR was electron donor limited in our experiments, a conclusion supported by the virtual absence of methane in the headspace of the cultures at the end of the experiment (data not shown).

DIR in the presence of HM alone was similar to that in the no HS addition treatments (Figure 2.1B, HM only vs. the inoculated control (-HM, -Glu)). As discussed below, the minor amount of reduction in these treatments was likely driven by metabolism of small amounts of OC in the inoculum. The fact that HM in the glucose-amended reactors increased the initial rate of DIR but did not serve as a source of electrons suggests this HM served only as an electron shuttle. Collectively our results show the ability of chemically-extracted humic

substances to function as electron shuttles as previously recognized (Melton et al., 2014), but also in the case of HA as a source of electrons for DIRB.

2.4.2 Microbial community composition

Shotgun metagenomic sequencing was carried out to analyze the microbial communities in the cultures and their potential to metabolize complex OC. Conserved phylogenetic marker genes from the metagenomes were used to estimate microbial community composition (Table S3). Reactors containing HA were dominated by a diverse group of microorganisms including *Spirochaetes*, *Proteobacteria*, *Euryarchaeota*, *Bacteroidetes*, *Ignavibacteriiae*, *Acidobacteria*, *Firmicutes* and *Chloroflexi*. Conversely, *Proteobacteria* and *Acidobacteria* were dominant taxa in both the HM and glucose-only reactors, with the abundance of *Acidobacteria* being higher in the presence of glucose. Overall, HA promoted the development of a more diverse microbial community compared to HM and/or glucose. In particular the high abundance of organisms from phyla such as *Spirochaetes*, *Euryarchaeota*, *Bacteroidetes*, *Ignavibacteriiae*, *Firmicutes* and *Chloroflexi*, all of which are known to degrade complex SOC materials (Nemergut et al., 2010; Coolen et al., 2011; Colombo et al., 2014; Mhuantong et al., 2015a), implies that these taxa played a role in HA metabolism. Of course, taxa from the *Proteobacteria* and *Acidobacteria* were also substantial components of the HA microbial community and were likely also involved in organic carbon metabolism. In contrast to the HA reactors, HM did not stimulate DIR activity, and did not substantially alter microbial community composition relative to the glucose-only treatments. Based on these results, we infer that HM did not stimulate the growth of any particular phyla thought to participate in the metabolism of HS.

2.4.3 DIR gene systems

Organisms responsible for DIR in the cultures were assessed by searching for PCC (Figure 2.2) and PilA (Figure 2.S2) gene systems. Contrary to expectations, the reactors with highest rates of DIR did not show significant increases in PCC or PilA gene abundance. Experiments with HM and/or glucose had PCC genes mainly assigned to *Geobacter* and *Geothrix*, whereas HA reactors had increases in *Ignavibacterium/Melioribacter* related taxa. The latter taxa are known to be capable of fermentative growth with polysaccharides as well as DIR(Iino et al., 2010; Podosokorskaya et al., 2013; Fortney et al., 2016); thus the presence of both PCC and CAZyme genes (Figure 2.3) attributable to these organisms in the HA cultures suggests that they played a role both in DIR and upstream production of fermentation end-products that served as substrates for DIR. It is notable that our findings reveal for the first time that the previously described DIRB *Geothrix*(Coates et al., 1999b) contains PCC gene systems for reduction of extracellular electron acceptors (see Figure 2.S1 and Supporting Text 1).

2.4.4 General trends in organic carbon metabolism gene abundance

The potential for HA and HM degradation to provide electrons for DIRB was evaluated by determining if genes coding for CAZymes were differentially present in cultures with or without HS at the end of the experiments.(Lin et al., 2014) A total of 42312 genes across all treatments were classified as one of 258 different CAZyme families. The metagenomes amended with HA had more sequences coding for GHs, GTs, CBMs, CEs, PLs, cohesin, dockerin and SLH compared to the metagenomes from cultures containing HM and/or glucose (Figure 2.3). Additionally, GHs, GTs and PLs showed slightly higher gene abundance in treatments containing HM compared to those with only glucose (Figure 2.3). The increase in CAZymes in

the HA experiments implies that these enzymes played an important role in the degradation of HS. CAZyme family gene abundance was similar in both HM-containing and glucose only cultures. These results indicate that HM did not undergo significant enzymatic transformation, which is consistent with the finding that HM did not serve as an electron donor for DIR (Fig. 2.1B). The significant abundance of CAZymes in HM cultures can be attributed to metabolism of small amounts of complex OC in the inoculum.

The taxonomic origin of each CAZyme was determined by BLASTP.

Bacteroidetes, *Firmicutes*, *Euryarchaeota*, *Chlorobi*, *Chloroflexi*, *Spirochaetes*, *Planctomycetes*, and unclassified bacteria dominated the taxonomic classification of CAZymes present in HA experiments (Figure 2.4A). On the contrary, the CAZymes detected in treatments containing HM and/or glucose were dominated by *Proteobacteria*, *Acidobacteria* and unclassified bacteria. GHs, CBMs, CEs and PLs were also dominated by *Euryarchaeota* and *Bacteroidetes* when HM and glucose were present (Figure 2.4B). The overall higher diversity of CAZyme taxonomic assignments present in the HA experiments was expected, since the phylogenetic marker genes revealed that these microbial communities were much more diverse (Table 2.S3). It should also be noted that the abundance of unclassified taxa was greater in the HA experiments, suggesting the presence of novel microorganisms capable of complex OC degradation.

The high abundance of GHs, GTs, CBMs, CEs, PLs, cohesin, dockerin and SLH in HA experiments indicates that a large portion of the HA resembles polysaccharides and is in fact biodegradable. This is in agreement with previous geochemical results and the higher (oxygen + nitrogen)/carbon ratio found in HA (0.94) compared to HM (0.56). (Yang et al., 2011a) Additionally, many of these CAZymes were assigned to *Bacteroidetes*, *Firmicutes*, *Chloroflexi* and *Spirochaetes*, which are important contributors to the degradation of

lignocellulosic material in various environments.(Warnecke et al., 2007; He et al., 2013; Liu et al., 2013; Wang et al., 2016) *Planctomycetes* and *Euryarchaeota* were also detected in the presence of HAs and have been associated with cellulose decomposition with GTs(Schellenberger et al., 2010; Howe et al., 2016), and metabolism of carbohydrates with GHs(Hu et al., 2013; Li et al., 2015), respectively.

2.4.5 Key enzymatic systems involved in humic substance metabolism

To determine which enzymes had the most important role in the metabolism of HS, we determined which CAZyme families and KEGG modules had gene copy numbers at least two times higher in HS metagenomes compared to glucose only metagenomes. 129 and 72 CAZyme families, and 13 and 6 KEGG KO assignments with this criterion were identified in the HA and HM metagenomes respectively (see Supporting Text 2 and Tables 2.S4-2.S7). The CAZyme classes with the highest gene copy numbers were GHs and CBMs, followed by CEs, GTs and PLs (Figure 2.5). AAs and ADs under this criterion were negligible. The most abundant GHs in HA treatments were categorized as cellulases and endohemicellulases and debranching and oligosaccharide degrading enzymes.(Liu et al., 2013; Munir et al., 2014; van Munster et al., 2014a; Cuskin et al., 2015b; Mhuantong et al., 2015a; Chang et al., 2016b) Identical analysis of the HM treatments revealed cellulases, endohemicellulases, debranching and oligosaccharide degrading enzymes, but also included pectinases and fungal cell wall degrading enzymes. The higher abundance of these CAZyme families in the presence of HA compared to HM and/or glucose treatments is another indication that alkali-extracted SOM contains polysaccharides that are bioavailable for microbial degradation into simpler organic acids utilized by DIRB.(Lovley and Phillips, 1988, 1989)

2.4.6 Aromatic carbon degradation

Aromatic carbon makes up more than 10% of the bulk carbon in the HA and HM used in this study.(Wang et al., 2011) This component of HS includes quinone moieties(Adhikari et al., 2016) that were likely involved in electron shuttling for DIR, yet these same compounds could undergo degradation coupled to DIR.(Kleber, 2010) The abundance of redox enzymes that act in conjunction with CAZymes in aromatic carbon degradation (referred to as AAs) was similar in all metagenomes, but highest when HAs were the only carbon source added (Figure 2.3). By looking at these trends, one might conclude that AAs were important in the degradation of HAs when glucose is not present. However, AAs were also found in the metagenomes amended with only glucose (Figure 2.3), suggesting that the inoculum contained aromatic carbon compounds, in addition to other complex OC components as described above, that were subject to degradation. The taxonomic origin of AAs found in the HA treatments (primarily *Firmicutes*, *Proteobacteria* and *Euryarchaeota*) differed from those in the glucose-only and/or HM cultures, which were dominated by *Proteobacteria* (Figure 2.4). These results demonstrate that diverse taxa with the capability of aromatic carbon degradation were enriched in the HA cultures.

Since AAs were not predominant in the CAZyme gene inventories, a search for genes involved in the degradation of smaller aromatic compounds using the KEGG Orthology (KO) database was carried out. A total of 2222 genes across all metagenomes were assigned to the Aromatics Degradation (AD) category(Kanehisa and Goto, 2000; Kanehisa et al., 2013; Kanehisa et al., 2016; Kanehisa et al., 2017), and their abundance was much lower compared to that of GHs, GTs, CBMs and CEs (Figure 2.3). Most of the AAs and ADs require oxygen to function and their low abundance in our anoxic incubations is not surprising. Moreover, the preferred OC substrate for microorganisms is usually composed of carbohydrates, not aromatic

structures.(Kalbitz et al., 2003) Therefore, the low abundance of AAs and ADs can be explained if the metabolism of more labile OC (i.e., carbohydrates) happens prior to the metabolism of aromatic moieties.

We have illustrated that HS can be broken down by hydrolytic and fermentative microorganisms. However, DIRB such as *Geobacter* and *Ignavibacter* also have the metabolic capacity to utilize monoaromatic compounds for growth.(Lovley et al., 1989; Lovley and Lonergan, 1990; Iino et al., 2010; Rotaru et al., 2011; Podosokorskaya et al., 2013) Fifteen draft genomes containing a complete EET system were recovered from the metagenomes, and were identified as *Geobacter*, *Geothrix*, *Holophaga*, *Ignavibacteriales*, *Melioribacter*, *Anaeromyxobacter* and *Prolixibacter*. Thirteen genomes within the same taxonomic categories were downloaded from the IMG database in order to have at least one reference for all taxa. All genomes were screened (see Supporting Text 3) for ADs, and while it was confirmed that the *Geobacter metallireducens* reference genome has a complete pathway for toluene degradation and benzoyl-CoA degradation, no such AD degradation pathways were observed in any other genome (Tables 2.S8-2.S12). Overall there was no correlation between DIR and AD gene abundance (Tables 2.S6 and 2.S7); thus, it seems unlikely that aromatic carbon degradation was a major pathway for HS metabolism in this experiment. However, the large increase in *Ignavibacterium*/*Melioribacter* related PCC gene systems in the HA vs. HM cultures (Figure 2.2) does suggest that these organisms played a role in upstream decomposition of complex OC.

2.4.7 Environmental Implications

Previous studies of the influence of HS on DIR in soils and sediments have focused on electron shuttling effects rather than coupling between DIR and HS (e.g. as a surrogate for SOM)

decomposition.(Lovley et al., 1996; Roden et al., 2010; Klüpfel et al., 2014) Through a combination of DIR incubation experiments and metagenomic sequence gene counts this study separated the simultaneous processes of electron shuttling and electron donating by HS recovered from peat soil by sodium pyrophosphate extraction. The analysis revealed that metabolism of the HA fraction recovered from the soil was directly coupled to DIR. After a 30-day period roughly 3% of the HA electron equivalents present in the reactors was coupled to DIRB metabolism (see calculation below). Evidence of this phenomenon was provided by the overrepresentation of putative genes coding for enzymes that break down complex lignocellulosic material. For example, 129 CAZy families were at least two times more abundant in experiments containing HA compared to those with glucose only. The potential for microorganisms to utilize a small but significant portion of HA (see below) is consistent with the emerging view of SOC as a continuum of variably decomposable organic compounds.(Lehmann and Kleber, 2015)

Limited information on the biological degradation of HS under anoxic conditions is currently available,(Ueno et al., 2016) and the potential contribution of HS degradation to overall SOC metabolism under Fe(III)-reducing and other anaerobic respiratory conditions remains an open question. Supplemental Text 4 provides calculations to estimate the abundance of metabolizable HA in a representative histosol similar the soil from which the extracts employed in this study were recovered. The calculations translate into a biodegradable SOC pool of over $300 \mu\text{mol cm}^{-3}$. This represents a surprisingly large pool of labile OC that could drive DIR while at the same time promoting that process via electron shuttling. In situations where Fe(III) oxides are not abundant, this large pool of labile OC would alternatively drive other anaerobic metabolic pathways such as sulfate reduction or methanogenesis. Although, due to the extraction

process HS do not directly represent SOM(Lehmann and Kleber, 2015), our own and other recent results(Ueno et al., 2016) affirm that chemically-extracted HS represent a pool of SOM that is indeed biodegradable. In addition, recent studies indicate that DIR can liberate OC compounds associated with Fe(III) oxide surfaces(Adhikari et al., 2017; Zhao et al., 2017) that are thought to represent a significant pool of preserved SOC in soils and sediments.(Wagai and Mayer, 2007; Lalonde et al., 2012) This raises the possibility of a positive feedback loop where DIR driven by SOM depolymerization/fermentation and accelerated by electron shuttling leads to destabilization of Fe-associated OC which in turn further accelerates anaerobic carbon metabolism.

2.5 Acknowledgements

This research was supported by the U.S. Department of Energy, Office of Biological and Environmental Research, Subsurface Biogeochemical Research (SBR) program through grants DE-SC0014275 and DE-SC0016217, and by the SBR Scientific Focus Area at the Pacific Northwest National Laboratory (PNNL). Jacqueline Mejia was supported by the Graduate Engineering Research Scholars and the Biotechnology Training Program (NIH 5T32-GM08349) from the University of Wisconsin-Madison. Funds for the metagenomic sequencing were provided by Office of the Vice Chancellor for Research and Graduate Education at UW-Madison.

2.6 Figures

2.6.1 Figure (2.1) Fe(II) production

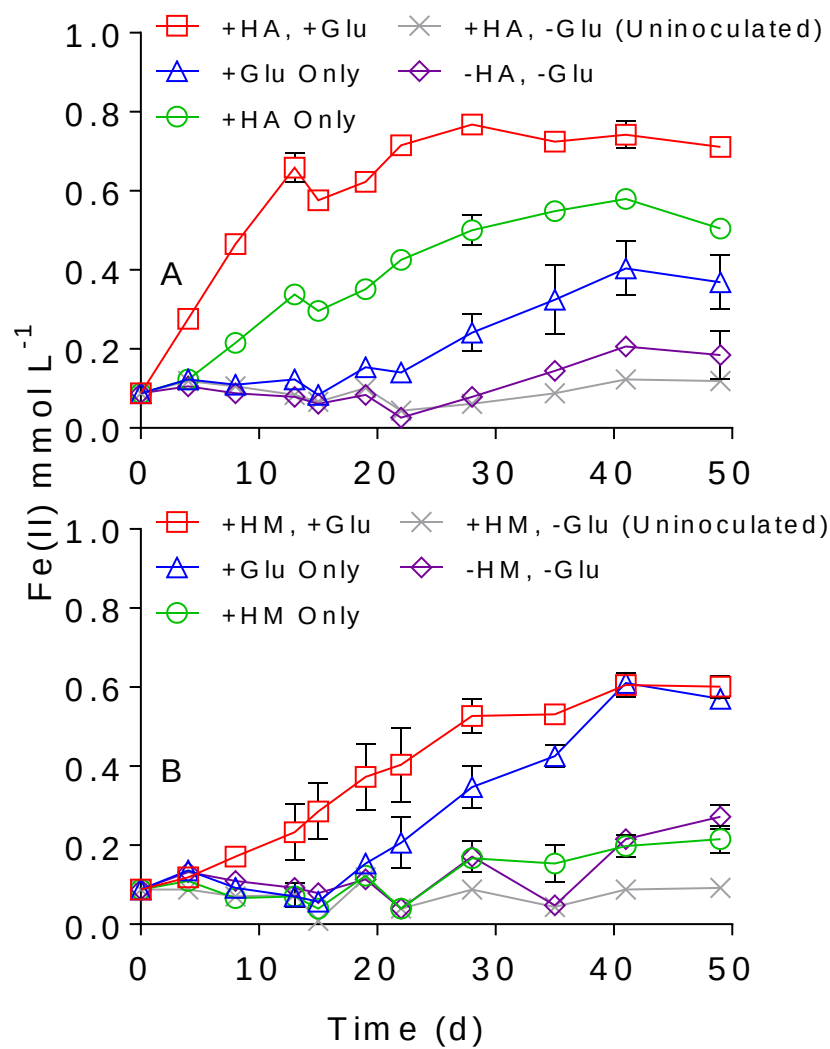


Figure 2.1. 0.5M HCl-extractable Fe(II) production for the HA (A) and HM (B) hematite reduction experiments. Data points represent the mean $\pm$ range of duplicate reactors.

2.6.2 Figure (2.2) PCC gene system abundance

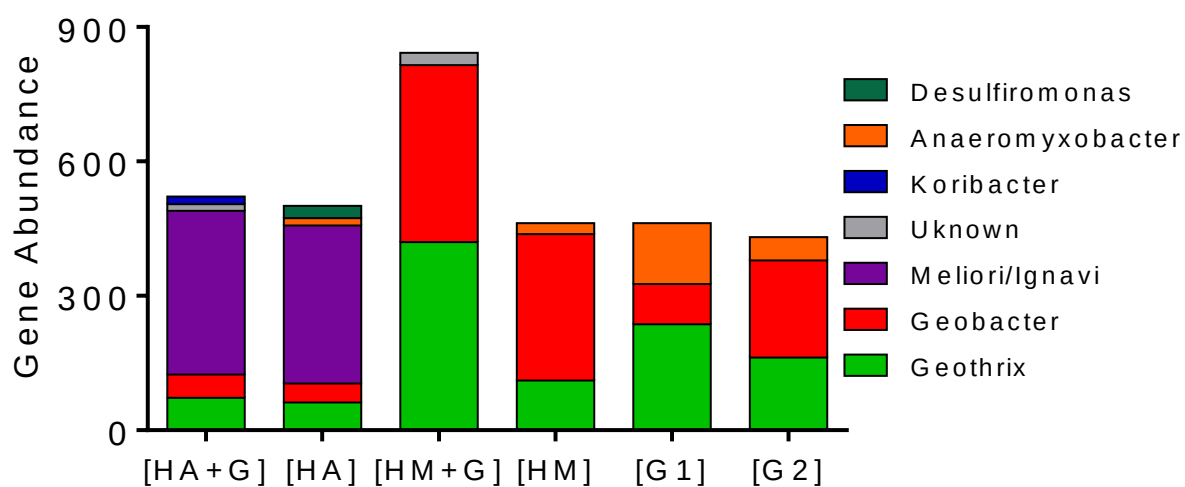


Figure 2.2. Abundance and taxonomic assignments of porin-cytochrome complex (PCC) genes found in the HA and HM cultures. The gene abundance was normalized by the total assembled base pairs. Abbreviations: HA = Humic Acid; G = Glucose; HM = Humin. G1 and G2 represent replicate glucose-only cultures.

2.6.3 Figure (2.3) Abundance of carbon degrading genes

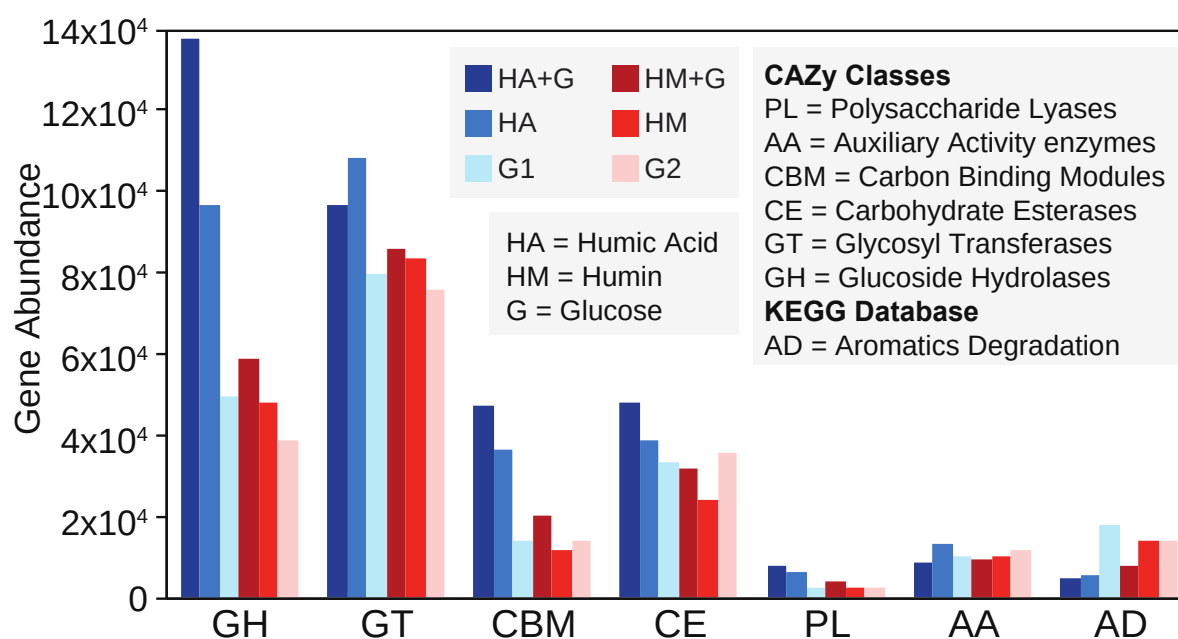


Figure 2.3. Abundance of genes categorized within a CAZyme class (GH, GT, CBM, CE, PL and AA) and KEGG Aromatics Degradation (AD) category, in the HA and HM data sets. The gene abundance was normalized by the total assembled base pairs.

2.6.4 Figure (2.4) Abundance of carbon degrading genes

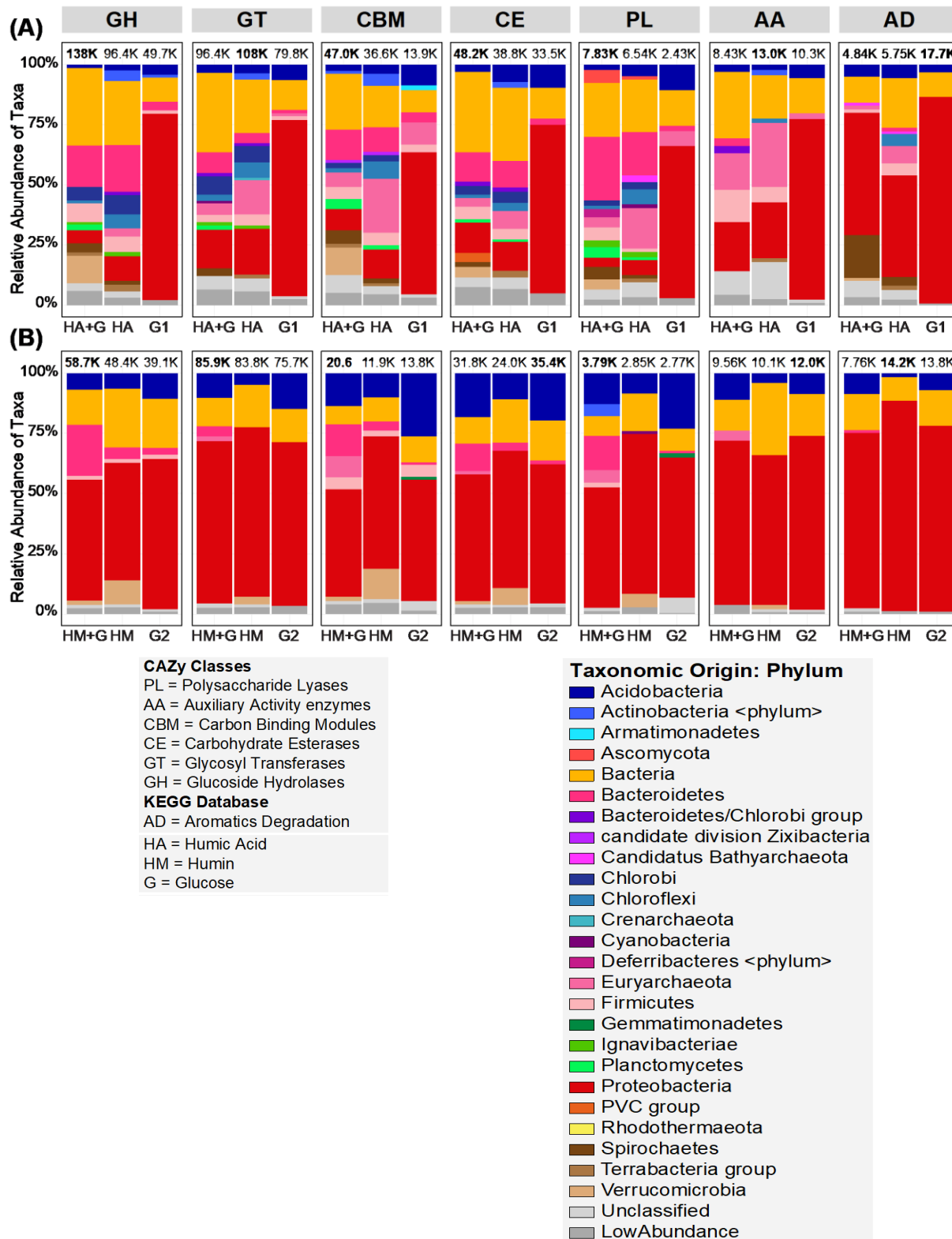


Figure 2.4. Taxonomic assignment of genes classified within a CAZyme class (GH, GT, CBM, CE, PL and AA) and KEGG Aromatics Degradation (AD) category, in the HA (A) and HM (B) data sets. The y-axis represents the relative abundance of phyla and the number at the top of the bars represents the sum of the gene abundances within a specific category and for a specific experiment. Abbreviations: HA = Humic Acid; G = Glucose; HM = Humin. G1 and G2 represent replicate glucose-only cultures.

2.6.5 Figure (2.5) Abundance of carbon degrading genes involved in HA metabolism

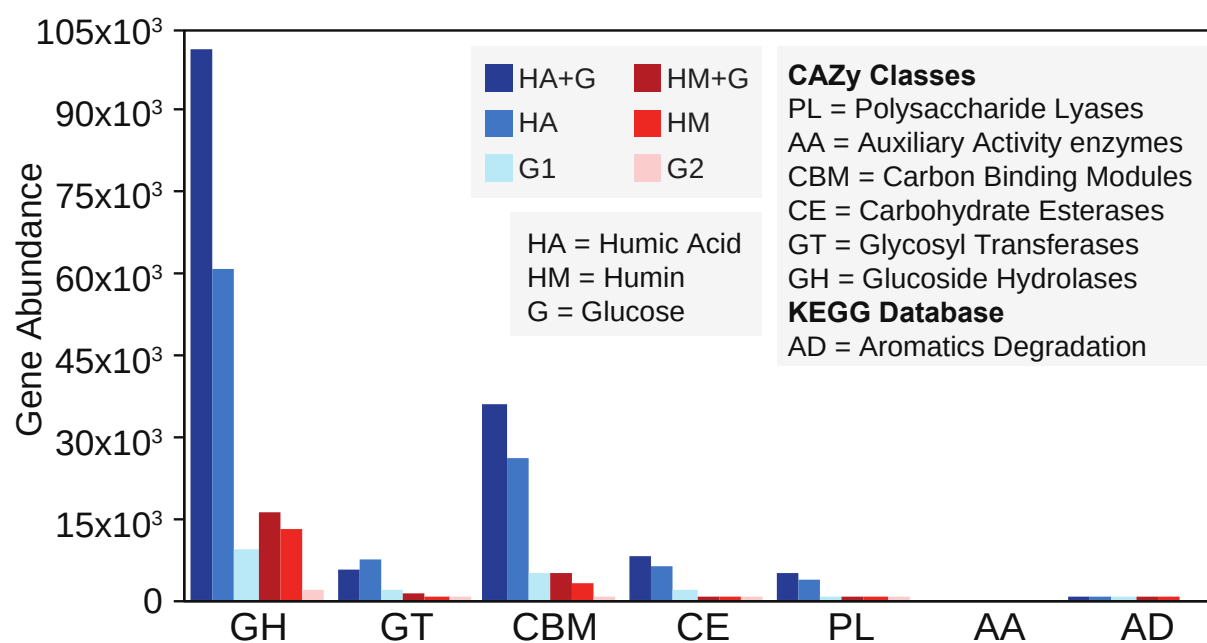


Figure 2.5. Sum of CAZyme and AD genes that had abundance values at least two times higher in metagenomes amended with HA or HM compared to those with only glucose.

2.7 Supplemental information

2.7.1 Supplemental text 1 *Geothrix fermentans* sp. PCC description

The PCC gene system was identified in *Geothrix fermentans* sp. nov., by hidden Markov model designed to target the beta-barrel porin protein (OmbB/OmbC) from *Geobacter sulfurreducens* PCA (details in Materials and Methods) (Figure 2.S3). Protein analysis for cellular location, beta-barrel porin trans-membrane motifs and c-type cytochrome heme-binding sites revealed that the PCC in *Geothrix fermentans* sp. nov., contains two periplasmic c-type cytochromes labeled OmaB1 and OmaB2 containing 5 and 6 heme-binding sites respectively (Figure 2.S3). The analogous OmcB extracellular c-type cytochrome contains 10 heme-binding sites. It is worth noting that the total number of heme-binding sites in the *Geobacter sulfurreducens* PCA OmaB-OmcB gene cluster is 20 and the total number of heme-binding sites in the *Geothrix fermentans* sp. nov., OmaB1-OmaB2-OmcB gene cluster is 21. Comparison between the PCC of *Geothrix fermentans* sp. nov., found in this experiment and the previously published whole genome for *Geothrix fermentans* DSM 14018 (Kyrpides, 2012) showed a matching PCC gene system with three additional extracellular decaheme c-type cytochromes.

The DIR capabilities of *Geothrix fermentans* are well understood (Coates et al., 1999a; Nevin and Lovley, 2002) however the EET gene system utilized to move electrons from inside the cell to electron acceptors outside the cell has not been documented. The *Geothrix fermentans* sp. nov., from this study was identified to contain the PCC first discovered in *Geobacter sulfurreducens* PCA and later found in organisms such as *Anaeromyxobacter dehalogenans* 2CP-1, *Denitrovibrio acetiphilus* DSM 12809, *Ignavibacterium album* JCM 16511 and *Desulfuromonas acetoxidans* DSM 684. (Shi et al., 2014) Although this study does provide

evidence that *Geothrix fermentans* sp. nov., contains the necessary genes for a complete PCC metabolism pathway further studies are needed to provide proof of this pathways functionality.

2.7.2 Supplemental text 2 key enzymatic systems involved in humic substance metabolism

CAZyme families and KEGG aromatic degradation genes with abundance values at least two times higher in metagenomes amended with humic substances (humic acids-HA or humins-HM) compared to glucose only treatments are illustrated in Tables 2.S4, 2.S5, 2.S6 and 2.S7. The known CAZyme classification was determined by previous studies,(Geisler-Lee et al., 2006; Blouzard et al., 2007; Allgaier et al., 2010; Suzuki et al., 2012; Liu et al., 2013; Zhao et al., 2013; Janeček et al., 2014; Munir et al., 2014; van Munster et al., 2014b; Cuskin et al., 2015a; Mhuantong et al., 2015b; Chang et al., 2016a; Suyotha et al., 2016) and is not specified for all CAZy families.

2.7.3 Supplemental text 3 aromatic carbon degradation analysis

Genome reconstruction (i.e. binning) from metagenomes was carried out by a coverage-composition algorithm using MaxBin.(Wu et al., 2014) The completeness and potential contamination of the bins was assessed using CheckM.(Parks et al., 2015b) The curated high quality bins (i.e., draft genomes) were searched for PCC genes, which resulted in classifications to *Geobacter*, *Geothrix*, *Ignavibacteriales*, *Anaeromyxobacter* and *Prolixibacter*. These draft genomes, and for comparison, genomes downloaded from IMG, were then screened for KEGG genes involved in aromatic degradation pathways (Tables 2.S8, 2.S9, 2.S10 and 2.S11).

2.7.4 Supplemental text 4 estimation of abundance of biodegradable HA in a representative histosol

The amount of DIR driven by HA degradation in our experiments was ca. 0.7 mmol Fe(III) L⁻¹ in the HA-only treatments (Figure 2.1A). This is equivalent to oxidation of ca. 0.175 mmol C L⁻¹, which represents 2.8 % of the total OC added to the reactors, based on the amount of HA added (180 mg C L⁻¹) and the OC content of the HA (42.4 %).(Yang et al., 2011b) Although this may seem like only a minor amount of OC degradation, it is important to consider how much SOC this represents in a typical OC-rich soil or sediment. Consider a soil with 15% dry weight SOC and a bulk density of 0.4 g dry mass cm⁻³, which represent typical values for histosols with a significant mineral content(Rabenhorst, 1999) such as the one from which the HA used in this study was extracted.(Yang et al., 2011b) The HA fraction of this histosol represented ca. 10% of total SOC (Yang et al., personal communication). Based on these values, the total HA content of the soil was on the order of 12,000 μmol per cm³ bulk soil. Assuming, based on our results, that ca. 3% of this material is subject to microbial degradation under anoxic conditions, this value translates into a biodegradable SOC pool of over 300 $\mu\text{mol cm}^{-3}$.

2.7.5 Supplemental figures

2.7.5.1 Figure (2.S1) Geothrix PCC gene system

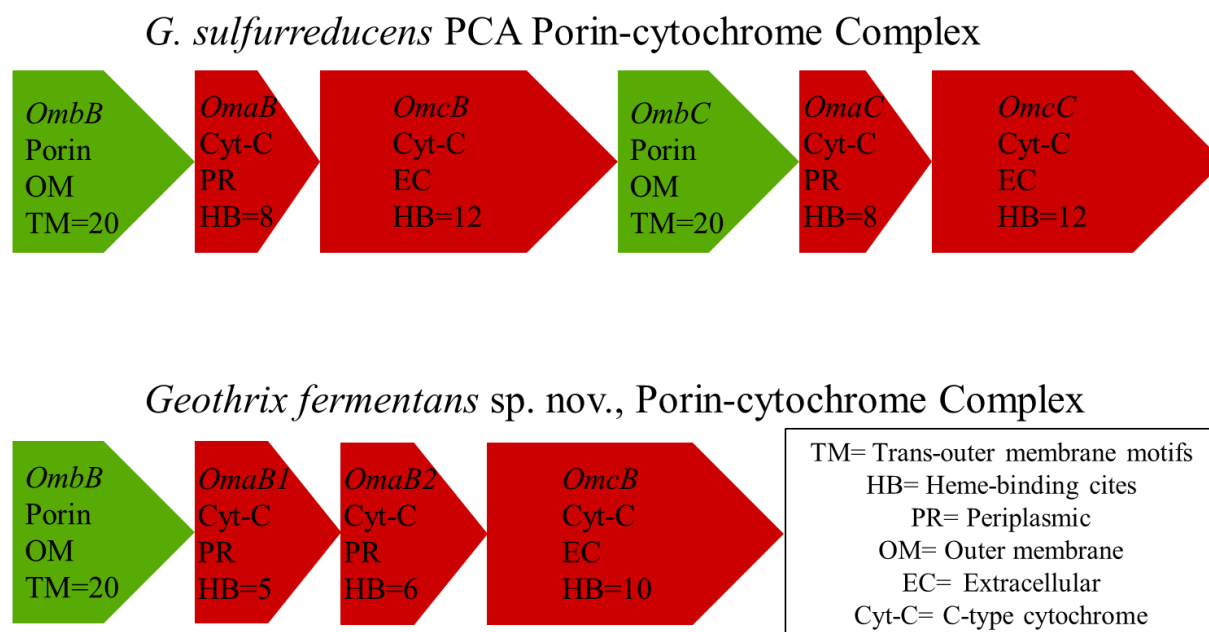


Figure 2.S1. Comparison of PCC in *Geobacter sulfurreducens* PCA and *Geothrix fermentans* sp. nov., identified from the metagenomes of this study. (Richardson et al., 2012)

2.7.5.2 Figure (2.S2) PilA gene abundance

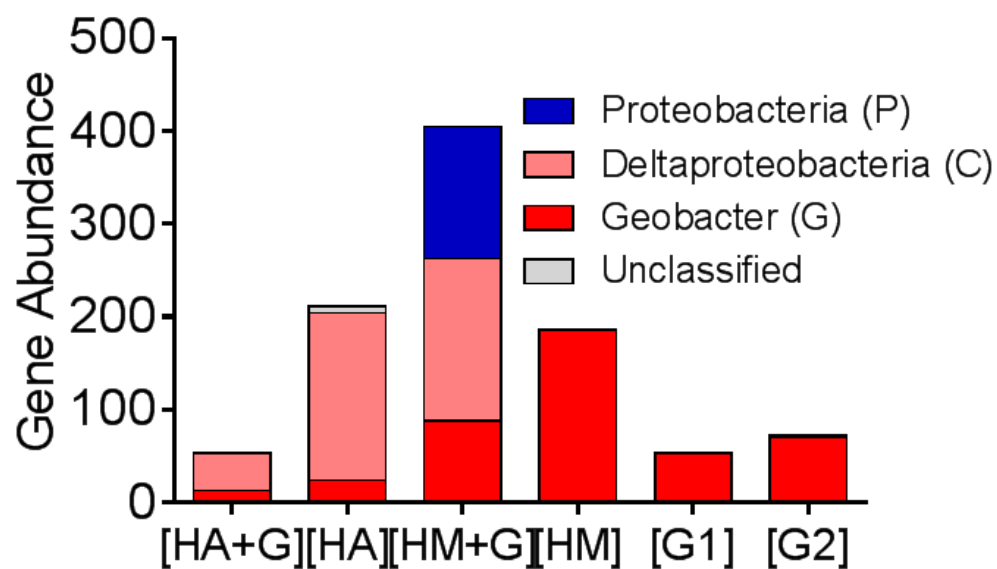


Figure 2.S2. PilA gene abundance for the HA and HM experiments. Assigned gene copies reflect the hits multiplied by the IMG scaffold read depth and normalized to the total base pairs in each metagenome. Abbreviations: HA = Humic Acid; G = Glucose; HM = Humin. G1 and G2 represent replicate glucose-only cultures.

2.7.5.3 Figure (2.S3) abundance of CAZyme modules

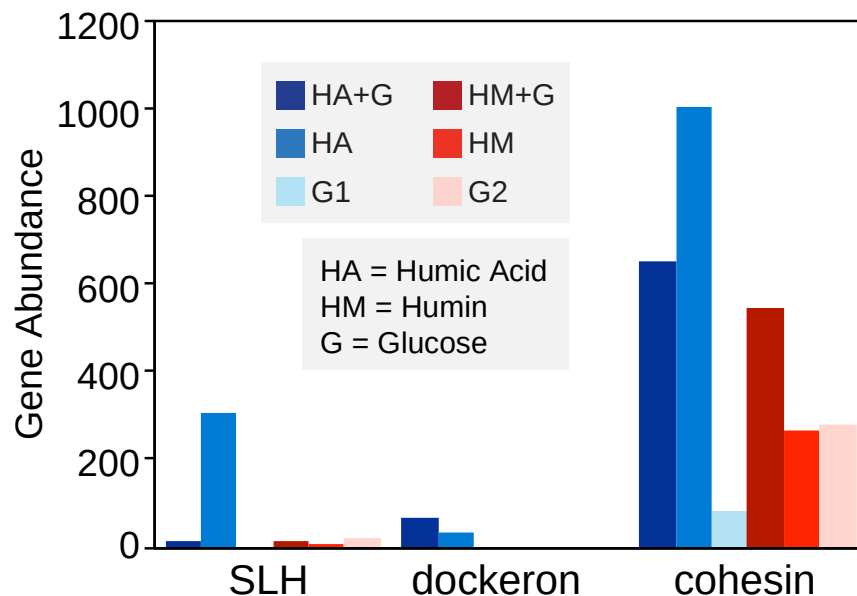


Figure 2.S3. Abundance of genes categorized within a carbohydrate active enzyme (CAZyme) module (SLH, dockeron and cohesin), in the HA and HM data sets. The gene abundance was normalized by the total assembled base pairs.

2.7.5.4 Figure (2.S4) taxonomy of CAZyme modules containing organisms

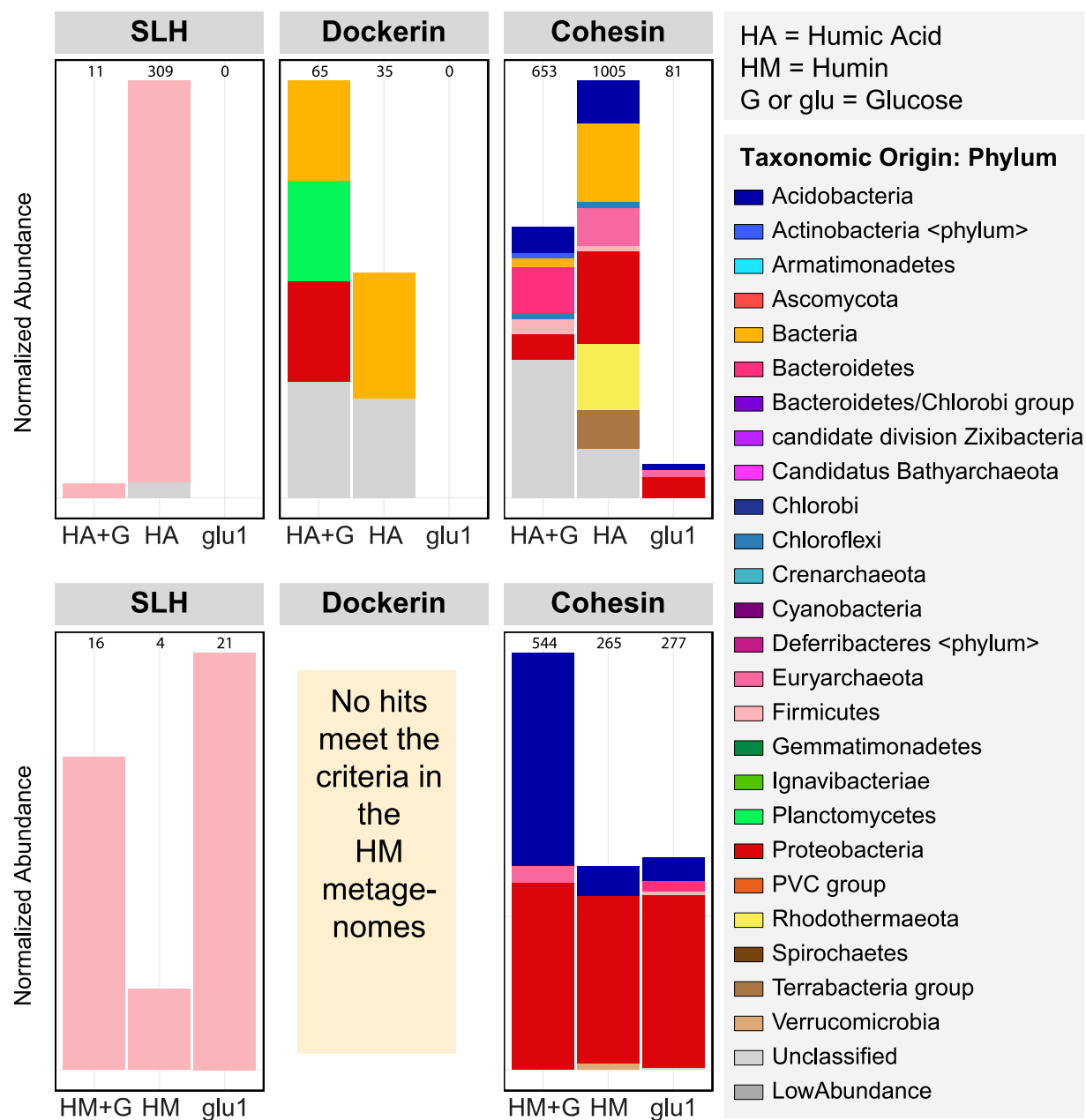


Figure 2.S4. Taxonomic origin of CAZy modules present in all treatments. The y-axis represents the relative abundance of the phyla and the number at the top of the bar represents the total abundance of a specific modules and for a specific treatment.

2.7.6 Supplemental tables

2.7.6.1 Table (2.S1) Metagenome assembly information

Table 2.S1. Metagenomes assembly with CLC Genomic Workbench information.

Metagenome Name	HA1 - Humic Acid and Glucose	HA2 - Glucose Only	HA3 - Humic Acid Only	HM1 - Humin and Glucose	HM2 - Glucose Only	HM3 - Humin Only
# raw reads	104473306	91226740	96492596	101724132	103934230	113463654
# reads after quality trimming	103265407	90181765	95508545	96399945	97833792	106864039
Avg length of reads matched after trimming	100.09	100.05	100.09	98.57	98.41	98.42
Total bases of reads matched after trimming	10335773099	9022764233	9559836211	9502323849	9628120889	10517032546
# reads matched after assembly	69236438	77944508	59796089	81074555	85566986	83953644
Avg length of reads matched after assembly	100.17	100.11	100.21	98.58	98.41	98.41
Total bases of reads matched after assembly*	6935661698	7802952571	5991905329	7992678301	8420617307	8261511278
# reads NOT matched after assembly	34028969	12237257	35712456	15325390	12266806	22910395
Avg length of reads NOT matched after assembly	99.92	99.68	99.91	98.51	98.44	98.45
Total bases of reads NOT matched after assembly	3400111401	1219811662	3567930882	1509645548	1207503582	2255521268
% raw reads assembled	67%	86%	63%	84%	87%	79%
Contig N75:	618	753	531	876	989	732
Contig N50:	1924	6160	1605	5870	5563	2717
Contig N25:	13420	65123	9535	82088	40778	25972
Contig Minimum length:	119	79	68	92	106	99
Contig Maximum length:	1042875	1235177	1262335	1804791	1786503	1581748
Contig Average length:	1010	1224	908	1312	1383	1110
Contig Total length	306895909	171117903	340747055	208247969	193292646	175066219
# Contigs	303938	139850	375441	158677	139783	157672

* This value was used to normalize the coverage among the different samples

2.7.6.2 Table (2.S2) Phylogenetic markers used for microbial community analysis

Table 2.S2. 32 COGs representing phylogenetic marker chosen for microbial community analysis.

COG ID	COG Name	Consensus COG length (amino acids)
COG0016	Phenylalanyl-tRNA synthetase alpha subunit	335
COG0072	Phenylalanyl-tRNA synthetase beta subunit	650
COG0081	Ribosomal protein L1	228
COG0244	Ribosomal protein L10	175
COG0080	Ribosomal protein L11	141
COG0102	Ribosomal protein L13	148
COG0093	Ribosomal protein L14	122
COG0200	Ribosomal protein L15	152
COG0197	Ribosomal protein L16/L10AE	146
COG0256	Ribosomal protein L18	125
COG0090	Ribosomal protein L2	275
COG0091	Ribosomal protein L22	120
COG0089	Ribosomal protein L23	94
COG0198	Ribosomal protein L24	104
COG0087	Ribosomal protein L3	218
COG0088	Ribosomal protein L4	214
COG0094	Ribosomal protein L5	180
COG0097	Ribosomal protein L6P/L9E	178
COG0051	Ribosomal protein S10	104
COG0100	Ribosomal protein S11	129
COG0048	Ribosomal protein S12	129
COG0099	Ribosomal protein S13	121
COG0184	Ribosomal protein S15P/S13E	89
COG0186	Ribosomal protein S17	87
COG0185	Ribosomal protein S19	93
COG0052	Ribosomal protein S2	252
COG0092	Ribosomal protein S3	233
COG0098	Ribosomal protein S5	181
COG0049	Ribosomal protein S7	148
COG0096	Ribosomal protein S8	132
COG0103	Ribosomal protein S9	130
COG0532	Translation initiation factor IF-2, a GTPase	509

2.7.6.3 Table (2.S3) Microbial community composition

Table 2.S3. Microbial community composition based on phylogenetic marker genes found in the metagenomes. Taxonomic assignments of these genes was carried out by BLASTP using the Ref_Seq protein database.

Phylogenetic Markers by MEGAN_RefSeq_COG len normalized							
Phylum	Humic Acid + Glucose	Humic Acid Only	Glucose Only 1	Humin + Glucose	Humin Only	Glucose Only 2	
Spirochaetes	 20.8	 5.2	0.0	0.0	0.0	0.1	
Proteobacteria	 13.7	 23.5	 84.2	 70.9	 86.5	 73.2	
Euryarchaeota	 11.6	 18.1	1.0	1.6	0.0	0.0	
Bacteroidetes	 11.2	 5.5	0.9	3.5	0.0	0.3	
Ignavibacteriae	 10.1	 10.5	0.0	0.0	0.0	0.0	
Acidobacteria	 8.9	 7.3	 11.7	 22.4	 9.1	 24.3	
Firmicutes	 4.7	 9.3	1.1	0.1	0.0	0.2	
Chloroflexi	 2.2	 7.3	0.0	0.0	0.0	0.0	
Lentisphaerae	 1.8	0.0	0.0	0.0	0.0	0.0	
Actinobacteria	 2.1	 5.8	0.0	0.0	0.0	0.0	
Verrucomicrobia	 1.7	0.5	0.2	 1.2	 4.0	0.2	
Candidatus Kryptonia	 1.3	 0.9	0.0	0.0	0.0	0.0	
Crenarchaeota	0.5	0.8	0.0	0.0	0.0	0.0	
Terrabacteria group	0.4	0.7	0.1	0.0	0.0	0.0	
Chrysiogenetes	0.3	0.0	0.0	0.0	0.0	0.0	
Thermotogae	0.3	0.0	0.0	0.0	0.0	0.0	
FCB group	0.2	0.7	0.0	0.0	0.0	0.0	
Deferribacteres	0.1	0.2	0.0	0.0	0.0	0.0	
Aquificae	0.1	0.0	0.0	0.0	0.0	0.0	
Thaumarchaeota	0.1	0.1	0.0	0.0	0.0	0.0	
Nitrospirae	0.1	0.0	0.0	0.0	0.0	0.0	
Planctomycetes	0.0	0.6	0.0	0.0	0.0	0.0	
Cyanobacteria	0.0	0.0	0.0	0.0	0.0	0.0	
Unclassified	 8.0	 2.8	0.8	0.2	0.3	1.7	
Total	 100.0	 100.0	 100.0	 100.0	 100.0	 100.0	

2.7.6.4 Table (2.S4) CAZy families involved in HA metabolism

Table 2.S4. 129 CAZy families with abundance values at least two times higher in the metagenomes containing humic acid (HA) versus those with only glucose (G).

Glycoside Hydrolases (GHs)					
CAZy Family	Known CAZyme Activities	Known CAZyme Classification	HA + G	HA	G1
GH74	endoglucanase; oligoxyloglucan reducing end-specific cellobiohydrolase; xyloglucanase	Cellulases and endohemicellulases	15960	14280	1160
GH39	α -L-iduronidase; β -xylosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	7385	619	78
GH2	β -galactosidase; β -mannosidase; β -glucuronidase; α -L-arabinofuranosidase; mannosylglycoprotein endo- β -mannosidase; exo- β -glucosaminidase	Debranching enzymes and Oligosaccharide-degrading enzymes	6228	3316	1473
GH5	endo- β -1,4-glucanase / cellulase; endo- β -1,4-xylanase; β -glucosidase; β -mannosidase; β -glucosylceramidase; glucan β -1,3-glucosidase; licheninase; exo- β -1,4-glucanase / cellodextrinase; glucan endo-1,6- β -glucosidase; mannan endo- β -1,4-mannosidase; cellulose β -1,4-cellobiosidase; steryl β -glucosidase; endoglycoceramidase; chitosanase; β -primeverosidase; xyloglucan-specific endo- β -1,4-glucanase; endo- β -1,6-galactanase; hesperidin 6-O- α -L-rhamnosyl- β -glucosidase; β -1,3-mannanase; arabinoxylan-specific endo- β -1,4-xylanase; mannan transglycosylase	Cellulases and endohemicellulases	6150	2394	957
GH33	sialidase or neuraminidase; trans-sialidase; 2-keto-3-deoxynononic acid hydrolase; anhydrosialidase; 3-deoxy-D-manno-octulosonic-acid hydrolase	Debranching enzymes and Oligosaccharide-degrading enzymes	4834	3301	649

GH78	α -L-rhamnosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	4824	2682	69
GH42	β -galactosidase; α -L-arabinopyranosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	4622	498	40
GH29	α -L-fucosidase; α -1,3/1,4-L-fucosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	4385	2311	87
GH130	β -1,4-mannosylglucose phosphorylase; β -1,4-mannooligosaccharide phosphorylase; β -1,4-mannosyl-N-acetyl-glucosamine phosphorylase; β -1,2-mannobiose phosphorylase; β -1,2-oligomannan phosphorylase; β -1,2-mannosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	3896	2353	419
GH28	polygalacturonase; exo-polygalacturonase; exo-polygalacturonosidase; rhamnogalacturonase; rhamnogalacturonan α -1,2-galacturonohydrolase; rhamnogalacturonan α -L-rhamnopyranohydrolase; endo-xylogalacturonan hydrolase	Pectinases	2494	1644	134
GH127	β -L-arabinofuranosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	2427	1947	470
GH110	α -galactosidase; α -1,3-galactosidase	NA	2403	233	5
GH43	β -xylosidase; α -L-arabinofuranosidase; arabinanase; xylanase; galactan 1,3- β -galactosidase; α -1,2-L-arabinofuranosidase; exo- α -1,5-L-arabinofuranosidase; [inverting] exo- α -1,5-L-arabinanase; β -1,3-xylosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	2381	1850	578
GH92	mannosyl-oligosaccharide α -1,2-mannosidase; mannosyl-oligosaccharide α -1,3-mannosidase; mannosyl-oligosaccharide α -1,6-mannosidase; α -mannosidase; α -1,2-mannosidase; α -1,3-mannosidase; α -1,4-mannosidase;	CAZy families processing fungal cell wall	2239	1723	86

	mannosyl-1-phosphodiester α -1,P-mannosidase				
GH20	β -hexosaminidase; lacto-N-biosidase; β -1,6-N-acetylglucosaminidase); β -6-SO ₃ -N-acetylglucosaminidase	CAZy families processing fungal cell wall	2236	1235	141
GH38	α -mannosidase; mannosyl-oligosaccharide α -1,2-mannosidase; mannosyl-oligosaccharide α -1,3-1,6-mannosidase; α -2-O-mannosylglycerate hydrolase; mannosyl-oligosaccharide α -1,3-mannosidase	CAZy families processing fungal cell wall	2225	1352	513
GH57	α -amylase; α -galactosidase; amylopullulanase; cyclomaltodextrinase; branching enzyme; 4- α -glucanotransferase	Debranching enzymes and Oligosaccharide-degrading enzymes	2073	2001	440
GH51	endoglucanase; endo- β -1,4-xylanase; β -xylosidase; α -L-arabinofuranosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	1838	1011	84
GH36	α -galactosidase; α -N-acetylgalactosaminidase; stachyose synthase; raffinose synthase	Debranching enzymes and Oligosaccharide-degrading enzymes	1798	616	70
GH106	α -L-rhamnosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	1716	908	18
GH105	unsaturated rhamnogalacturonyl hydrolase; d-4,5-unsaturated β -glucuronyl hydrolase	Pectinases	1655	986	380
GH95	α -L-fucosidase; α -1,2-L-fucosidase; α -L-galactosidase	Cellulases and endohemicellulases	1473	997	43
GH16	xyloglucan:xyloglucosyltransferase; keratan-sulfate endo-1,4- β -galactosidase; endo-1,3- β -glucanase; endo-1,3(4)- β -glucanase; licheninase; β -agarase; κ -carrageenase; xyloglucanase; endo- β -1,3-galactanase; β -porphyranase; hyaluronidase; endo- β -1,4-galactosidase; chitin β -1,6-glucanosyltransferase; endo- β -1,4-galactosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	1422	1400	451

GH50	β -agarase	NA	1372	470	30
GH99	glycoprotein endo- α -1,2-mannosidase; mannan endo-1,2- α -mannanase	CAZy families processing fungal cell wall	1066	216	19
GH35	β -galactosidase; exo- β -glucosaminidase; exo- β -1,4-galactanase; β -1,3-galactosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	941	462	92
GH30	endo- β -1,4-xylanase; β -glucosidase; β -glucuronidase; β -xylosidase; β -fucosidase; glucosylceramidase; β -1,6-glucanase; glucuronoarabinoxylan endo- β -1,4-xylanase; endo- β -1,6-galactanase; [reducing end] β -xylosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	842	795	308
GH116	β -glucosidase; β -xylosidase; acid β -glucosidase/ β -glucosylceramidase; β -N-acetylglucosaminidase	Debranching enzymes and Oligosaccharide-degrading enzymes	819	639	42
GH27	α -galactosidase; α -N-acetylgalactosaminidase; isomaltodextranase; β -L-arabinopyranosidase; galactan:galactan galactosyltransferase	Debranching enzymes and Oligosaccharide-degrading enzymes	799	570	37
GH53	endo- β -1,4-galactanase	Debranching enzymes and Oligosaccharide-degrading enzymes	766	285	23
GH10	endo-1,4- β -xylanase; endo-1,3- β -xylanase; tomatinase; xylan endotransglycosylase	Cellulases and endohemicellulases	750	763	55
GH9	endoglucanase; endo- β -1,3(4)-glucanase / lichenase-laminarinase; β -glucosidase; lichenase / endo- β -1,3-1,4-glucanase; exo- β -1,4-glucanase / cellodextrinase; cellobiohydrolase; xyloglucan-specific endo- β -1,4-glucanase / endo-xyloglucanase; exo- β -glucosaminidase	Cellulases and endohemicellulases	646	743	94
GH93	exo- α -L-1,5-arabinanase	Debranching enzymes and Oligosaccharide-degrading enzymes	613	853	0
GH71	α -1,3-glucanase	Debranching enzymes and Oligosaccharide-degrading enzymes	528	13	0

GH108	N-acetylmuramidase	NA	515	1153	229
GH129	α -N-acetylglactosaminidase	NA	511	21	0
GH97	glucoamylase; α -glucosidase; α -galactosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	496	453	66
GH123	β -N-acetylglactosaminidase; glycosphingolipid β -N-acetylglactosaminidase	NA	491	254	0
GH12	endoglucanase; xyloglucan hydrolase; β -1,3-1,4-glucanase; xyloglucan endotransglycosylase	Cellulases and endohemicellulases	473	204	4
GH117	α -1,3-L-neoagarooligosaccharide hydrolase; α -1,3-L-neoagarobiase/neoagarobiose hydrolase	NA	453	337	4
GH26	β -mannanase; exo- β -1,4-mannobiohydrolase; β -1,3-xylanase; lichenase / endo- β -1,3-1,4-glucanase; mannobiose-producing exo- β -mannanase	Cellulases and endohemicellulases	410	375	37
GH89	α -N-acetylglucosaminidase	CAZy families processing fungal cell wall	378	310	5
GH44	endoglucanase; xyloglucanase	Cellulases and endohemicellulases	350	189	23
GH125	exo- α -1,6-mannosidase	CAZy families processing fungal cell wall	332	169	0
GH64	β -1,3-glucanase	Debranching enzymes and Oligosaccharide-degrading enzymes	173	217	10
GH25	lysozyme	NA	145	458	63
GH47	α -mannosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	142	168	0
GH17	glucan endo-1,3- β -glucosidase; glucan 1,3- β -glucosidase; licheninase; ABA-specific β -glucosidase; β -1,3-glucanosyltransglycosylase	Cell wall elongation	137	86	8
GH100	alkaline and neutral invertase	Disaccharide Degrading	129	223	19
GH76	α -1,6-mannanase	CAZy families processing fungal cell wall	105	164	17

GH120	β -xylosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	93	164	25
GH128	β -1,3-glucanase	CAZy families processing fungal cell wall	50	59	0
GH66	cycloisomaltooligosaccharide glucanotransferase; dextranase	NA	48	231	24
GH55	exo- β -1,3-glucanase; endo- β -1,3-glucanase	CAZy families processing fungal cell wall	46	148	0
GH62	α -L-arabinofuranosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	38	55	6
GH11	endo- β -1,4-xylanase; endo- β -1,3-xylanase	Cellulases and endohemicellulases	36	199	0
GH54	α -L-arabinofuranosidase; β -xylosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	35	100	0
GH67	α -glucuronidase; xylan α -1,2-glucuronidase	Debranching enzymes and Oligosaccharide-degrading enzymes	23	35	0
GH79	β -glucuronidase; hyaluronoglucuronidase; heparanase; baicalin β -glucuronidase; β -4-O-methyl-glucuronidase	Pectinases	22	16	0
GH59	β -galactosidase; galactocerebrosidase	Cellulases and endohemicellulases	18	55	0
GH84	N-acetyl β -glucosaminidase; hyaluronidase; [protein]-3-O-(GlcNAc)-L-Ser/Thr β -N-acetylglucosaminidase	NA	12	52	0
Glycosyl Transferases (GTs)					
CAZy Family	Known CAZyme Activities		HA + G	HA	G1
GT5	UDP-Glc: glycogen glucosyltransferase; ADP-Glc: starch glucosyltransferase; NDP-Glc: starch glucosyltransferase; UDP-Glc: α -1,3-glucan synthase; UDP-Glc: α -1,4-glucan synthase		2376	2233	1102
GT39	Dol-P-Man: protein α -mannosyltransferase		1051	799	335
GT66	dolichyl-diphosphooligosaccharide—protein glycotransferase; undecaprenyl-diphosphooligosaccharide—protein glycotransferase		718	1731	257

GT81	NDP-Glc: glucosyl-3-phosphoglycerate synthase; NDP-Man: mannosyl-3-phosphoglycerate synthase; ADP-Glc: glucosyl-2-glycerate synthase	461	884	80
GT3	glycogen synthase	249	400	37
GT94	GDP-Man: GlcA- β -1,2-Man- α -1,3-Glc- β -1,4-Glc- α -1-PP-undecaprenol β -1,4-mannosyltransferase	167	371	42
GT10	galactoside α -1,3/1,4-L-fucosyltransferase; galactoside α -1,3-L-fucosyltransferase; glycoprotein α -1,3-L-fucosyltransferase	145	127	0
GT75	UDP-Glc: self-glucosylating β -glucosyltransferase; UDP-L-arabinopyranose mutase	103	134	0
GT89	β -D-arabinofuranosyl-1-monophosphoryldecaprenol : arabinan β -1,2-arabinofuranosyltransferase	92	406	37
GT74	α -1,2-L-fucosyltransferase	88	71	9
GT13	α -1,3-mannosyl-glycoprotein β -1,2-N-acetylglucosaminyltransferase	64	42	0
GT92	UDP-Gal: N-glycan core α -1,6-fucoside β -1,4-galactosyltransferase; UDP-Gal: β -galactoside β -1,4-galactosyltransferase	40	28	0
GT77	α -xylosyltransferase; α -1,3-galactosyltransferase; arabinosyltransferase; arabinosyltransferase	15	11	0
GT33	GDP-Man: chitobiosyldiphosphodolichol β -mannosyltransferase	7	180	0
Carbon Binding Modules (CBMs)				
CAZy Family	Known CAZyme Activities	HA + G	HA	G1
CBM9	Binds to cellulose	10344	3130	849
CBM44	Binds to cellulose and xyloglucan	5329	9754	1808
CBM32	Binds to galactose, lactose, polygalacturonic acid and LacNAc (β -D-galactosyl-1,4- β -D-N-acetylglucosamine)	4933	2900	613
CBM67	Binds to L-rhamnose	3918	1841	208
CBM16	Binds to cellulose and glucomannan	2437	757	187
CBM66	Binds to the terminal fructoside residue of fructans	1815	2373	658
CBM4	Binds to xylan, β -1,3-glucan, β -1,3-1,4-glucan, β -1,6-glucan and amorphous cellulose but not with crystalline cellulose.	1653	447	67
CBM51	Attached to various enzymes from families GH2, GH27, GH31, GH95, GH98 and GH101. Binds to galactose and to blood group A/B-antigens	1076	443	30

CBM40	Attached to various enzymes from GH 33 family. Binds to sialic acid	609	448	71
CBM57	binds to various glycosidases	567	987	28
CBM35	Binds to xylan and β -galactan, and decorated soluble mannans and mannoooligosaccharides	462	223	6
CBM20	Binds to granular starch and interacts strongly with cyclodextrins	405	653	124
CBM22	Binds to xylan and has affinity with mixed β -1,3/ β -1,4-glucans	346	71	4
CBM8	Binds to cellulose	336	83	0
CBM13	Binds to galactose residues, glycoside hydrolases and glycosyltransferases, xylan and GalNAc	235	533	87
CBM56	Binds to β -1,3-glucan	221	161	24
CBM23	Binds to mannan	212	66	8
CBM6	Bnds to cellulose, β -1,3-glucan, β -1,3-1,4-glucan and β -1,4-glucan.	209	276	63
CBM11	Binds to β -1,4-glucan and β -1,3-1,4-mixed linked glucans	155	93	0
CBM38	Binds to inulin	113	86	5
CBM61	Attached to various enzymes from families GH16, GH30, GH31, GH43, GH53 and GH66. Binds to β -1,4-galactan	111	61	22
CBM47	Binds to fucose	106	254	6
CBM5	Binds to chitin	70	71	9
CBM12	Binds to chitin	70	55	8
CBM26	Binds to starch	62	21	0
CBM30	Binds to cellulose	50	85	18
CBM62	Binds to galactose moieties found on xyloglucan, arabinogalactan and galactomannan	47	14	0
CBM59	Binds to mannan, xylan, and cellulose	46	17	0
CBM45	Binds to starch	29	38	0
CBM37	Broad binding specificity to xylan, chitin, microcrystalline and phosphoric-acid swollen cellulose, as well as more heterogeneous substrates, such as alfalfa cell walls, banana stem and wheat straw.	24	65	0

CBM70	Binds to hyaluronan		22	79	8	
CBM77	Binds to pectin		15	16	0	
CBM2	Binds to cellulose, chitin or xylan.		13	161	4	
CBM42	Attached to various enzymes from GH54 family. Binds to arabinofuranose (present in arabinoxylan)		11	34	0	
CBM54	Binds to xylan, yeast cell wall glucan and chitin		10	80	0	
CBM63	Binds to cellulose		7	35	0	
Carbohydrate Esterases (CEs)						
CAZy Family	Known CAZyme Activities		Known CAZyme Classification	HA + G	HA	G1
CE14	N-acetyl-1-D-myo-inosityl-2-amino-2-deoxy- α -D-glucopyranoside deacetylase; diacetylchitobiose deacetylase; mycothiol S-conjugate amidase		Debranching enzymes and Oligosaccharide-degrading enzymes	2683	3497	1108
CE12	pectin acetylesterase; rhamnogalacturonan acetylesterase; acetyl xylan esterase		Pectinases	1686	446	6
CE8	pectin methylesterase		Pectinases	1596	1120	469
CE6	acetyl xylan esterase		Debranching enzymes and Oligosaccharide-degrading enzymes	1123	362	68
CE15	4-O-methyl-glucuronoyl methylesterase		CAZy families processing fungal cell wall	791	666	38
CE2	acetyl xylan esterase		Debranching enzymes and Oligosaccharide-degrading enzymes	332	128	41
Polysaccharide Lyases (PLs)						
CAZy Family	Known CAZyme Activities			HA + G	HA	G1
PL9	pectate lyase; exopolygalacturonate lyase; thiopeptidoglycan lyase			1729	2083	190
PL12	heparin-sulfate lyase			1710	1124	116
PL8	hyaluronate lyase; chondroitin AC lyase; xanthan lyase; chondroitin ABC lyase			536	157	0
PL10	pectate lyase			363	176	10

PL11	rhamnogalacturonan lyase; exo-unsaturated rhamnogalacturonan lyase	304	71	0
PL17	alginate lyase; oligoalginate lyase	290	85	8
PL21	heparin lyase; heparin-sulfate lyase; acharan-sulfate lyase	40	4	0
PL7	poly(β -mannuronate) lyase / M-specific alginate lyase; α -L-guluronate lyase / G-specific alginate lyase; poly-(MG)-lyase / MG-specific alginate lyase	34	7	0
PL24	ulvan lyase	34	8	0
Other Modules				
CAZy Family		HA + G	HA	G1
cohesin		653	1005	81
dockerin		65	35	0
SLH		11	309	0

2.7.6.5 Table (2.S5) CAZy families abundant in HM reactors

Table 2.S5. 72 CAZy families with abundance values at least two times higher in the metagenomes containing humin (HM) versus those with only glucose (G).

Glycoside Hydrolases (GHs)					
CAZy Family	Known CAZyme Activities	Known CAZyme Classification	HM + G	HM	G2
GH43	β -xylosidase; α -L-arabinofuranosidase; arabinanase; xylanase; galactan 1,3- β -galactosidase; α -1,2-L-arabinofuranosidase; exo- α -1,5-L-arabinofuranosidase; [inverting] exo- α -1,5-L-arabinanase; β -1,3-xylosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	2181	1670	186
GH2	β -galactosidase; β -mannosidase; β -glucuronidase; α -L-arabinofuranosidase; mannosylglycoprotein endo- β -mannosidase; exo- β -glucosaminidase	Debranching enzymes and Oligosaccharide-degrading enzymes	2040	1567	193
GH5	endo- β -1,4-glucanase / cellulase; endo- β -1,4-xylanase; β -glucosidase; β -mannosidase; β -glucosylceramidase; glucan β -1,3-glucosidase; licheninase; exo- β -1,4-glucanase / cellodextrinase; glucan endo-1,6- β -glucosidase; mannan endo- β -1,4-mannosidase; cellulose β -1,4-cellobiosidase; steryl β -glucosidase; endoglycoceramidase; chitosanase; β -primeverosidase; xyloglucan-specific endo- β -1,4-glucanase; endo- β -1,6-galactanase; hesperidin 6-O- α -L-rhamnosyl- β -glucosidase; β -1,3-mannanase; arabinoxylan-specific endo- β -1,4-xylanase; mannan transglycosylase	Cellulases and endohemicellulases	1771	1684	579

GH28	polygalacturonase; exo-polygalacturonase; exo-polygalacturonosidase; rhamnogalacturonase; rhamnogalacturonan α -1,2-galacturonohydrolase; rhamnogalacturonan α -L-rhamnopyranohydrolase; endo-xylogalacturonan hydrolase	Pectinases	832	626	178
GH127	β -L-arabinofuranosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	660	305	24
GH105	unsaturated rhamnogalacturonyl hydrolase; d-4,5-unsaturated β -glucuronyl hydrolase	Pectinases	600	322	34
GH78	α -L-rhamnosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	571	599	80
GH92	mannosyl-oligosaccharide α -1,2-mannosidase; mannosyl-oligosaccharide α -1,3-mannosidase; mannosyl-oligosaccharide α -1,6-mannosidase; α -mannosidase; α -1,2-mannosidase; α -1,3-mannosidase; α -1,4-mannosidase; mannosyl-1-phosphodiester α -1,P-mannosidase	CAZy families processing fungal cell wall	501	228	49
GH20	β -hexosaminidase; lacto-N-biosidase; β -1,6-N-acetylglucosaminidase); β -6-SO ₃ -N-acetylglucosaminidase	CAZy families processing fungal cell wall	499	245	31
GH9	endoglucanase; endo- β -1,3(4)-glucanase / lichenase-laminarinase; β -glucosidase; lichenase / endo- β -1,3-1,4-glucanase; exo- β -1,4-glucanase / cellodextrinase; cellobiohydrolase; xyloglucan-specific endo- β -1,4-glucanase / endo-xyloglucanase; exo- β -glucosaminidase	Cellulases and endohemicellulases	413	349	66
GH37	α , α -trehalase	Disaccharide Degrading	412	71	33
GH27	α -galactosidase; α -N-acetylgalactosaminidase; isomaltodextranase; β -L-arabinopyranosidase; galactan:galactan galactosyltransferase	Debranching enzymes and Oligosaccharide-degrading enzymes	394	437	8

GH95	α -L-fucosidase; α -1,2-L-fucosidase; α -L-galactosidase	Cellulases and endohemicellulases	366	228	16
GH88	d-4,5-unsaturated β -glucuronyl hydrolase	Debranching enzymes and Oligosaccharide-degrading enzymes	364	142	39
GH8	chitosanase; cellulase; licheninase; endo-1,4- β -xylanase; reducing-end- xylose releasing exo-oligoxylanase	Cellulases and endohemicellulases	349	170	45
GH51	endoglucanase; endo- β -1,4- xylanase; β -xylosidase; α -L- arabinofuranosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	327	544	61
GH42	β -galactosidase; α -L- arabinopyranosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	318	200	78
GH97	glucoamylase; α -glucosidase; α - galactosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	316	177	18
GH29	α -L-fucosidase; α -1,3/1,4-L- fucosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	296	372	37
GH35	β -galactosidase; exo- β - glucosaminidase; exo- β -1,4- galactanase; β -1,3-galactosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	285	320	29
GH53	endo- β -1,4-galactanase	Debranching enzymes and Oligosaccharide-degrading enzymes	276	197	93
GH65	α,α -trehalase; maltose phosphorylase; trehalose phosphorylase; kojibiose phosphorylase; trehalose-6- phosphate phosphorylase; nigerose phosphorylase; 3-O- α - glucopyranosyl-L-rhamnose phosphorylase; 2-O- α - glucopyranosylglycerol: phosphate β - glucosyltransferase; α -glucosyl-1,2- β -galactosyl-L-hydroxylysine α - glucosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	269	151	6
GH26	β -mannanase; exo- β -1,4- mannobiohydrolase; β -1,3-xylanase; lichenase / endo- β -1,3-1,4- glucanase; mannobiose-producing exo- β -mannanase	Cellulases and endohemicellulases	266	343	0

GH55	exo- β -1,3-glucanase; endo- β -1,3-glucanase	CAZy families processing fungal cell wall	203	263	15
GH39	α -L-iduronidase; β -xylosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	190	360	67
GH10	endo-1,4- β -xylanase; endo-1,3- β -xylanase; tomatinase; xylan endotransglycosylase	Cellulases and endohemicellulases	189	286	33
GH99	glycoprotein endo- α -1,2-mannosidase; mannan endo-1,2- α -mannanase	CAZy families processing fungal cell wall	179	14	0
GH106	α -L-rhamnosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	166	126	0
GH76	α -1,6-mannanase	CAZy families processing fungal cell wall	161	56	6
GH115	xylan α -1,2-glucuronidase; α -(4-O-methyl)-glucuronidase	Debranching enzymes and Oligosaccharide-degrading enzymes	146	322	7
GH87	mycodextranase; α -1,3-glucanase	CAZy families processing fungal cell wall	130	257	0
GH54	α -L-arabinofuranosidase; β -xylosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	89	48	0
GH100	alkaline and neutral invertase	Disaccharide Degrading	76	52	19
GH67	α -glucuronidase; xylan α -1,2-glucuronidase	Debranching enzymes and Oligosaccharide-degrading enzymes	67	117	14
GH89	α -N-acetylglucosaminidase	CAZy families processing fungal cell wall	67	77	0
GH117	α -1,3-L-neoagarooligosaccharide hydrolase; α -1,3-L-neoagarobiase/neoagarobiose hydrolase	NA	66	30	7
GH125	exo- α -1,6-mannosidase	CAZy families processing fungal cell wall	64	29	8
GH110	α -galactosidase; α -1,3-galactosidase	NA	39	8	0
GH79	β -glucuronidase; hyaluronoglucuronidase; heparanase; baicalin β -	Pectinases	34	8	0

	glucuronidase; β -4-O-methyl-glucuronidase				
GH123	β -N-acetylgalactosaminidase; glycosphingolipid β -N-acetylgalactosaminidase	NA	27	7	0
GH66	cycloisomaltooligosaccharide glucanotransferase; dextranase	NA	24	8	0
GH62	α -L-arabinofuranosidase	Debranching enzymes and Oligosaccharide-degrading enzymes	8	8	0
Glycosyl Transferases (GTs)					
CAZy Family	Known CAZyme Activities		HM + G	HM	G2
GT81	NDP-Glc: glucosyl-3-phosphoglycerate synthase; NDP-Man: mannosyl-3-phosphoglycerate synthase; ADP-Glc: glucosyl-2-glycerate synthase		591	204	99
GT94	GDP-Man: GlcA- β -1,2-Man- α -1,3-Glc- β -1,4-Glc- α -1-PP-undecaprenol β -1,4-mannosyltransferase		249	191	91
GT66	dolichyl-diphosphooligosaccharide—protein glycotransferase; undecaprenyl-diphosphooligosaccharide—protein glycotransferase		205	69	25
GT89	β -D-arabinofuranosyl-1-monophosphoryldecaprenol : arabinan β -1,2-arabinofuranosyltransferase		47	51	0
GT74	α -1,2-L-fucosyltransferase		17	70	0
GT92	UDP-Gal: N-glycan core α -1,6-fucoside β -1,4-galactosyltransferase; UDP-Gal: β -galactoside β -1,4-galactosyltransferase		16	73	0
GT22	Dol-P-Man: Man6GlcNAc2-PP-Dol α -1,2-mannosyltransferase; Dol-P-Man: Man8GlcNAc2-PP-Dol α -1,2-mannosyltransferase; Dol-P-Man: Man2-GlcNAc-phosphatidylinositol α -1,2-mannosyltransferase; Dol-P-Man: Man3-GlcNAc-phosphatidylinositol α -1,2-mannosyltransferase		4	7	0
Carbon Binding Modules (CBMs)					
CAZy Family	Known CAZyme Activities		HM + G	HM	G2
CBM32	Binds to galactose, lactose, polygalacturonic acid and LacNAc (β -D-galactosyl-1,4- β -D-N-acetylglucosamine)		1502	643	229
CBM6	Bnds to cellulose, β -1,3-glucan, β -1,3-1,4-glucan and β -1,4-glucan.		858	269	4
CBM67	Binds to L-rhamnose		427	472	56
CBM35	Binds to xylan and β -galactan, and decorated soluble mannans and mannoooligosaccharides		416	117	13

CBM16	Binds to cellulose and glucomannan	358	196	80	
CBM12	Binds to chitin	284	83	33	
CBM57	binds to various glycosidases	227	167	0	
CBM22	Binds to xylan and has affinity with mixed β -1,3/ β -1,4-glucans	189	13	0	
CBM51	Attached to various enzymes from families GH2, GH27, GH31, GH95, GH98 and GH101. Binds to galactose and to blood group A/B-antigens	160	80	22	
CBM56	Binds to β -1,3-glucan	151	188	39	
CBM4	Binds to xylan, β -1,3-glucan, β -1,3-1,4-glucan, β -1,6-glucan and amorphous cellulose but not with crystalline cellulose.	148	306	5	
CBM40	Attached to various enzymes from GH 33 family. Binds to sialic acid	68	130	28	
CBM30	Binds to cellulose	32	68	0	
CBM53	Binds to starch	16	71	0	
CBM54	Binds to xylan, yeast cell wall glucan and chitin	11	4	2	
CBM38	Binds to inulin	9	12	0	
Carbohydrate Esterases (CEs)					
CAZy Family	Known CAZyme Activities	Known CAZyme Classification	HM + G	HM	G2
CE2	acetyl xylan esterase	Debranching enzymes and Oligosaccharide-degrading enzymes	248	235	61
CE12	pectin acetylesterase; rhamnogalacturonan acetylesterase; acetyl xylan esterase	Pectinases	218	281	25
CE15	4-O-methyl-glucuronoyl methylesterase	CAZy families processing fungal cell wall	106	228	15
Polysaccharide Lyases (PLs)					
CAZy Family	Known CAZyme Activities		HM + G	HM	G2
PL9	pectate lyase; exopolygalacturonate lyase; thiopeptidoglycan lyase		292	223	77
PL11	rhamnogalacturonan lyase; exo-unsaturated rhamnogalacturonan lyase		71	9	0
PL10	pectate lyase		63	67	0

PL8	hyaluronate lyase; chondroitin AC lyase; xanthan lyase; chondroitin ABC lyase	47	106	0
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2.7.6.6 Table (2.S6) Aromatic carbon degrading genes in HA reactors

Table 2.S6. 13 aromatic degradation genes had abundance values at least two times higher in the metagenomes containing humic acid (HA) versus those with only glucose (G).

KEGG belonging to the Aromatics Degradation (AD) category			
KEGG Module	HA + G	HA	G1
Benzoyl-CoA degradation, benzoyl-CoA => 3-hydroxypimeloyl-CoA [PATH:map01220 map00362]	166	563	14
Cumate degradation, p-cumate => 2-oxopent-4-enoate + 2-methylpropanoate [PATH:map01220 map00622]	38	96	0
KEGG KO	HA + G	HA	G1
KO:K13954===yiaY===alcohol dehydrogenase [EC:1.1.1.1]	1430	1313	644
KO:K05710===hcaC===3-phenylpropionate/trans-cinnamate dioxygenase ferredoxin subunit	858	1107	313
KO:K00483===hpaB===4-hydroxyphenylacetate 3-monooxygenase [EC:1.14.14.9]	529	1920	16
KO:K07535===badH===2-hydroxycyclohexanecarboxyl-CoA dehydrogenase [EC:1.1.1.-]	265	237	87
KO:K00055===E1.1.1.90===aryl-alcohol dehydrogenase [EC:1.1.1.90]	201	470	5
KO:K00002===AKR1A1, adh===alcohol dehydrogenase (NADP+) [EC:1.1.1.2]	70	80	11
KO:K04115===bcrD, badG===benzoyl-CoA reductase subunit D [EC:1.3.7.8]	47	121	0
KO:K04112===bcrC, badD===benzoyl-CoA reductase subunit C [EC:1.3.7.8]	41	45	3
KO:K16050===hsaD===4,5:9,10-diseco-3-hydroxy-5,9,17-trioxoandrost- 1(10),2-diene-4-oate hydrolase [EC:3.7.1.17]	29	63	5
KO:K07539===oah===6-oxocyclohex-1-ene-carbonyl-CoA hydrolase [EC:3.7.1.21]	25	83	5
KO:K04114===bcrA, badF===benzoyl-CoA reductase subunit A [EC:1.3.7.8]	15	97	0

KO:K01612===E4.1.1.61===4-hydroxybenzoate decarboxylase [EC:4.1.1.61]	11	65	0
KO:K04113===bcrB, badE===benzoyl-CoA reductase subunit B [EC:1.3.7.8]	11	48	2

2.7.6.7 Table (2.S7) Aromatic carbon degrading genes in HM reactors

Table 2.S7. Six aromatic degradation genes had abundance values at least two times higher in the metagenomes containing humin (HM) versus those with only glucose (G).

KEGG belonging to the Aromatics Degradation (AD) category				
KEGG Module	HM + G	HM	G2	
Benzoyl-CoA degradation, benzoyl-CoA => 3-hydroxypimeloyl-CoA [PATH:map01220 map00362]	14	39	0	
KEGG KO	HM + G	HM	G2	
KO:K13953===adhP===alcohol dehydrogenase, propanol-preferring [EC:1.1.1.1]	167 9	129 7	50 0	
KO:K14748===etbAa===ethylbenzene dioxygenase alpha subunit [EC:1.14.12.-]	56	30	0	
KO:K10676===tfdB===2,4-dichlorophenol 6-monooxygenase [EC:1.14.13.20]	27	47	1	
KO:K16049===hsaC===3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione 4,5-dioxygenase [EC:1.13.11.25]	19	14	4	
KO:K07534===badK===cyclohex-1-ene-1-carboxyl-CoA hydratase [EC:4.2.1.-]	15	17	0	
KO:K14749===etbAb===ethylbenzene dioxygenase beta subunit [EC:1.14.12.-]	13	7	0	

2.7.6.8 Table (2.S8-2.S12) Screening of PCC containing bins for aromatic carbon degrading genes

Table 2.S8. Screening of *Geobacter* published and draft genomes from this study for aromatic degradation gene pathways.

KO Module ID	KO Module Name	KO Term ID	Geobacter metallireducens GS 15	Geobacter sulfurreducens AM 1	Geobacter sulfurreducens KN400	HA34 Bin15 Geobacter	HM12 Bin4 Geobacter	HM56 Bin7 Geobacter sulfurreducens
M00548	Benzene degradation benzene => catechol [PATH:map01220 map00362]	KO:K16249	0	0	0	0	0	0
		KO:K16250	0	0	0	0	0	0
		KO:K16251	0	0	0	0	0	0
		KO:K16252	0	0	0	0	0	0
		KO:K16253	0	0	0	0	0	0
		KO:K16254	0	0	0	2	0	0
M00418	Toluene degradation, anaerobic toluene => benzoyl-CoA [PATH:map01220 map00623]	KO:K16255	3	0	0	0	0	0
		KO:K16256	1	0	0	0	0	0
		KO:K16257	1	0	0	0	0	0
		KO:K16258	1	0	0	0	0	0
		KO:K16259	1	0	0	0	0	0
		KO:K16260	1	0	0	0	0	0
		KO:K16261	1	0	0	0	0	0
		KO:K16262	1	0	0	0	0	0
		KO:K16263	1	0	0	0	0	0
M00541	Benzoyl-CoA degradation benzoyl-CoA => 3-hydroxypimeloyl-CoA [PATH:map01220 map00362]	KO:K16264	0	0	0	0	0	0
		KO:K16265	0	0	0	0	0	0
		KO:K16266	0	0	0	0	0	0
		KO:K16267	0	0	0	0	0	0
		KO:K16268	1	0	0	0	0	0
		KO:K16269	2	0	0	0	0	0
		KO:K16270	1	0	0	0	0	0
		KO:K16271	1	0	0	0	0	0
		KO:K16272	2	0	0	0	0	0

Table 2.S9. Screening of *Geothrix* published and draft genomes from this study for aromatic degradation gene pathways.

KO Module ID	KO Module Name	KO Term ID	Geothrix fermentans DSM 14018	HA34 Bin7 Geothrix fermentans	HM34 Bin13 Geothrix fermentans	Holophaga foetida TMBS4 DSM 6591	HM12 Bin10 Holophaga foetida	HM12 Bin22 Holophaga foetida	HM34 Bin11 Holophaga foetida	HM56 Bin28 Holophaga foetida
M00538	Toluene degradation toluene => benzoate [PATH:map01220 map00623]	KO:K15760	0	0	0	0	0	0	0	0
		KO:K15761	0	0	0	0	0	0	0	0
		KO:K15762	0	0	0	0	0	0	0	0
		KO:K15763	0	0	0	0	0	0	0	0
		KO:K15764	0	0	0	0	0	0	0	0
		KO:K15765	0	0	0	0	0	0	0	0
		KO:K00055	0	0	0	1	0	0	0	0
		KO:K00141	0	0	0	0	0	0	0	0
M00537	Xylene degradation xylene => methylbenzo	KO:K15757	0	0	0	0	0	0	0	0
		KO:K15758	0	0	0	0	0	0	0	0
		KO:K00055	0	0	0	1	0	0	0	0
		KO:K00141	0	0	0	0	0	0	0	0
M00568	Catechol ortho-cleavage catechol => 3-oxoadipate	KO:K03381	0	0	0	0	0	0	0	0
		KO:K01856	0	0	0	0	0	0	0	0
		KO:K03464	0	0	0	0	0	0	0	0
		KO:K01055	2	2	2	0	1	3	1	1
		KO:K14727	0	0	0	0	0	0	0	0
M00569	Catechol meta-cleavage catechol => acetyl-CoA / 4-methylcatechol => propanoyl-CoA [PATH:map01220]	KO:K00446	0	0	0	0	0	0	0	0
		KO:K07104	0	0	0	0	0	0	0	0
		KO:K10217	0	0	0	0	0	0	0	0
		KO:K01821	1	3	1	1	1	1	1	0
		KO:K01617	0	0	0	0	0	0	0	0
		KO:K10216	0	0	0	0	0	0	0	0
		KO:K18364	0	0	0	0	0	0	0	0
		KO:K02554	0	0	0	0	0	0	0	0
		KO:K18365	0	0	0	0	0	0	0	0
		KO:K01666	0	0	0	0	0	2	1	1
		KO:K18366	0	0	0	0	0	0	0	0
		KO:K04073	0	0	0	0	1	0	0	0

Table 2.S10. Screening of *Ignavibacterium*/*Melioribacter* published and draft genomes from this study for aromatic degradation gene pathways.

KO Module ID	KO Module Name	KO Term ID	<i>Ignavibacterium</i> album Mat9 16 JCM 16511	HA12 Bin10 <i>Ignavibacteriales</i>	HA12 Bin4 <i>Ignavibacteriales</i>	<i>Melioribacter</i> roseus P3M	HA12 Bin5 <i>Melioribacter</i> roseus
M00569	Catechol meta-cleavage catechol => acetyl-CoA / 4-methylcatechol => propanoyl-CoA [PATH:map01220 map00362 map00622]	KO:K00446	0	0	0	0	0
		KO:K07104	1	0	0	0	0
		KO:K10217	0	0	0	0	0
		KO:K01821	0	0	0	0	0
		KO:K01617	0	0	0	0	0
		KO:K10216	0	0	0	0	0
		KO:K18364	0	0	0	0	0
		KO:K02554	0	0	0	0	0
		KO:K18365	0	0	0	0	0
		KO:K01666	0	0	0	0	0
		KO:K18366	0	0	0	0	0
		KO:K04073	0	0	0	0	0

Table 2.S11. Screening of *Anaeromyxobacter* published and draft genomes from this study for aromatic degradation gene pathways.

KO Module ID	KO Module Name	KO Term ID	Anaeromyxobacter dehalogenans 2CP 1	Anaeromyxobacter dehalogenans 2CP C	Anaeromyxobacter sp Fw109 5	Anaeromyxobacter sp K	HA34 Bin5 s Anaeromyxobacter dehalogens	HA56 Bin37 s Anaeromyxobacter dehalogens
M00537	Xylene degradation xylene => methylbenzoate [PATH:map01220 map00622]	KO:K15757	0	0	0	0	0	0
		KO:K15758	0	0	0	0	0	0
		KO:K00055	0	0	0	0	0	0
		KO:K00141	0	0	0	0	0	1
M00538	Toluene degradation toluene => benzoate [PATH:map01220 map00623]	KO:K15760	0	0	0	0	0	0
		KO:K15761	0	0	0	0	0	0
		KO:K15762	0	0	0	0	0	0
		KO:K15763	0	0	0	0	0	0
		KO:K15764	0	0	0	0	0	0
		KO:K15765	0	0	0	0	0	0
		KO:K00055	0	0	0	0	0	0
		KO:K00141	0	0	0	0	0	1
M00568	Catechol ortho-cleavage catechol => 3-oxoadipate [PATH:map01220 map00362]	KO:K03381	0	0	0	0	0	0
		KO:K01856	0	0	0	0	0	1
		KO:K03464	0	0	0	0	0	0
		KO:K01055	0	0	0	0	0	1
		KO:K14727	0	0	0	0	0	0
M00569	Catechol meta-cleavage catechol => acetyl-CoA / 4-methylcatechol => propanoyl-CoA [PATH:map01220 map00362 map00622]	KO:K00446	0	0	0	0	0	0
		KO:K07104	0	0	0	0	0	0
		KO:K10217	0	0	0	0	0	0
		KO:K01821	1	1	1	1	0	0
		KO:K01617	0	0	0	0	0	0
		KO:K10216	0	0	0	0	0	0
		KO:K18364	0	0	0	0	0	0
		KO:K02554	0	0	0	0	0	0
		KO:K18365	0	0	0	0	0	0
		KO:K01666	0	0	0	0	0	1
		KO:K18366	0	0	0	0	0	0
		KO:K04073	0	0	0	0	0	0

Table 2.S12. Screening of *Prolixibacter* published and draft genomes from this study for aromatic degradation gene pathways.

KO Module ID	KO Module Name	KO Term ID	Prolixibacter bellariivorans ATCC BAA 1284	Prolixibacter bellariivorans JCM 13498	HM12 Bin18 Prolixibacter bellariivorans
M00569	Catechol meta-cleavage catechol => acetyl-CoA / 4-methylcatechol => propanoyl-CoA [PATH:map01220 map00362 map00622]	KO:K00446	0	0	0
		KO:K07104	0	0	0
		KO:K10217	0	0	0
		KO:K01821	0	0	0
		KO:K01617	0	0	0
		KO:K10216	0	0	0
		KO:K18364	0	0	0
		KO:K02554	0	0	0
		KO:K18365	0	0	0
		KO:K01666	0	0	1
		KO:K18366	0	0	0
		KO:K04073	0	0	0

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**Chapter 3. Colonization habitat controls biomass, composition, and metabolic activity of
attached microbial communities in the Columbia River hyporheic corridor**

3.1 Abstract

Hydrologic exchange plays a critical role in biogeochemical cycling within the hyporheic zone (interface between river water and groundwater) of riverine ecosystems. Such exchange may set limits on rates of microbial metabolism and impose deterministic selection on microbial communities that adapt to dynamically changing dissolved organic carbon (DOC) sources. This study examined the response of attached microbial communities (*in situ* colonized sand packs) from groundwater, hyporheic, and riverbed habitats within the Columbia River hyporheic corridor to “cross-feeding” with either groundwater, river water, or DOC-free artificial fluids. Our working hypothesis was that deterministic selection during *in situ* colonization would dictate the response to cross feeding, with communities displaying maximal biomass and respiration when supplied with their native fluid source. In contrast to expectations, the major observation was that the riverbed colonized sand had much higher biomass and respiratory activity, as well as a distinct community structure, compared to the hyporheic and groundwater colonized sand. 16S rRNA gene amplicon sequencing revealed a much higher proportion of certain heterotrophic taxa as well as significant numbers of eukaryotic algal chloroplasts in the riverbed colonized sand. Significant quantities of DOC were released from riverbed sediment and colonized sand, and separate experiments showed that the released DOC stimulated respiration in the groundwater and piezometer colonized sand. These results suggest that accumulation and degradation of labile POC within the riverbed is likely to release DOC which may enter the hyporheic corridor during hydrologic exchange, thereby stimulating microbial activity and imposing deterministic selective pressure on microbial community composition.

3.2 Introduction

The interface between river water and groundwater (i.e., the hyporheic zone) represents a zone of elevated microbial metabolism and biogeochemical cycling in lotic ecosystems. During periods of high river flow, fluid exchange between groundwater and river water can occur outside of the local riverbed hyporheic zone, extending into and beyond the riparian zone. The term hyporheic corridor (HC) (Stanford and Ward, 1993) is used to encompass this broader ecotone, which represents a temporally variable domain where groundwater and river waters mix in response to hydrologic dynamics. The HC is an example of a hot moment (McCracken et al., 2002; Gu et al., 2012), where mixing of river water and groundwater constituents occurs in high flow zones in which river water-groundwater exchange is most intense. The HC is a zone of confluence of organic materials and nutrients that has the potential to remove nitrogen inputs and processes large amounts of organic carbon (Trimmer et al., 2012), as well as environmental pollutants and even microbial pathogens (Tufenkji et al., 2002). A scientific understanding of the linkage between microbial communities and biogeochemical cycling within the HC is fundamental to forecasting how these critical systems will respond to dynamic changes in watershed and local-scale hydrology.

General theories for mechanisms that control the composition of microbial communities were recently expanded by Vellend et al. (2010) and Hanson et al. (2012), detailing the key components as speciation, organism dispersal, ecological selection, and ecological drift. These factors have been further classified into two classes, deterministic and stochastic. Deterministic selection is imposed by the environmental conditions that a microbial community is exposed to (Dumbrell et al., 2010; Ofiteru et al., 2010; Langenheder and Székely, 2011), whereas stochastic selection occurs due to perturbations such as probabilistic dispersal, random birth-death events,

and unpredictable disturbances (Chase and Myers, 2011). Determining how deterministic versus stochastic selection processes influence microbial community assembly is still poorly understood, and few studies have investigated these influences simultaneously. Stegen et al. (2012; 2013; 2015) conducted such an analysis on subsurface microbial communities at the U.S. Department of Energy Hanford 300 Area site in eastern Washington, USA (see Materials and Methods). Broadly speaking, the results suggested that microbial community assembly was controlled by deterministic selection linked to environmental conditions, dispersal related to hydrological transport, and random ecological drift as a result of stochastic population shifts. In another recent study at the Hanford 300 Area site, Stegen et al. (2016) found that during river water intrusion into the Columbia River HC, there was a loss of DOC linked to microbial metabolism in hyporheic sediments. This phenomenon triggered a shift from stochastic to deterministic selection and was associated with elevated abundances of microbial taxa that can degrade a broad suite of organic compounds. Parallel work by Graham et al. (2016, 2017) provided further evidence for the importance of hydrologic mixing-induced deterministic selection in controlling Hanford 300 Area HC microbial community composition, and linked such processes to changes in rates of microbial metabolism. However, these studies were performed *in situ* and hence the impact of dynamic mixing versus homogeneous environmental selection (where the selective environment remains spatially homogeneous within each successional stage, Dini-Andreote et al., 2015) is unknown.

This study employed an experimental approach to gain insight into how river water-groundwater mixing might be expected to impact Hanford 300 Area HC microbial communities. As discussed in Graham et al. (2016), the Hanford Reach of the Columbia River represents a model system in which to integrate community ecology and microbial metabolism in

a hydrobiogeochemical context. The biomass, composition, and metabolic activity of attached communities from three different habitats were investigated, including ones obtained (via *in situ* colonization of sterile sand; see below) from groundwater, riverbed, and hyporheic zone environments. To simulate river water-groundwater mixing events, each microbial community was “cross fed” with either sterile groundwater, sterile river water, or sterile DOC-free artificial versions of these fluids. Key to the experimental design was the exclusion of dispersal effects, and limitation of stochastic processes to disturbance during isolation and sampling of the colonized sand. Microbial biomass (ATP content), respiratory activity (resazurin reduction), and community composition (16S rRNA gene amplicon sequencing) were followed over time to assess the temporal responses to cross feeding.

Several prior studies have demonstrated habitat-driven selection of hyporheic microbial communities, including ones in systems with different hydrology, substratum properties, organic matter inputs, or gradients in heavy metal contamination (Feris et al., 2003; Findlay et al., 2003; Feris et al., 2004; Gao et al., 2005; Findlay et al., 2008; Mosher and Findlay, 2011; Febria et al., 2012; Febria et al., 2015). Based on this work and recent microbial community assembly studies in the Hanford 300 Area HC (Graham et al., 2016; Stegen et al., 2016; Graham et al., 2017), deterministic selection imposed by the varying DOC sources/amounts in the three different habitats was expected to lead to distinct microbial assemblages in the colonized sand materials. Stegen et al. (2016) showed (using high resolution Fourier transform-ion cyclotron resonance mass spectrometry) significant differences in the properties of DOC among groundwater, river water, and hyporheic zone fluids, which were correlated with both microbial respiration and community composition. We hypothesized that the microbial community response to experimental manipulation during the laboratory incubations would positively reflect such

deterministic selection, such that groundwater-derived microbial communities would show increased biomass and metabolism when exposed to filtered natural groundwater compared to filtered river water (or artificial carbon-free groundwater), whereas riverbed microbial communities would have higher biomass and metabolism when given filtered river water compared to filtered groundwater (or artificial carbon-free river water). The response of hyporheic zone microbial communities was an unknown, since that habitat experiences frequent mixing of groundwater and river water during flow reversals (see below). A secondary hypothesis was that microbial communities in the microcosms would undergo a temporal shift in response to cross feeding, i.e. a community exposed to a non-native fluid source would become similar to communities present in the corresponding non-native community. Experimental evaluation of these hypotheses provides new insight into the direct connection between habitat-driven deterministic selection and biogeochemical function in a large river HC.

3.3 Materials and methods

3.3.1 Hanford 300 Area hyporheic corridor

The Hanford 300 Area site is located on the north side of the Hanford Reach of the Columbia River, a ca. 80-km free-flowing section of river below Priest Rapids Dam. At this location the Columbia River flows through Pleistocene flood gravels of the Hanford formation which are underlain by Miocene-Pliocene fluvial and lacustrine sediments of the Ringold formation (Thorne et al., 2006). The Hanford formation sands and gravels have a high conductivity, and daily power generating operations at the Priest Rapids Dam can cause river stage fluctuations > 3 m within a 6 hour period (Tiffan et al., 2002) and common vertical fluctuations of > 1.5 m in a 24 hr period (Waichler et al., 2005). Larger seasonal changes in river stage can cause river water

intrusion for extended periods of time extending far into the groundwater zone traveling through underground areas of high conductivity known as paleo-channels (Arntzen et al., 2006; Johnson et al., 2012; Zachara et al., 2016, see Figure S1). Because the river stage is almost always changing, the Hanford Reach HC is extensive and dynamic with groundwater and river water mixing zones constantly changing spatially and temporally.

3.3.2 *In situ* sand pack incubation

Sand pack microcosms were constructed using approximately 80 cm³ of Hanford formation medium grade sand (Central Pre-Mix, Pasco, WA) in a modified 2 × 4.5", 18 mesh (1 mm) stainless steel infusers (R.S.V.P. International Inc.) plugged with Pyrex fiber glass (Corning Inc.). The sand packs were combusted at 450°C for 8 hr to remove any OC and to render them sterile. Sterile sand packs were incubated *in situ* for a six-week period in three distinct locations (habitats) within Hanford Area 300 corridor (see Fig. 3.S2): (1) within inland groundwater zone well 2-32 (screened within the upper 15 m of the Hanford formation) located ca. 200 m from the north edge of the river (denoted "Groundwater sand", or "GWS"); (2) within hyporheic zone piezometer P3 in the riverbed (screened over the upper 2 m of sediment) ca. 5 m from the river edge (denoted "Piezometer sand", or "PZS"); and (3) just below riverbed surface, a few meters from the river edge (denoted "Riverbed sand", or "RBS"). The photos in Fig. 3.S4 illustrate the Area 300 near-shore environment, including the placement of piezometer T3 and the gravel-covered riverbed. The GWS and PZS packs were incubated in a vertical array attached to stainless steel cable. The RBS packs were contained within perforated (1 mm holes) stainless steel tubes, which were subsequently emplaced within a shallow (ca. 10 cm deep) trench dug into the riverbed and covered by a layer of riverbed gravel to prevent illumination. Periodic visual

observation indicated that the tubes remained covered during the incubation period. The RBS packs were placed at an elevation below the seasonal low water level, and remained under water throughout the colonization period.

Based on continuous (hourly) measurements of specific conductivity (SpC) in the groundwater well compared to river water (see Fig. 3.S5), river water did not intrude into the groundwater well during the *in situ* sand pack colonization periods. Thus, the GWS microbial communities were not influenced by exposure to intruded river water during *in situ* colonization. This was an important to the experimental design, because it ensured that any response of GWS microbial communities to experimental fluid source manipulation was not confounded by fluid source changes during *in situ* incubation. In contrast to the groundwater well, the piezometers experienced frequent changes in SpC because of river stage driven flow reversals and associated river water-groundwater mixing (Fig. 3.S4).

3.3.3 Water sampling

Groundwater was collected from Hanford 300 Area well 2-32 and RW was collected adjacent to Hanford Area 300 Piezometer P3. The fluids were collected on the same day that the sand packs were retrieved after their six-week *in situ* incubation period. A peristaltic pump connected to 0.22 μ M polyethersulfone Sterivex filters (Millipore Co.) was used to collect water.

Approximately 2 L of fluid was passed through the filter prior to sample collection. The fluids were collected and stored in sterile, acid-washed Nalgene 2 L Teflon FEP bottles (Thermo Fisher). Testing showed that the containers did not leach significant amounts of DOC. All water feedstock solutions were stored at 4°C and measured for DOC at each time point to ensure no

loss of DOC during storage. Although no loss of DOC was observed, we did not monitor the composition of the DOC or check for bacterial growth.

3.3.4 Colonized sand incubation experiments

A conceptual overview of the colonized sand incubation experiments is provided in Fig. 3.S3. Two replicate “cross feeding” experiments were conducted with sand pack materials that were colonized *in situ* during September/October of 2014 or July/August of 2015. After the *in situ* colonization period, sand packs were retrieved and stored at 4°C. In the laboratory, replicate sand pack of each type were homogenized at room temperature. For Cross feed experiment 1 (“CF1”), 100 g portions of wet sand were placed in sterile (autoclaved) 150 ml glass jar reactors. The second cross feed experiment (“CF2”) was conducted with 50 g portions of wet sand. Most of the reactors were prepared in triplicate, with a few in duplicate. Reactors were loosely capped but not sealed to allow for an aerobic environment and stored in the dark. Feedstock solutions consisted of filtered-sterilized (0.2 µm) groundwater (GW), river water (RW), artificial river water (ARW) and artificial groundwater (AGW). The ARW and AGW compositions matched the inorganic contents of their natural counterparts but did not contain any DOC. A 30 mL volume of feed stock solution was added to each reactor at the start of the experiment. At evenly spaced time points over a 5-7 week period, all of the fluid phase was extracted with a carbon-free glass pipette and analyzed for DOC concentration. After fluid phase extraction, the sand was homogenized using a sterile metal spatula and weighed subsamples (0.5-1.5 g) were collected for ATP biomass, resazurin reduction, and 16S rRNA gene amplicon sequencing (see below). Reactors were then provided with fresh feed solution and stored in the dark at room temperature until the next sampling point. The volume of fluid added to the reactors decreased by 2 mL at

each sampling point in order to maintain a constant sand:water ratio relative to the amount of sand left in the reactor.

A third experiment denoted Desorption/Cross feeding (“DCF”) was conducted in March/April 2015 to test if DOC released from riverbed sediment or RBS packs could influence microbial biomass, respiratory activity and community structure. The concept underlying the DCF experiment was that deposition/accumulation of OC at the riverbed surface could lead to release of DOC that could subsequently move into the HC and stimulate microbial metabolism during periods of groundwater intrusion, which happen on a daily basis in the Hanford 300 Area (Fig. S5). Sand types and incubation techniques were identical to the cross feed experiments. Feed stock solutions used were river sediment desorbed DOC (denoted “Sed DOC”), RBS desorbed DOC (denoted “RBS DOC”), RW, and ARW. Desorbed DOC was obtained by adding 500 g of river sediment or RBS pack material to 500 mL of ARW. The sediment or sediment slurries were vortexed gently for 30 s and allowed to settle for 24 hr at 4°C. After settling, the fluid phase was removed and passed through a GF/F filter (Whatman) to remove particulates. We assumed that by maintaining the slurries at 4 °C (10-15 °C lower than *in situ* temperatures), most of the released DOC came from desorption as opposed to microbial attack on particulate organic carbon (POC) in the sediment or sand. The released DOC solutions were stored at 4°C and tested at each time point to ensure no loss of DOC content throughout the duration of the experiment.

3.3.5 Analytical methods

A detailed description of the analytical methods employed in this study is provided in Supplementary Text 1. Briefly, DOC concentrations were determined with a high-temperature

combustion carbon analyzer. ATP content and resazurin reduction were used as measures of microbial biomass and aerobic respiration, respectively. Recent studies have demonstrated the utility of resazurin reduction to assess aerobic respiration as a function of microbial biomass and community composition in stream and HC environments (Stanaway et al., 2012; Graham et al., 2016). The linear mixed modeling (LMM) package in R (TeamR, 2016) was used to analyze the biomass and respiration data. DNA was extracted from sand microcosm samples using standard commercial kits as previously described (Converse et al., 2015). 16S rRNA gene amplicon libraries (107 total) were generated using the 515F-806R universal prokaryotic primers (Caporaso et al., 2011). The amplicons were sequenced on an Illumina MiSeq instrument at Pacific Northwest National Laboratory. Raw sequence data have been deposited in the NCBI Sequence Read Archive (SRA) under accession numbers SAMN07191787-SAMN07191821 (CF1), SAMN07191822-SAMN07191859 (CF2), and SAMN07191860-SAMN07191893 (DCF). The sequences were processed through the QIIME pipeline (Caporaso et al., 2010). The software package Primer (version 7.0) was used for non-metric dimensional scaling (NMDS) analysis of microbial community composition, and for analysis of similarity (ANOSIM) comparisons of community composition among different reactor treatments. Dried colonized sand materials were examined using a Hitachi S-3400 variable pressure scanning electron microscope (SEM).

3.4 Results

3.4.1 Microbial biomass and respiration

The initial cross feeding experiment (CF1) showed that biomass (ATP content) and aerobic respiratory activity (resazurin reduction) of the RBS community were consistently higher than communities associated with the other two sand types irrespective of the fluid source (Fig. 3.1). This was true even for the ARW and AGW reactors that received negligible DOC (< 0.1 ppm) input. Similar results were observed in the second experiment (CF2; see Fig. 3.S6). No consistent increases or decreases in biomass values related to feed solution were observed throughout the experiments. The GWS and PZS reactors that received RW with higher DOC content (ca. 1.2 ppm) showed very little separation in biomass and respiration from those receiving GW (ca. 0.3 ppm DOC), AGW, or ARW. Aerobic respiration rates remained relatively stable throughout both experiments, except for the RBS reactors where a decreasing trend was observed over time (Figs. 3.1E and 3.S5E). The same relative order of biomass and respiration for the different sand types was observed in the DCF experiment (Fig. 3.2), with RBS showing much higher values than PZS and GWS, and PZS showing higher values compared to GWS. Biomass values in reactors exposed to Sed DOC or RBS DOC were not systematically higher than in reactors receiving RW or ARW (Fig. 3.2A-C). However, respiration rates were elevated in GWS and (to a lesser extent) PZS exposed to Sed DOC and RBS DOC compared to RW or ARW (Fig. 3.2E,F).

Statistical (LMM) comparison of the two replicate cross feeding experiments using the lme4 package in R (Table 3.1) confirmed that sand type was the greatest source of variation for both biomass and respiration. F-tests with p values lower than 0.05 were considered significant. Time was a significant factor only for CF2 microbial respiration rates (which decreased with

time); this was not the case for CF2 microbial biomass or for CF1 respiration. The significance of year for the biomass data was due to CF2 (2015) having higher overall values than CF1 (2014). Although fluid type was a statistically significant factor for the combined CF1/CF2 biomass data and the CF2 respiration data, these differences neither supported nor refuted the overall hypothesis of this study (see below). LMM results for the DCF experiment revealed that sand type and time were again the most significant sources of variation for biomass content. For respiration, fluid type was a marginally significant source of variation, with sand type and time and still the leading sources of variation.

Pairwise comparisons revealed a few cases where fluid type had a statistically significant impact on microbial biomass or respiration. For the two cross feeding experiments (Table 3.S1), biomass values showed small (typically less than 2-fold) but significant differences among water types that were in general unexpected relative to the working hypothesis of the study that a given sand type would perform best when provided fluid from its native environment. For example, for CF2, the biomass for RBS receiving GW was higher than RBS receiving RW; and GWS receiving RW was higher than GWS receiving GW. Likewise, for respiration in CF2, rates were higher for GWS with RW than GW. Other significant differences were related to differential responses to AGW or ARW compared to non-artificial fluids. For the DCF experiment (Table 3.S2), GWS reactors had significantly higher biomass when exposed to Sed DOC and RBS DOC compared ARW. Also, both GWS and PZS respiration rates were significantly higher in the presence of Sed DOC and RBS DOC compared to RW.

3.4.2 DOC dynamics

DOC data for CF1 and CF2 showed an unexpected trend for the RBS reactors (Fig. 3.3), where DOC concentrations exceeded values expected for the given feed stocks. These results indicate that DOC was released into solution from the RBS between sampling points. PZS reactors containing GW and AGW displayed a smaller but detectable amount of DOC release. In contrast, GWS and PZS reactors containing RW showed a slight loss of DOC. Substantial release of DOC from RBS was again observed in the DCF experiment (Fig. 3.4). A loss of DOC occurred in GWS exposed to RW, Sed DOC and RBS DOC; likewise, PZS exposed to Sed DOC and RBS DOC showed loss of DOC.

3.4.3 Microbial community composition

NMDS analysis was conducted on the 16S rRNA gene amplicon libraries to assess differences in microbial community structure among the different sand reactor types for the three cross feeding experiments. Libraries from different fluid treatments were grouped together for each time point during each experiment, because ANOSIM analysis showed that (with the exception of GWS in the DCF experiment; see below) fluid type did not significantly influence community composition (Table 3.2). The results revealed large differences in community structure among the different reactor types for each of the three experiments (Fig. 3.5). Although the RBS and PZS communities grouped closer to one each other than either did to the GWS, ANOSIM nevertheless showed that the RBS and PZS communities were distinct (Table 3.2). This finding is not surprising in light of the difference in alpha diversity among the sand reactor communities (Table 3.S3).

Phylum-level analysis revealed major differences in sand reactor community composition for each of the three experiments (Fig. 3.6; Table 3.3). We found it insightful to approach this analysis by examining Proteobacteria and non-Proteobacteria phyla in two separate groupings. Proteobacteria were more abundant in the GWS (70-75 % of total reads) compared to the RBS (50-55 %) and PZS (40-45 %) reactors, particularly in CF2 and DCF. However, non-Proteobacterial phyla increased over time in the GWS reactors, whereas the relative abundance of Proteobacteria vs. non-Proteobacteria showed only minor change over time in the RBS and PZS reactors. *Alpha*, *Beta*, and *Gammaproteobacteria* were the dominant Proteobacterial classes in all reactors, with smaller but substantial amounts of *Deltaproteobacteria* in the RBS and PZS reactors (Fig. 3.7).

The families represented within the main Proteobacterial classes and selected non-Proteobacterial phyla for each sand type were summarized for each experiment (Tables 3.S4-3.S6). Based on the phylum-level breakdown in Fig. 6, the major non-Proteobacterial phyla selected for analysis included *Acidobacteria*, *Bacteroidetes*, *Nitrospirae*, *Planctomycetes*, and *Verrumicrobia*, together with *Crenarchaeota* from the class *Thaumarchaeota*. For the family-level analysis, the number of amplicon reads for each family was summed across all water types for a given sand type and divided by the total number of reads for the corresponding phylum. Only families that comprised $\geq 0.1\%$ of total phylum reads were considered in the analysis.

A detailed treatment of the phylum/family level analysis is provided in Supplementary Text 2. A key result is that certain taxa were much more abundant in the RBS and (to a lesser extent) PZS reactors compared to GWS, including non-Proteobacterial phyla with known heterotrophic physiologies (e.g. *Acidobacteria*, *Planctomycetes*, and *Verrumicrobia*), and *Deltaproteobacteria* with unique metabolic properties (fermentative *Syntrophobacteraceae*,

anaerobic respiratory *Geobacteraceae*, and various diverse heterotrophic taxa from the order *Myxococcales*). Another major distinction was the presence of different types of “*Cyanobacteria*” sequences in RBS and PZS versus GWS. BLAST analysis revealed that the dominant “*Cyanobacteria*” OTUs in RBS (90-95 % of total “*Cyanobacteria*” sequences) and PZS (72-84 %) actually corresponded to chloroplasts from the order stramenopiles, a large group of eukaryotic algae that includes diatoms. In contrast, the dominant (88-94 %) “*Cyanobacteria*” OTU in GWS was related to class ML635J-21, which Soo et al. (2014) showed is related to the *Melainabacteria*, a novel group of non-photosynthetic (non-PS) *Cyanobacteria* recently identified in the human gut and in groundwater from Rifle, CO (DiRienzi et al., 2013). The role of non-PS *Cyanobacteria* in GWS is unknown, whereas the chloroplast 16S rRNA gene sequences (as well as SEM imaging; Fig. 3.S7) provide unambiguous evidence for the presence of eukaryotic algal biomass in the RBS and PZS.

Although ANOSIM indicated that fluid source in general did not have a significant influence on overall microbial community composition, we examined the major Proteobacterial and non-Proteobacterial taxa mentioned above to look for possible case-by-case differences in abundance as a function of fluid source. The search revealed no systematic variations in the relative abundance of different Proteobacteria classes in CF1 and CF2 (data not shown). For non-Proteobacteria in CF1 and CF2, GWS fed with RW showed a modest increase in *Planctomycetes* and *Verrumicrobia* over time (Fig. 3.S8). For DCF, a similar stimulation of *Planctomycetes* and *Verrumicrobia* occurred in GWS reactors in response to RW, Sed DOC, and RBS DOC compared to ARW (Fig. 3.S9B,C). Even more striking was the several-fold increase in *Bacteroidetes* abundance in GWS in response to Sed DOC and RBS DOC (Fig. 3.S9A). The latter results likely account for the significant ANOSIM R value for GWS in DCF (Table 3.2).

No systematic variations in Proteobacterial class abundance were observed for DCF (data not shown).

3.5 Discussion

3.5.1 Habitat selection of attached HC microbial communities

Although the sand packs did not reproduce *in situ* groundwater, riverbed, or hyporheic zone sediment properties, the sand is from the same geological (Hanford) formation, and provided a clean substratum on which microbial community development as a function of *in situ* habitat could be assessed (Graham et al., 2016). NMDS analysis of the 16S rRNA gene amplicon libraries indicated that distinct attached microbial communities did in fact arise in the three different *in situ* colonization habitats (Fig. 3.5). The RBS and PZS were more diverse (Table 3.S3), and contained a much larger proportion of non-Proteobacterial taxa (Fig. 3.6). These results clearly demonstrate that habitat imposed deterministic selection on attached microbial communities. This basic finding agrees with a prior high-throughput (pyrosequencing) 16S rRNA gene amplicon analysis of microbial communities in contrasting field and laboratory hyporheic zone sediments (Li et al., 2012), and with a wealth of ongoing studies in various lotic ecosystems (see Battin et al. (2016) for an excellent review). However, our results do not support the hypothesis that, because of deterministic selection during colonization, microbial communities would show maximal biomass and metabolic activity when exposed to their native fluid source as opposed to alternative DOC sources (Figs. 3.1, 3.2, and 3.S5). Instead, it appears that colonized sand type, as a reflection of habitat-driven deterministic selection during colonization, exerted primary control on biomass and metabolic activity (see LMM results in Table 3.1). Although in some cases fluid source had a significant influence on biomass or

respiration, the differences did not indicate that a given sand type had higher biomass or respiration when provided with fluid from its native environment (see pairwise comparisons in Table 3.S1). The overwhelming influence of *in situ* colonization habitat was also reflected by the fact that (with a few notable exceptions; see below) microbial community composition generally showed no change in response to fluid source manipulation (see ANOSIM results in Table 3.2). Despite some internal reorganization of communities in the sand reactors (in particular GWS; see Figs. 3.6 and 3.7), ANOSIM analysis showed that the three different sand type communities remained distinct (p values < 0.004) over time during each of the experiments (data not shown).

Patterns in the relative abundance of dominant taxa provide insight into potential linkages between community composition and the physiological function of those communities in the different HC habitats. A subset of these taxa was present in comparable numbers in all three habitats, whereas others showed major variations connected to differential organic matter input and metabolism. Metabolically diverse heterotrophic Proteobacteria (Kerstens et al., 2006) were abundant in all the colonized sands, which is consistent with other hyporheic microbial communities (Feris et al., 2003; Li et al., 2012; Battin et al., 2016) as well as previous Hanford 300 Area studies (Graham et al., 2016; Stegen et al., 2016; Graham et al., 2017), and indicates a central role of these organisms in OC decomposition across the HC. The abundance of *Pseudomonadaceae* relative to all *Gammaproteobacteria* was particularly high in the GWS, which is consistent with a previous sand colonization study in Hanford 300 Area groundwater (Converse et al., 2015), where *Pseudomonadaceae* accounted for 60-80 % of all Proteobacteria. In addition, incubation experiments with Hanford 300 Area sediments indicated a key role for *Pseudomonadaceae* and other heterotrophic taxa in OC metabolism (Percak-Dennett and Roden, 2014). Non-Proteobacterial heterotrophs from class *Bacteroidetes* were also abundant in all sand

types (3-14 % of all reads across the three experiments), suggesting these organisms are well-adapted to the diverse suite of OC sources present in the Hanford 300 Area HC (Stegen et al., 2016). Likewise, organisms from the genus *Nitrospira*, a well known aerobic nitrite-oxidizing taxon (Daims, 2014), accounted for a few percent of total reads in each sand type and are likely to have been involved in nitrogen cycling across the HC. A similar conclusion applies also to the (presumed) aerobic ammonium-oxidizing *Thaumarchaeota* families *Cenarchaeaceae* and *Nitrososphaeraceae* (Oshiki et al., 2016), which is consistent with a recent study that revealed substantial numbers of *Thaumarchaeota* and inferred their role in nitrogen cycling within the Hanford 300 Area HC (Graham et al., 2016).

The distribution of other taxa showed clear differences across the three HC habitats. The dominance of aerobic predatory (Bdellovibrionaceae, ref. Rotem et al., 2014) and bacteriolytic (Haliangiaceae, ref. Garcia and Müller, 2014) within GWS *Deltaproteobacteria* suggest that these taxa likely thrive in Hanford 300 Area groundwater through consumption of other heterotrophic microbial biomass. The much higher abundance of other types of *Deltaproteobacteria* in RBS (and to a lesser extent PZS) compared to GWS is an obvious distinction that is almost certainly related to *in situ* metabolic function. In particular, the presence of fermentative (e.g. *Syntrophobacteraceae*) and anaerobic respiratory (e.g. *Geobacteraceae*) point to microbial populations in RBS and PZS that thrive under low-O₂ or anoxic conditions, e.g. in anaerobic microsites within an otherwise bulk aerobic environment. This idea is consistent with the conclusion that the high biomass and respiration of RBS and (to a lesser extent) PZS compared to GWS resulted from the input of fresh river-derived OC (see below); and with the results of an earlier study that provided geochemical evidence for anoxic conditions and cultivation-based evidence for the presence of considerable numbers of anaerobic respiratory

taxa in Hanford 300 Area riverbed sediments (Moser et al., 2003). Among non-Proteobacterial phyla, organisms from the *Acidobacteria*, *Planctomycetes*, and *Verrumicrobia* phyla were several-fold more abundant in RBS and PZS compared to GWS (Table 3.3). The dominant taxa detected within these phyla (see Supplementary Text 2 and Tables 3.S4-3.S6) were all relatives of heterotrophic organisms whose prominence points to enhanced (relative to GWS) OC decomposition in RBS and PZS reactors by these phyla.

3.5.2 Riverbed hotspot for organic carbon processing

In addition to RBS displaying 5-10 fold higher microbial biomass and respiration compared to GWS and PZS (Figs. 3.1, 3.2, and 3.S5), reactor fluid phase analyses indicated that DOC was released into solution from RBS during the incubation experiments (Figs. 3.3 and 3.4). We speculate that the released DOC originated from DOC and/or particulate organic carbon (POC) that accumulated in the RBS (e.g., via natural depositional and sorption processes at the riverbed surface) during *in situ* incubation. The input of fresh POC was evidenced by the presence of eukaryotic algal chloroplast sequences in the 16S rRNA gene amplicon libraries (Fig. 3.6), as well direct microscopic observation of diatom frustules in the RBS (Fig. 3.S7). We were unable to detect (by high-temperature combustion in an elemental analyzer) differences in the POC content of the different colonized sands from CF1 (all values were near the detection limit of ca. 0.1 % C; data not shown); however, the presence of bioavailable OC in the RBS was clearly reflected by their much higher biomass and respiration rates. In addition, visual inspection of the RBS reactors at the start of CF1 revealed the presence of layer of fine-grained materials that settled onto the sand surface after mixing and addition of filtered RW at the start of the experiment (Fig. 3.S10B). Such materials were absent in the GWS and present in much lower

abundance in the PZS reactors (Fig. 3.S10A,C). Although we cannot exclude the possibility that some of the fresh POC in RBS came from photosynthesis during colonization, the sand packs were contained within a perforated metal tube that remained covered with riverbed sand and gravel during *in situ* incubation. Based on this, we conclude that it is unlikely that the algal biomass that entered the RBS came from the water column and/or from periphyton material that was mobilized by turbulence near the riverbed surface. Even if some of the algal biomass came from *in situ* photosynthesis within the buried, tube-enclosed sand packs, it seems likely that such photosynthesis would also take place within the upper several cm of the native riverbed material. The key point is that fresh primary production entered into the upper several cm of the riverbed surface and had a major impact on microbial community composition and respiratory activity.

The release of DOC in the RBS cross feeding reactors motivated us to test whether or not riverbed surface sediment and riverbed colonized sand would release DOC during short-term (24 hr) “desorption” experiments, and if so whether such released DOC would stimulate microbial biomass and metabolism in colonized sand packs. Previous studies have documented the influence of labile DOC addition on the composition and/or metabolism of stream hyporheic microbial communities (e.g. Findlay et al., 2003; Foreman and Covert, 2003; Sinsabaugh and Covert, 2003; Li et al., 2012; Wiegner et al., 2015), although only one of these studies (Li et al., 2012) benefited from the large-scale 16S rRNA gene amplicon sequencing approach employed here. Two potential mechanisms for DOC release during the “desorption” experiments include (i) actual desorption from surface binding sites on sediment/sand particles, and (ii) release associated with POC decomposition. There is ample evidence for sorption of DOC to soil and sediment particles surfaces (Dunnivant et al., 1992; Tao and Lin, 2000; McCracken et al., 2002; Moore and Turunen, 2004; Remington et al., 2007; Perez et al., 2011), and for the release of

sorbed DOC during hydrologic disturbance (Komada and Reimers, 2001; Koelmans and Prevo, 2003; Schelker et al., 2013). And numerous studies have documented the release of DOC during POC decomposition in soils and sediments (e.g. Lehman and Mills, 1994; Godde et al., 1996; Kalbitz et al., 2002; Bengtson and Bengtsson, 2007; Blodau et al., 2007; Zhao et al., 2007; Planer-Friedrich et al., 2012). Although POC decomposition cannot be ruled out as a mechanism for DOC release, because the experiments were conducted at 4 °C and for a relatively short time period, it seems likely that much of the DOC release was via desorption. Given the relatively long-term nature of the cross feeding experiments compared to the desorption experiment, it is possible that at least some of the DOC released during the former originated from POC decay. Unfortunately, the temporal resolution of reactor fluid sampling during the cross feeding experiments was insufficient to assess the time frame on which DOC was released from the RBS, i.e. whether it was released quickly via desorption after sampling and fluid replacement, or more slowly as a result of POC decay. However, the key point is that the RBS contained sources of OC that were mobilized into solution during incubation.

The addition of “desorbed” DOC had no significant impact on microbial biomass or respiration in the RBS reactors (Fig. 3.2B,E). However, respiration rates in both the GWS and PZS reactors showed a significant positive response (2-3 fold increase) to freshly desorbed DOC sources compared to RW (Fig. 3.2E,F; Table 3.S2). In addition, taxa such as *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia* – that were abundant members of the non-Proteobacterial phyla in RBS and PZS – showed an increase in GWS in response to RW, Sed DOC, and RBS DOC (Figs. 3.S7 and 3.S8). The stimulation of the such types of heterotrophic taxa by increased OC input agrees with hyporheic sediment column experiments where different levels of DOC input were used to assess the influence of OC loading on microbial assemblages (Li et al., 2012),

although the stimulated phyla differed with *Verruimicrobia* being the only major overlap. The response by GWS and PZS to river-derived DOC input is also consistent with Stegen et al. (2016) who showed that rising river stage transported bioavailable DOC into the hyporheic zone, thereby stimulating respiration. Likewise, the stimulation of selected heterotrophs by DOC input agrees with the inference by Graham et al. (2016) that river water intrusion into the Hanford 300 Area HC selected for increased abundance of heterotrophic taxa (although not the same ones observed in this study). However, despite the microbial community and biogeochemical response of GWS and PZS to the freshly desorbed DOC, these respiration rates were far below those in the RBS reactors. This finding, together with the insignificant impact of desorbed DOC on RBS respiration rates, suggest that OC accumulation and metabolism played a key role in RBS biomass accumulation during *in situ* colonization and respiration during laboratory incubation.

The DCF results support the idea that input of OC from the overlying riverbed was at least partially responsible for the relatively high biomass and respiration of the PZS compared to GWS in the cross feeding experiments. The PZS packs were incubated in the hyporheic zone ca. 1 m below the riverbed surface, and therefore had less contact with materials in the river water. However, some fine-grained material was evident in the PZS reactors (Fig. 3.S10C), less than in the RBS reactors, but more than in the GWS reactors which showed no such layer (Fig. 3.S10A). The PZS 16S rRNA gene amplicon libraries also contained detectable amounts of chloroplast sequences (Fig. 3.6). These results suggest that the near-surface hyporheic zone received at least some input of POC from the river, which could account for the presence of a microbial community more similar to RBS than GWS (Figs. 3.6 and 3.7). Although we cannot completely exclude the possibility that some of the algal biomass in RBS came from photosynthesis during colonization, the sand packs were contained within a (perforated) metal tube that remained

covered with riverbed sand and gravel during *in situ* incubation. Thus it seems likely that the algal biomass that entered the RBS came from the water column and/or from periphyton material (see Fig. 3.S4C,D) that was mobilized by turbulence near the riverbed surface. In the case of PZS, the algal biomass detected could not have come from *in situ* photosynthesis, but rather must have entered the hyporheic zone via downward transport from the riverbed.

3.5.3 Biogeochemical implications

The Columbia River represents a premier example of how river stage variations can lead to large-scale mixing of river water and groundwater. Forecasting models predict an increase in large rain events (>50 mm/d) and total precipitation in North America (see Trenberth, 2011 and references therein). Such increases in precipitation are expected to escalate the occurrence and intensity of river water-groundwater mixing events (Jones and Holmes, 1996; Boulton et al., 1998; Sophocleous, 2002), leading to a higher potential for development of hot spots and moments within the HC. Although progress has been made in linking microbial community dynamics with biogeochemistry in hyporheic zone environments (see Graham et al., 2016; Stegen et al., 2016 and references therein), mixing-induced variations in microbial community structure and function remain a poorly understood component of riverine biogeochemical function (Marmonier et al., 2012).

The central goal of this study was to gain experimental insight (through fluid source manipulation, or “cross feeding”) into how hydrologic exchange may be expected to influence the size, activity, and composition of microbial communities within the nearshore hyporheic zone and the broader HC. The initial hypothesis underlying the work was related to the potential influence of deterministic selection imposed by DOC sources (during *in situ*

colonization) on the community response to fluid source manipulation. Our results, however, did not concur, and instead pointed to different a first-order impact of hydrodynamics on HC microbial communities, namely the potential for depositional processes and hydrologic exchange to drive input of labile OC to the riverbed. This process led to much higher microbial biomass and respiration in riverbed colonized sand compared to sand colonized in the hyporheic zone or in groundwater adjacent to the river. In retrospect these results are perhaps not surprising, as it is well-recognized that on a volume-normalized basis by far the largest amount of riverine biogeochemical activity takes place either at or just below the riverbed surface (see ref. Trimmer et al., 2012 for review). This is because the concentration and residence time of organic matter and associated microbial communities in riverbed sediments is typically several orders of magnitude greater than in the overlying water (Findlay, 2010; Tank et al., 2010). From a hydrological perspective, permeable riverbed sediments can be considered as reactive sieves or filters which can capture entrained suspended particles and dissolved materials during river water infiltration (Findlay, 1995; Battin and Sengschmitt, 1999; Hatch et al., 2010; Battin et al., 2016). Thus, riverbed sediments are a focal point for material carried by the river, concentrating both allochthonous and autochthonous organic matter (Webster et al., 1999; Cole et al., 2007; Tank et al., 2010). Such organic matter inputs provide electron donors to support an array of respiratory and fermentative reactions, as reflected by the distinct RBS microbial communities reported in this study, and the results of an earlier cultivation-based study in Hanford 300 Area hyporheic sediments (Moser et al., 2003).

Microbial biomass and respiration in the PZS, though much lower than in the RBS, were higher than in GWS, and our experimental results (i.e. the DCF experiment) suggest that DOC released from riverbed sediment and colonized sand could stimulate the respiration of PZS and

GWS microbial communities. This finding agrees with seminal studies of hyporheic zone metabolism (Jones, 1995; Jones et al., 1995; Jones and Holmes, 1996), and highlights a key biogeochemical implication of this study (illustrated in Fig. 3.S11), that input of POC and its degradation within the near-surface riverbed is likely to drive an influx of labile DOC into the hyporheic zone, and potentially the broader HC depending on the intensity of river water-groundwater exchange. Our results are consistent with the conclusion of Graham et al. (2016) that microbial community structure and function in the Hanford 300 Area HC is the result of deterministic selective pressures that come into play during hydrologic exchange. It seems likely that the broad range of physiologies in the RBS and PZS communities means they have the potential to take advantage of frequent shifts in prevailing biogeochemical conditions, such as changes in OC supply and attendant shifts in redox conditions. In comparison, the GWS had a less diverse range of physiologies represented and was likely adapted to relatively stable in-shore biogeochemical conditions. In light of these observations, one might hypothesize that hyporheic zone (including riverbed) communities are more resilient to environmental perturbation compared to those in more stable groundwater systems. Further studies in HC environments such as the Hanford 300 Area are required to assess the relationship between environmental stability and microbial community/biogeochemical resilience in large river ecosystems. In particular, the potential influence of colonization time on the response of attached communities to environmental perturbation needs to be assessed over a range of time scales that encompasses those of the perturbations, e.g. the large seasonal shifts in hydrobiogeochemical conditions that are known to take place in the Hanford Reach of the Columbia River (Graham et al., 2016; Stegen et al., 2016) and other large river ecosystems.

3.6 Acknowledgements

We thank Emily Graham (PNNL) for review of the manuscript and acknowledge Shaomei He and Jacqueline Meija (UW-Madison) for helpful discussions.

3.7 Funding information

This work was supported by the U.S. Department of Energy, Office of Biological and Environmental Research, Subsurface Biogeochemical Research Program through the SBR Scientific Focus Area at the Pacific Northwest National Laboratory (PNNL).

3.8 Figures

3.8.1 Figure (3.1) ATP and resazurin reduction data for Cross feed experiment 1

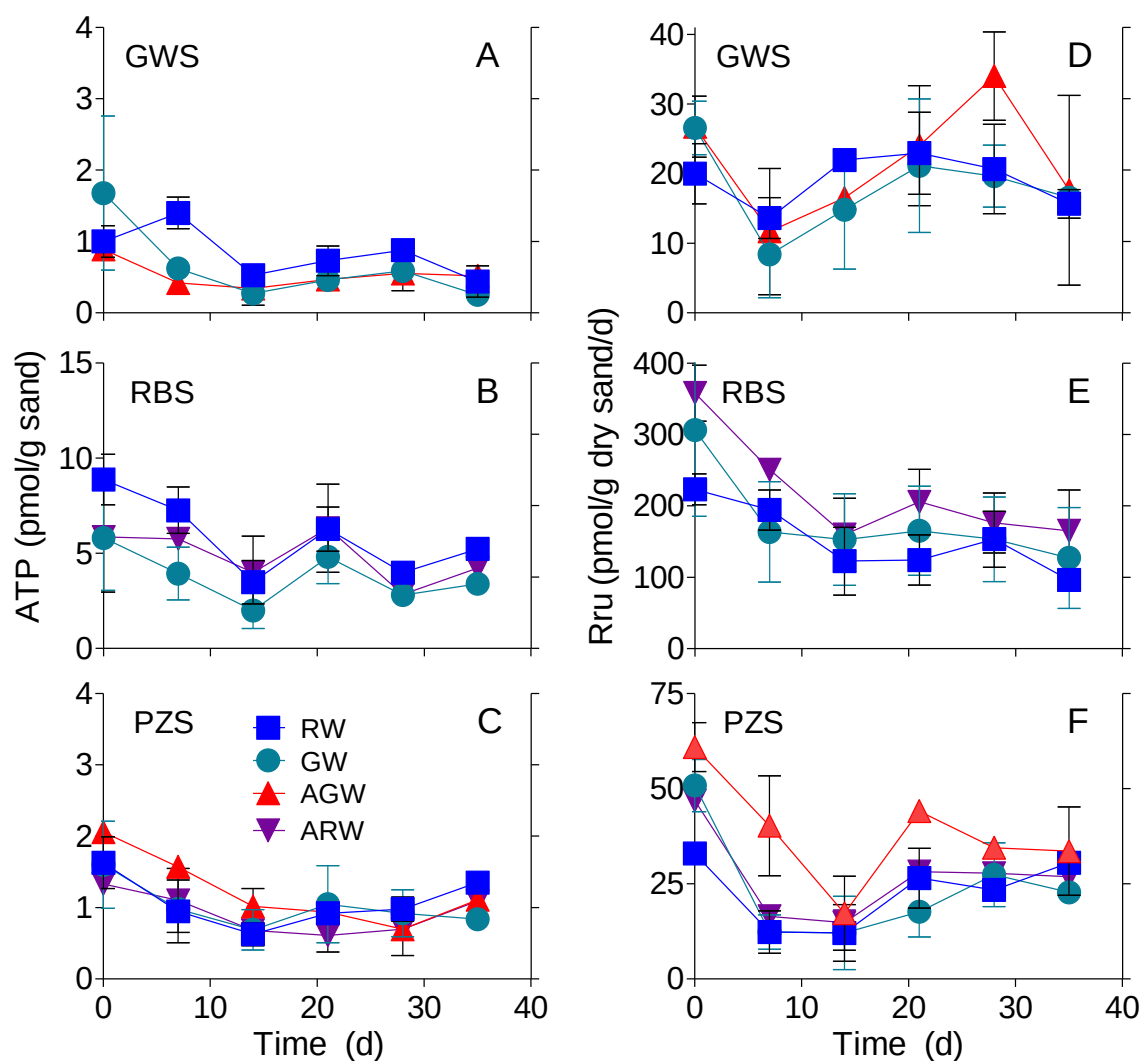


Figure 3.1 ATP content and rates of resazurin transformation to resorufin for the groundwater (A, D), riverbed (B, E), and piezometer (C, F) sand colonized reactors for Cross feed experiment

1. Each data point shows the mean $\pm$ SD or range of triplicate or duplicate

3.8.2 Figure (3.2) ATP and resazurin reduction data for Desorption/Cross feed experiment

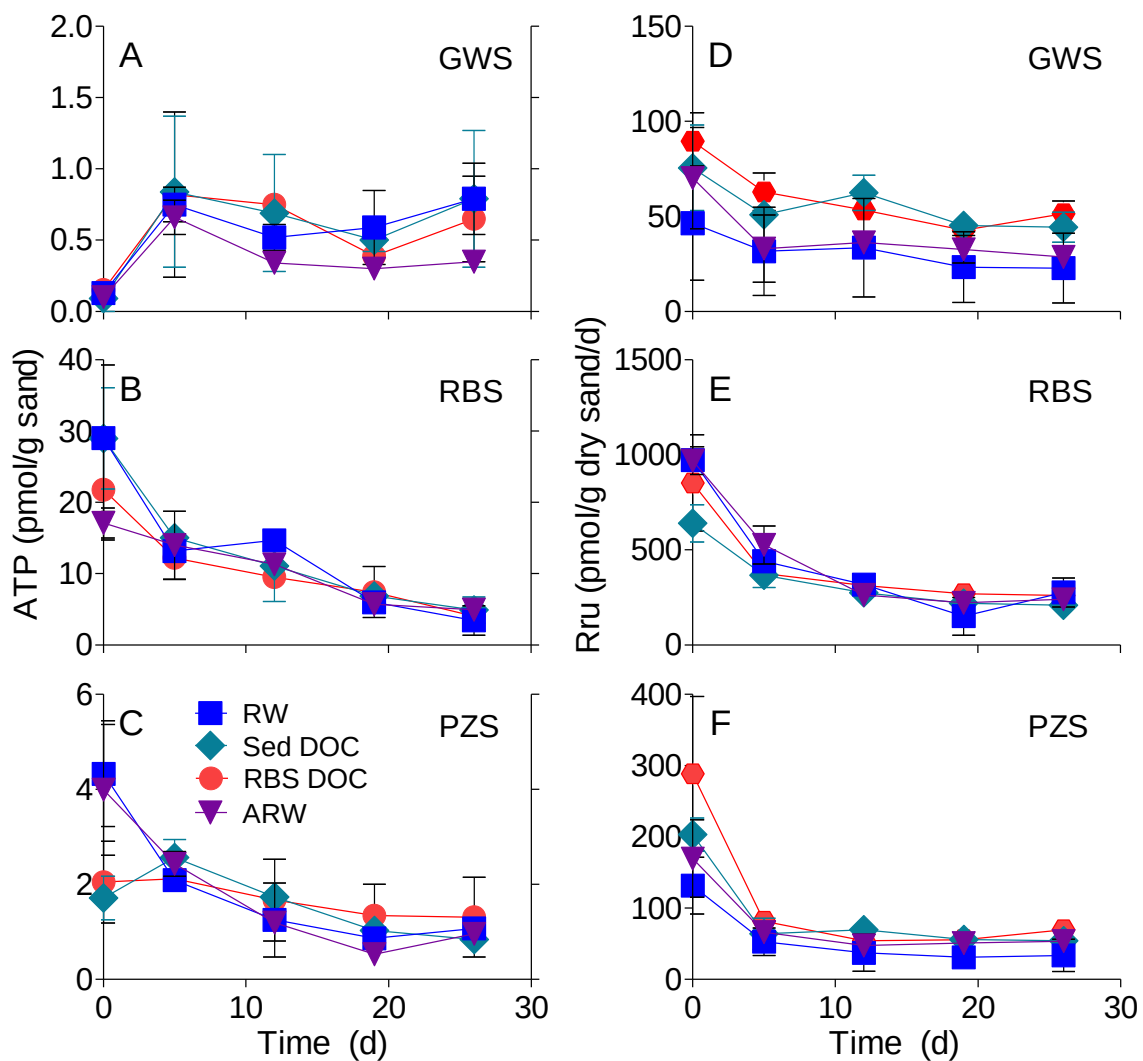


Figure 3.2 ATP content (A-C) and rates of resazurin transformation to resorufin (D-F) for the Desorption/Cross feed experiment.

3.8.3 Figure (3.3) DOC data for Cross feed 1 and 2 experiments

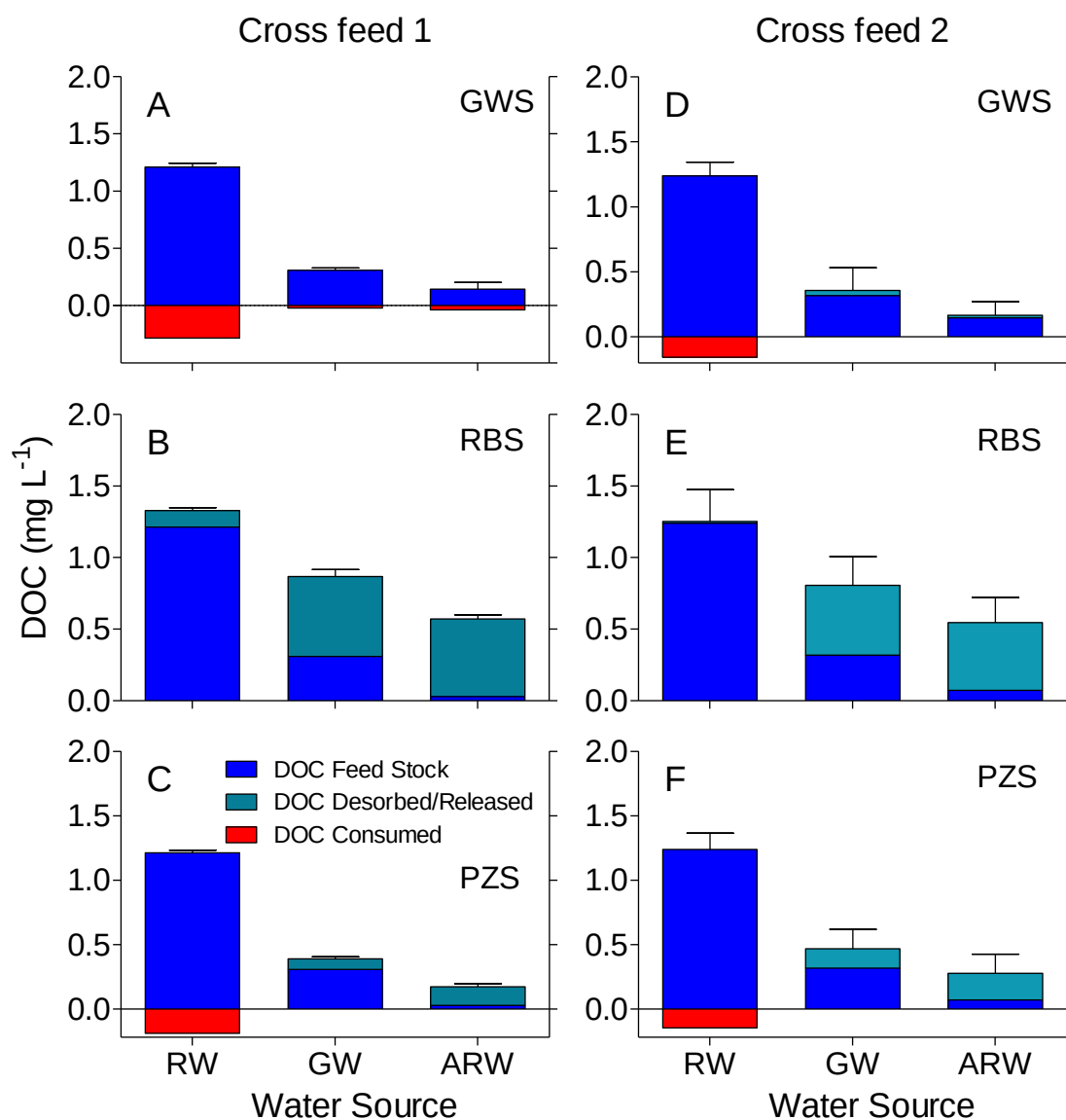


Figure 3.3 DOC concentration in the fluid phase of groundwater (A, D), riverbed (B, E), and piezometer (C, F) sand reactors for the two replicate Cross feed experiments. The green and red sections show the release or consumption of DOC based on the difference between reactor DOC and the expected DOC content of the feed stock solution (blue sections). Values are the mean $\pm$ SD of 3 time points for triplicate reactors for experiment 1, and the mean $\pm$ SD of 6 time points for triplicate reactors for experiment 2.

3.8.4 Figure (3.4) DOC data for the Desorption/Cross feed experiment

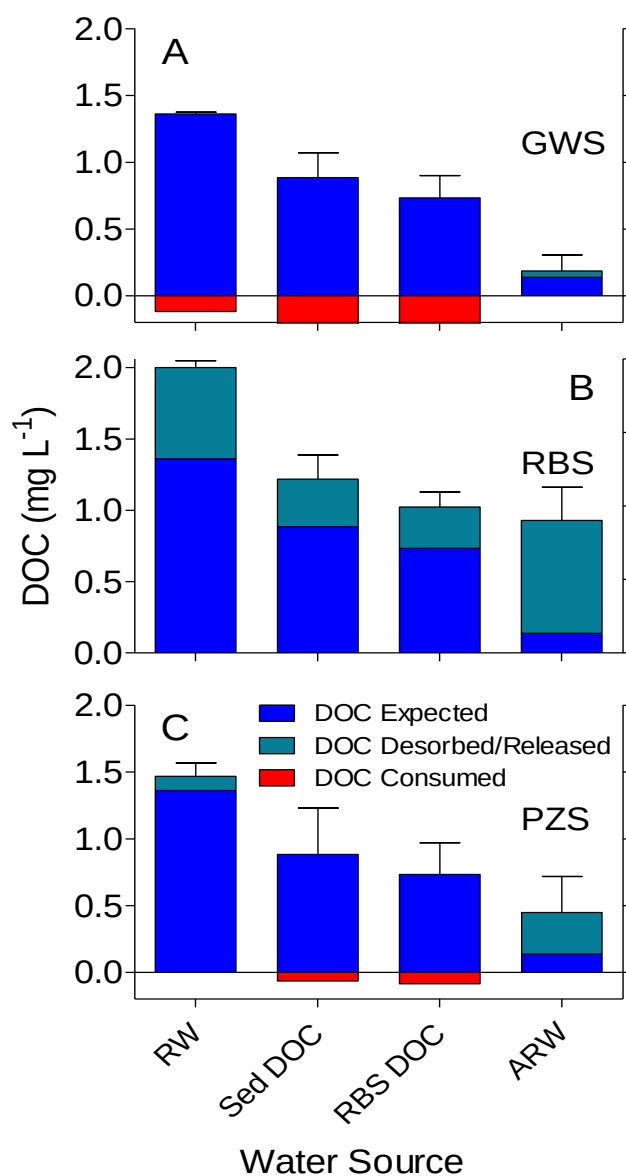


Figure 3.4 DOC concentration in the fluid phase of riverbed (A), groundwater (B), and piezometer (C) sand reactors for the Desorption/Cross feed experiment. The green and red sections show the release or consumption of DOC based on the difference between reactor DOC and the DOC content of the feed stock solution (indicated on x-axis). Values show mean $\pm$ SD of 3 time points for triplicate reactors.

3.8.5 Figure (3.5) NMDS plots for the Cross feeding and Desorption/Cross feed experiments

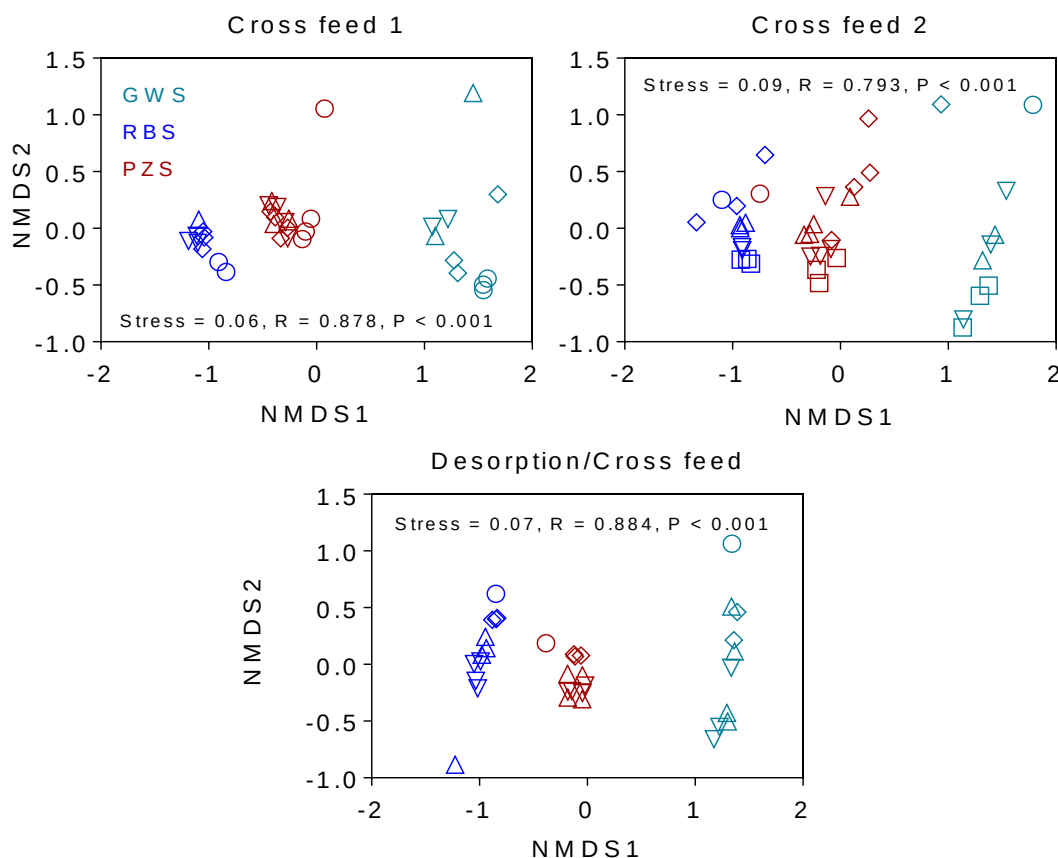


Figure 3.5 NMDS analysis of microbial community composition for the Cross feeding and Desorption/Cross feed experiments. R and P values in each panel are results of ANOSIM analysis of 16S rRNA gene amplicon dissimilarity. Results for reactors with different fluid sources are shown by the same symbol color because ANOSIM analysis showed that fluid type did not significantly influence community composition (see Table 2). Different time points (days) are shown with the following symbols: Cross feed 1, $\circ$ = 0, $\diamond$ = 14, $\triangle$ = 28, ∇ = 35; Cross feed 2, $\circ$ = 0, $\diamond$ = 7, $\triangle$ = 21, ∇ = 35, $\square$ = 41; Desorption/Cross feed, $\circ$ = 0, $\diamond$ = 5, $\triangle$ = 19, ∇ = 26, $\square$.

3.8.6 Figure (3.6) Phylum-level microbial community composition

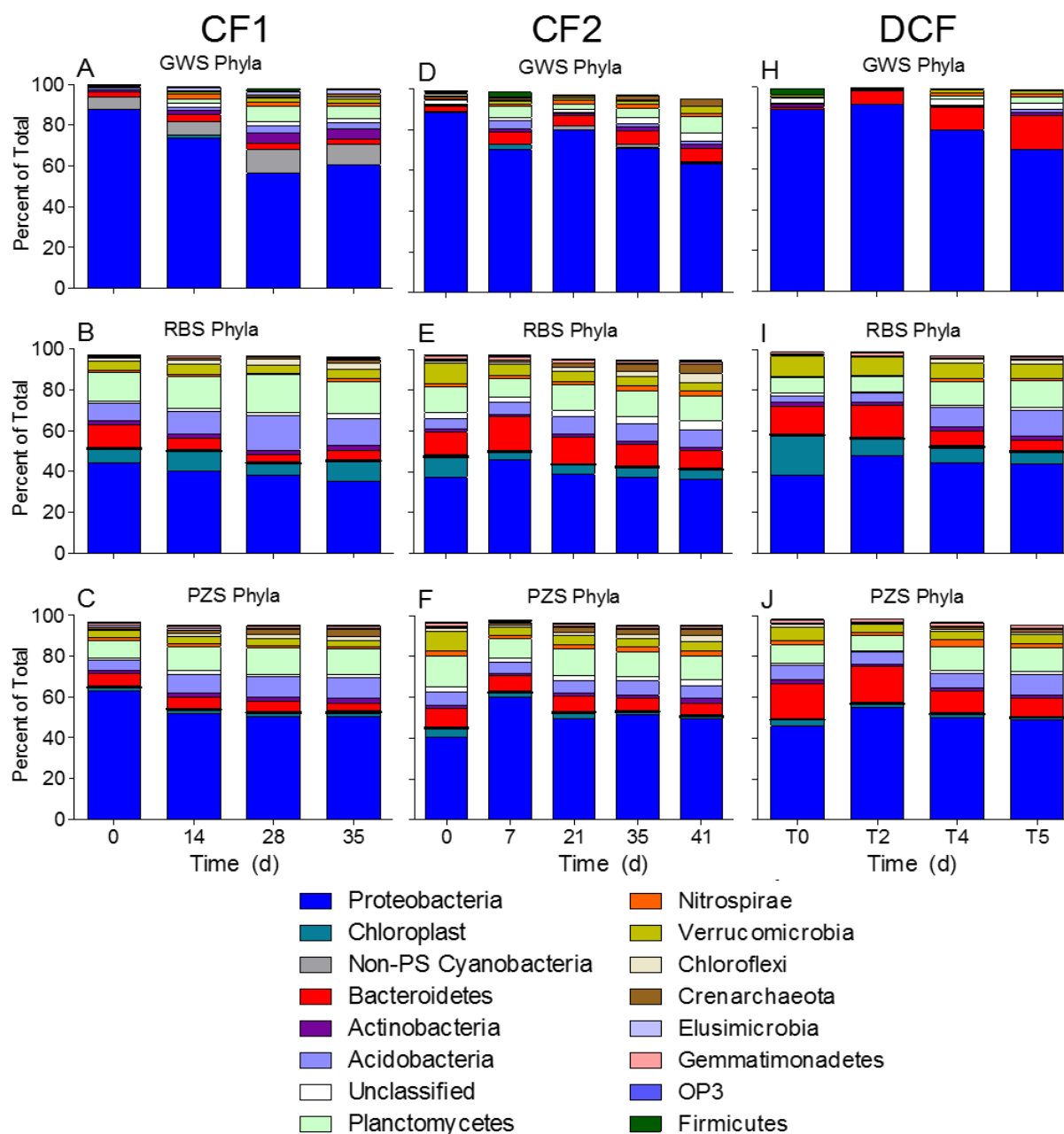


Figure 3.6 Phylum-level microbial community composition in the colonized sand reactors. Each bar shows the average result for 2-4 reactors that received different fluid sources during the incubation experiments. Results for reactors with different fluid sources were combined for each time point during each experiment because ANOSIM analysis showed that fluid type did not significantly influence community composition (see Table 2). Chloroplast refers to the

chloroplast from eukaryotic algae; Non-PS cyanobacteria refers non-photosynthetic cyanobacteria from the Class ML635J-21 in the Green genes and Silva 16S rRNA gene databases.

3.8.7 Figure (3.7) Proteobacteria class-level composition

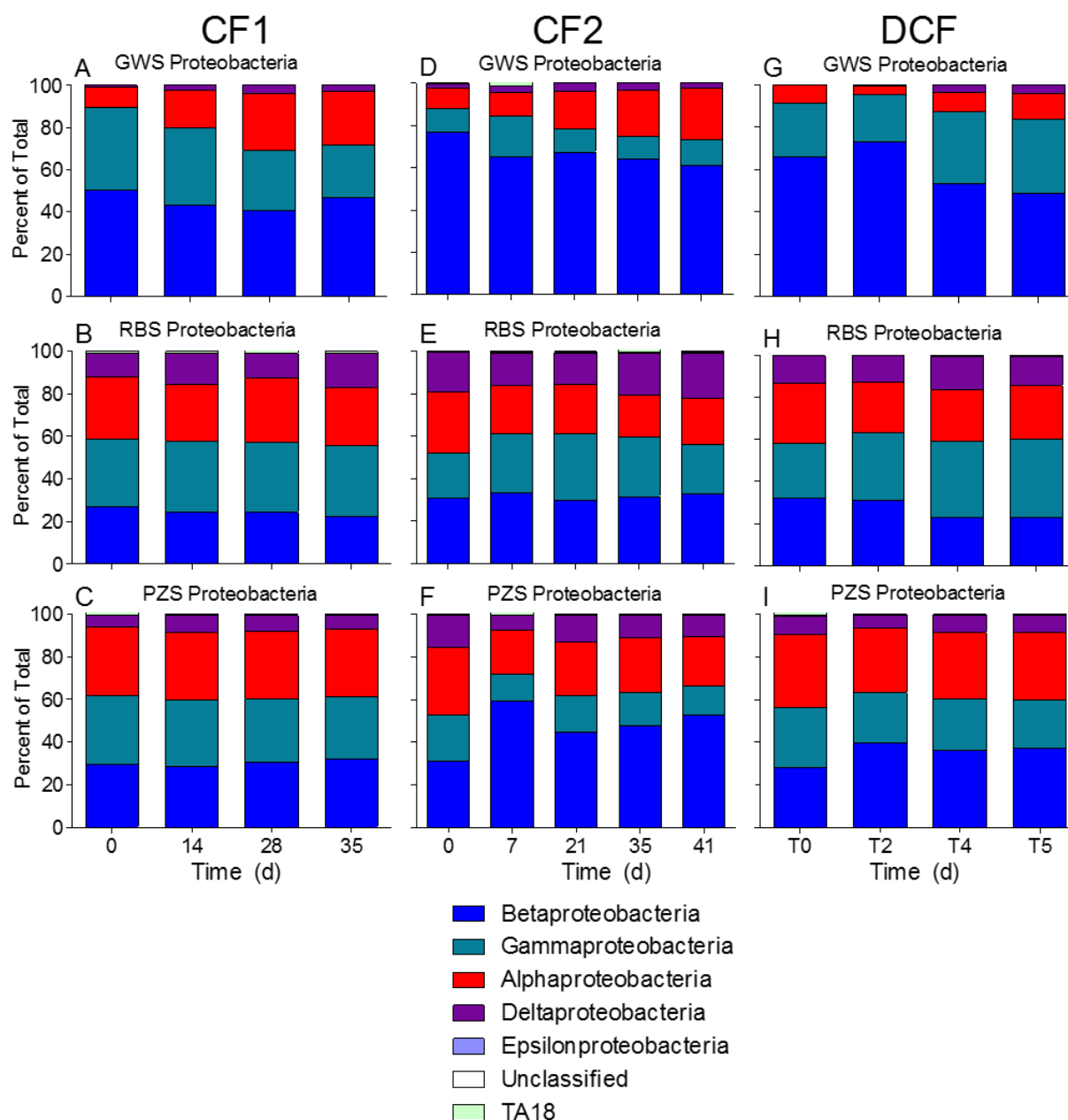


Figure 3.7 Proteobacterial class composition in the colonized sand reactors. Each bar shows the average result for 2-4 reactors that received different fluid sources during the incubation experiments. Results for reactors with different fluid sources were combined for each time point during each experiment because ANOSIM analysis showed that fluid type did not significantly influence community composition (see Table 3.2).

3.9 Tables

3.9.1 Table (3.1) Linear Mixed Model ANOVA for the cross feeding experiments

Dependent Variable	Factor	df	F	p
Biomass CF1 and CF2	Year	1	131.63	2.20E-16
	Sand	2	287.67	2.20E-16
	Water	3	3.77	0.01
	Time	1	2.56	0.11
Respiration CF1 ^a	Sand	2	44.89	2.95E-14
	Water	3	0.73	0.54
	Time	1	3.74	0.06
Respiration CF2 ^a	Sand	2	156.56	5.43E-13
	Water	3	6.62	2.70E-03
	Time	1	81.22	1.67E-08
Biomass DCF	Sand	2	86.58	7.10E-09
	Water	3	1.39	0.29
	Time	1	82.26	9.94E-13
Respiration DCF	Sand	2	62.09	5.16E-08
	Water	3	4.07	0.027
	Time	1	30.11	1.07E-06

^aRespiration data for CF1 contained a several measurements that were just above the detection limit; such data were not present in the CF2 data and thus the two data sets are modeled separately.

3.9.2 Table (3.2) ANOSIM analysis of microbial community composition

Experiment	Factor	R	p
CF1	Sand type ^a	0.878	0.001
CF2	Sand type	0.793	0.001
DCF	Sand type	0.884	0.001
CF1	Water type (GWS) ^b	0.115	0.216
CF1	Water type (RBS)	0.120	0.140
CF1	Water type (PZS)	0.090	0.317
CF2	Water type (GWS)	-0.096	0.233
CF2	Water type (RBS)	-0.062	0.706
CF2	Water type (PZS)	-0.187	0.968
DCF	Water type (GWS)	0.579	0.024
DCF	Water type (RBS)	-0.235	0.987
DCF	Water type (PZS)	0.196	0.148

^a Pooled across all time points and water types.

^b Pooled across all time points for each water type.

3.9.3 Table (3.3) Relative abundance of selected phyla summed across all water types

Experiment	Phylum	GWS % Total Reads	RBS % Total Reads	PZS % Total Reads
CF1	Proteobacteria	72.3	39.8	53.3
	Acidobacteria	1.9	11.7	8.8
	Bacteroidetes	3.0	6.7	5.5
	Cyanobacteria	8.3 ^a	9.4 ^b	2.1 ^b
	Nitrospirae	1.6	1.1	1.4
	Planctomycetes	3.4	15.5	11.5
	Verrucomicrobia	1.1	4.6	3.4
	Crenarchaeota	0.6	1.1	2.2
CF2	Proteobacteria	70.1	40.0	50.7
	Acidobacteria	1.1	7.5	6.2
	Bacteroidetes	6.3	13.0	7.4
	Cyanobacteria	1.3 ^a	5.4 ^b	2.5 ^b
	Nitrospirae	1.8	1.7	2.2
	Planctomycetes	5.5	11.6	12.0
	Verrucomicrobia	2.1	5.5	4.9
	Crenarchaeota	2.3	2.1	2.1
DCF	Proteobacteria	75.8	44.3	50.2
	Acidobacteria	0.7	8.1	7.7
	Bacteroidetes	13.7	10.4	13.4
	Cyanobacteria	0.4 ^a	9.3 ^b	1.8 ^b
	Nitrospirae	1.1	0.9	2.5
	Planctomycetes	1.9	10.5	10.4
	Verrucomicrobia	1.3	8.1	4.4
	Crenarchaeota	0.1	0.2	0.9

*See discussion section for rational of which phylum were selected and why summation across all water types was applicable.

^a Taxa were dominated (82-94 %) by non-photosynthetic taxa from Class ML635J-21.

^b Taxa were dominated (70-94 %) by chloroplasts of eukaryotic algae of the order Stramenopiles.

3.10 Supplemental material

3.10.1 Supplemental text 1 – analytical methods

3.10.1.1 DOC measurements

Dissolved organic carbon (DOC) was determined with a Shimadzu combustion carbon analyzer TOC-Vcsh with ASI-V auto sampler. DIC was removed in the instrument by acidification of the sample with 2M HCl (Fisher) with a 5 minute sparging time. The sample was then injected into the furnace set to 680 °C. Organic carbon standards were made from potassium hydrogen phthalate solid, (Acros Organics). The calibration range for the DOC determinations was from 0.10 to 10 mg C L⁻¹.

3.10.1.2 ATP biomass measurements

ATP content was used as a measure of microbial biomass in the reactors. The methods employed were adapted from those reported in Graham et al. (2016), using the Promega Glomax 20/20 Luminator with Bactiter-Glo Microbial Cell Viability Assay (Promega). Approximately 0.5 g of wet sand sample was mixed with 0.5 mL of sterile (autoclaved) 20 mM EDTA (Fluka) in 2 mL Nalgene Cryogenic Vials (Thermoscientific) and stored at -20°C. Samples were returned to room temperature prior to analysis and measured after 5 min incubation with Bactiter-Glo reagent and 25 µL of 0.4M MgCl₂. Standard curves were prepared with Adenosine 5'-triphosphate magnesium salt (Sigma) in 20 mM EDTA and ranged from 0 – 10 nM.

3.10.1.3 Resazurin reduction assay measurements

Resazurin reduction assays were conducted with samples from the microcosms as a measure of aerobic respiratory activity (Haggerty et al., 2008). The methods employed were adapted from those reported in Graham et al. (2016). Assays were started by adding approximately 2.5 g of wet sand to 100 μ l of 30 μ M resazurin and 2.5 mL of feed stock solution to vials, gently mixing and then incubating at room temperature in the dark on an angle at 50 rpm. After 48 hr the vials were weighed and 3 mL of acetonitrile (ACN) (HPLC Grade, Fischer Chemical) was added. After ACN addition, vials were sealed, vortexed, and reweighed before being placed in a sonicator bath for 10 min. After sonication, vials were put back on the 50 rpm shaker. After a 1 hr extraction time, vials were vortexed and the sand was allowed to settle. The screw cap was removed and 5 mL of extract was passed through a 17 mm, 0.22 μ m Teflon syringe filter (Thermoscientific) into pre-labeled 12 mL glass amber vials and stored at 4°C. The extraction vial with the remaining sand was dried at 75 °C for 72 hr then weighed to obtain dry sand weight. Fluorescence emission maxima for resazurin (630 nm) and resorufin (585 nm) were measured on resazurin sample extracts using a Horiba Fluorolog 3 fluorimeter. Resazurin reduction extracts were analyzed in quartz cuvettes and diluted 1:1 with ACN:ARW when above the calibration range. Fluorescence intensity was quantified by comparison to standards of resazurin ranging from 0 to 1.0 μ M (Sigma) and resorufin ranging from 0 to 0.2 μ M (Sigma) prepared in ACN:ARW (1:1).

3.10.1.4 Linear mixed model analysis

Linear mixed model (LMM) analysis was used to determine the influence of sand type, feed stock solution, and time on biomass and respiration values in the reactors. The LMM analysis

was implemented using packages lme4 and lmerTest in the R statistical package (TeamR, 2016). Additional R packages ggplot, tidyr, dplyr, and viridis were also used (Wickham, 2009; Bates et al., 2015; Francois, 2015; Christensen, 2016; Garnier, 2016; Wickham, 2016). Implementation included an adaptation of the original linear fit equation to use the final time point from each experiment to calculate the modeled intercepts; this allowed for comparison of the final status of the reactors based on the fitted model intercept. For the cross feed biomass LMM, both three-way and four-way interactions were significant. In all other cases three-way and four way-factor interactions were not significant and were therefore removed from the models. Biomass data for the two replicate cross feeding experiments (CF1 and CF2) were consistent and were modeled together. Due to the presence of a handful of CF1 respiration data points that were close to the detection limit, separate models were required for the CF1 and CF2 respiration data. Pair wise comparisons were performed using standard t-test on the difference between the log of the final time point for the two reactors being compared.

3.10.1.5 DNA extraction, sequencing, and processing methods

Samples from replicate reactor types at each time point were pooled for DNA extraction. This approach was required to obtain sufficient DNA for 16S rRNA gene sequencing. DNA was extracted from the pooled materials as described in Graham et al. (2016). DNA extracts were amplified using the universal 16S rRNA gene primers (515F-806R) described in Caporaso et al. (2011), adapted for the Illumina MiSeq platform by adding nine extra bases in the adapter region of the forward amplification primer that support paired-end sequencing. The reverse amplification primer contained a twelve base barcode sequence. Each 25 μ L PCR reaction contained 12 μ L of MoBio PCR Water (Certified DNA472 Free), 10 μ L of 5 Prime

HotMasterMix (1x), 1 μ L of forward primer (5 μ M concentration, 200 pM final), 1 μ L Golay Barcode Tagged Reverse Primer (5 μ M concentration, 200 pM final), and 1 μ L of template DNA. The conditions for PCR were: 94°C for 3 min to denature DNA, with 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s; with a final extension of 10 min at 72°. Amplicons were quantified using PicoGreen (Invitrogen) and a plate reader. Appropriate volumes of PCR product from each sample were then pooled so that each sample was represented with an equal amount of product. This pool was cleaned using the UltraClean® PCR Clean-Up Kit (MoBio), and quantified using the Qubit (Invitrogen). After quantification, the molarity of the pool was determined and diluted to 2 nM, denatured, and then diluted to a final concentration of 6.7 5pM with a 10% PhiX spike for sequencing on the Illumina MiSeq.

The 16S rRNA gene amplicon sequences were processed in QIIME 1.8.0. (Caporaso et al., 2010). First, the function `split_libraries_fastq.py` was used to demultiplex the fastq-formatted sequences, with a Phred quality cutoff of 20. To identify chimeras the function `identify_chimeric_seqs.py` was used to call USEARCH v6.1 with ‘non_chimera_retention’ set to ‘intersection.’ After removing identified chimeras (with function `filter_fasta.py`) the function `pick_open_reference_otus.py` was used to call USEARCH v6.1, pick operational taxonomic units (OTUs), and generate the associated phylogeny (97% similarity) using the SILVA (Release 111) database. The number of reads per library (107 total) ranged from ca. 13,000 to 125,000, with a mean of $51,726 \pm 28,079$. The software package Primer (version 7.0) was used for non-metric dimensional scaling (NMDS) analysis of microbial community composition, and for analysis of similarity (ANOSIM) comparisons of community composition among different reactor treatments.

3.10.2 Supplemental text 2 - comparative analysis of Proteobacterial and non-Proteobacterial in the colonized sand reactors

Proteobacteria. Several basic patterns were evident within the dominant Proteobacterial classes. For the *Alphaproteobacteria* (ca. 9 to 28 % of all Proteobacteria), there was considerable variation among sand types across the three experiments, but in general the dominant taxa were heterotrophs (Kersters et al., 2006) from the *Hyphomicrobiaceae*, *Hyphomonadaceae*, *Rhodospirillaceae*, *Rhodobacteraceae*, and *Sphingomonadaceae*. Similarly, for all sand types the *Betaproteobacteria* (ca. 27 to 65 % of all Proteobacteria) were dominated by the heterotrophic families *Comamonadaceae*, *Methylophilaceae*, *Oxalobacteraceae*, and *Rhodocyclaceae*. Likewise, the *Gammaproteobacteria* (ca. 12 to 34 % of all Proteobacteria) were dominated by a common group of heterotrophic taxa from the *Alteromonadaceae*, *Coxiellaceae*, *Moraxellaceae*, *Piscirickettsiaceae*, *Pseudomonadaceae*, *Sinobacteraceae*, and *Xanthomonadaceae*.

By far the largest distinction among Proteobacteria across sand type was the much higher abundance of *Deltaproteobacteria* in RBS and PZS, both initially and throughout the incubation experiments (Figure 3.8). Across all sand types and time points, *Deltaproteobacteria* accounted for 2-3 % of total *Proteobacteria* in GWS, 14-17 % for RBS, and 7-11 % for PZS (Table 3.3). For GWS, the dominant families were *Bdellovibrionaceae* (and the related family OM27, Rappe et al., 1997) and *Haliangiaceae*, which represent gram-negative, obligate predators of other gram-negative bacteria (Rotem et al., 2014); and bacteriolytic- and non-cellulolytic-type myxobacteria (Garcia and Müller, 2014), respectively. Although these families (along with other myxobacteria and predatory families such as *Myxococcaceae* and *Bacteriovoracaceae*; Tables 3.S2-3.S4) were present in RBS and PZS, *Deltaproteobacteria* in these two sand types also contained significant numbers of anaerobic fermentative and respiratory taxa such as

Syntrophobacteraceae (Kuever, 2014) and *Geobacteraceae* (Röling, 2014).

Syntrophobacteraceae accounted for 1-2 % of total Proteobacteria in RBS and PZS, whereas *Geobacteraceae* were ca. 10-fold more abundant in RBS (1-2 %) compared to PZS (ca. 0.1 %). It is also significant that the majority (60-70 %) of the unclassified *Delatproteobacterial* families in RBS and PZS (3-8 % of total Proteobacteria) were from in the order *Myxococcales*, which includes a wide variety of heterotrophic taxa, including the well-known anaerobic respiratory family *Anaeromyxobacteraceae* (Sanford et al., 2002; Yamamoto et al., 2014).

Non-Proteobacteria. Several broad trends in the dominant non-Proteobacterial taxa were also evident (Table 3.3). The abundance of *Bacteroidetes* and *Nitrospirae* was consistent across all three sand types. Although there were differences in the distribution of families within the *Bacteroidetes* (Tables 3.S4-3.S6), in general the dominant ones (e.g. *Chitinophagaceae*, *Flavobacteriaceae*, *Saprospiraceae*, as well as unassigned (at the family level) organisms from class *Sphingobacteria*, order *Sphingobacteriales*, which includes the genus *Sphingobacterium*) are all metabolically versatile heterotrophs known to occupy soil, aquatic, sewage sludge, and compost habitats (Campbell, 2014; Lambiase, 2014; McIlroy and Nielsen, 2014). For the phylum *Nitrospirae*, organisms from the nitrite-oxidizing genus *Nitrospira* (Daims, 2014) accounted for 1-2 % of total reads and for more than 80% of all reads in the phylum. Similarly, *Crenarchaeota* from the families *Cenarchaeaceae* or *Nitrososphaeraceae* (class *Thaumarchaeota*), both of which include aerobic ammonium-oxidizing organisms (Oshiki et al., 2016) were detected in each of the sand types at ca. 2-4 % of all reads.

In contrast to *Bacteroidetes*, *Nitrospirae*, and the *Crenarchaeota*, organisms from the *Acidobacteria*, *Planctomycetes*, and *Verrumicrobia* phyla were several-fold more abundant in RBS and PZS compared to GWS (Table 3.3). The *Acidobacteria* were dominated by families

within classes *Chloracidobacteria* and *Acidobacteria*, which have been identified in soil habitats and are known for their ability to degrade plant and microbial polysaccharides (Sait et al., 2002; Quaiser et al., 2003; Campbell, 2014). The *Planctomycetes* taxa were affiliated with the *Gemmataceae*, *Isosphaeraceae*, *Pirellulaceae*, and *Planctomycetaceae* families, all of which are aerobic heterotrophic budding bacteria with unique intracellular compartmental (organelle) structures (Lindsay et al., 2001; Pinos et al., 2016). The *Verrumicrobia* were dominated by taxa related to cultivated and uncultivated organisms from classes *Opitutae*, *Pedosphaerae*, *Spartobacteria*, and *Verrucomicrobiae*. Organisms within these families are mainly aerobes common in soils or other organic-rich environments (e.g. Sangwan et al., 2004; Eichorst et al., 2007; Kant et al., 2011; Yoon, 2014; Hernandez et al., 2015; Navarrete et al., 2015).

3.10.3 Figure (3.S1) The stages of river water intrusion at the Hanford Area 300 site

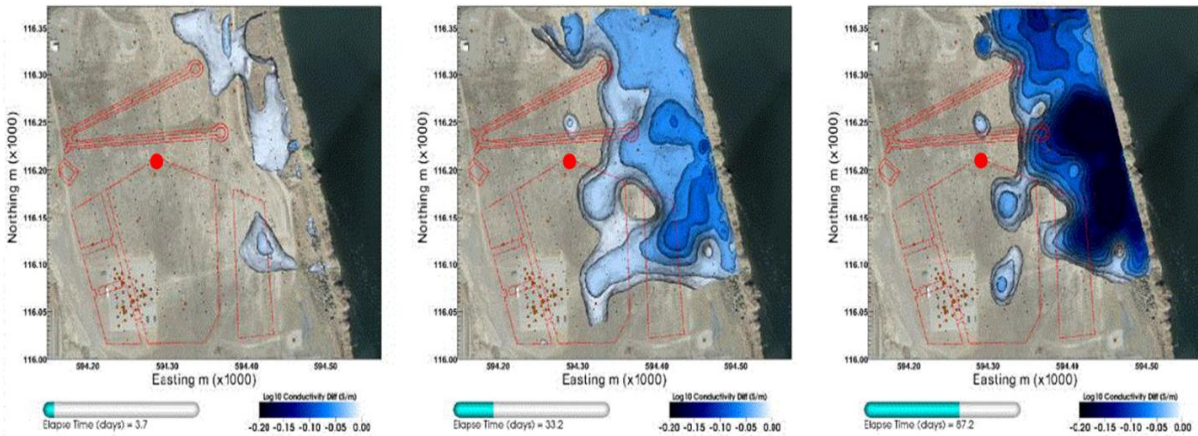


Figure 3.S1. The varying stages of river water intrusion at the Hanford Area 300 site (modified from Figs. 11 and 12 in Johnson et al., 2015, Water Resour. Res., 51, doi:10.1002/2014WR016129; used by permission). Darker blue coloration shows decrease in conductivity associated with higher DOC in river water. Elapsed time shown for a period of approximately 54 days. The red dot shows the location of groundwater well 2-32, from which water was obtained for the experiments reported in this paper.

3.10.4 Figure (3.S2) Diagrammatic representation of river stage driven hydrological dynamics

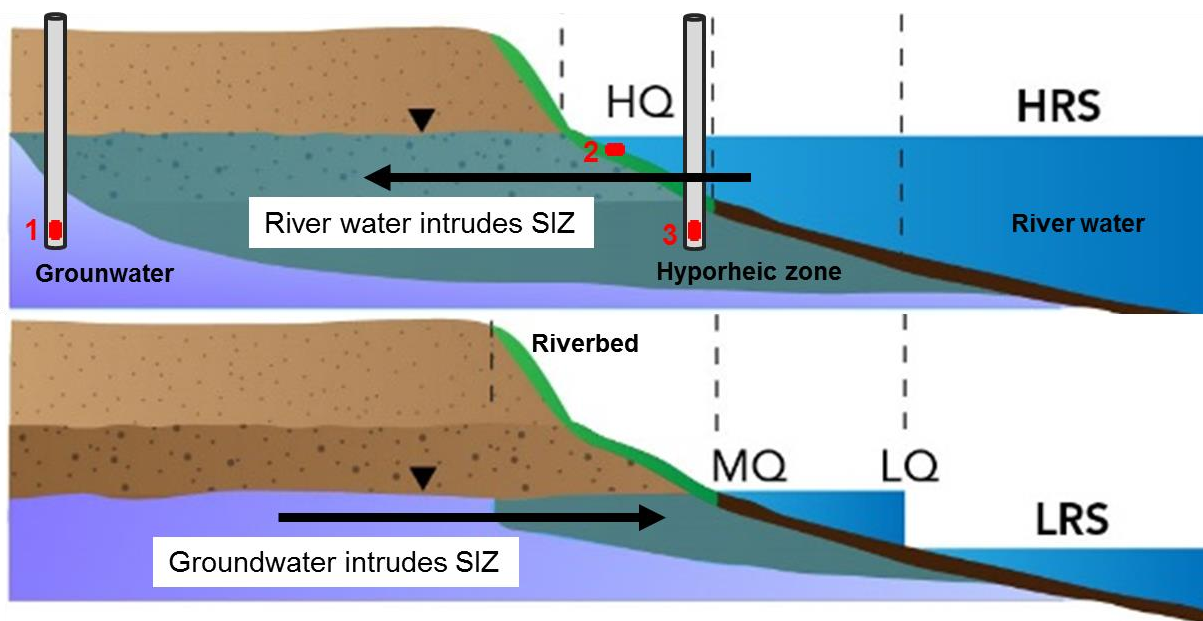
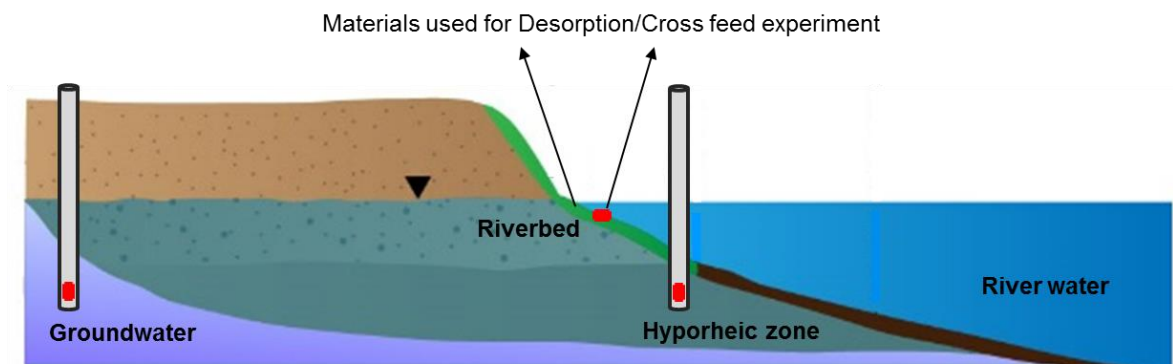


Figure 3.S2. Diagrammatic representation of river stage driven hydrological dynamics within the Hanford 300 Area SIZ. During high river stage (HRS), large river water-groundwater exchange (HQ) takes place during river water intrusion into the SIZ, whereas during low river stage (LRS), medium (MQ) or low (LQ) river water-groundwater exchange takes place and net groundwater release into the SIZ may take place. The red dots correspond to the three habitats examined in this study (1 = groundwater; 2 = riverbed; 3 = hyporheic zone piezometer).

3.10.5 Figure (3.S3) Conceptual explanation of the cross feeding and desorption/cross feeding experiments



Cross feed experiments 1 and 2:

Step 1: Incubate sand packs (red ovals) *in situ* within groundwater, riverbed, and hyporheic zone environments for 6 weeks to allow for microbial colonization.

Step 2: Expose the different colonized sand materials to either (1) filter-sterilized groundwater, (2) filter-sterilized river water, or (3) autoclaved (DOC-free) artificial groundwater and/or artificial river water.

Desorption/Cross feed experiment:

Step 1: Incubate sand packs (red ovals) *in situ* within groundwater, riverbed, and hyporheic zone environments for 6 weeks to allow for microbial colonization.

Step 2: Collect “desorbed” DOC from either riverbed sediment or riverbed colonized sand packs.

Step 2: Expose the different colonized sand materials to either (1) filter-sterilized river water, (2) filtered-sterilized “desorbed” DOC from riverbed sediment, (3) filtered-sterilized “desorbed” DOC from riverbed colonized sand packs, or (4) autoclaved (DOC-free) artificial river water.

Figure 3.S3. Conceptual explanation of the cross feeding and desorption/cross feeding experiments. See Materials and Methods for details.

3.10.6 Figure (3.S4) Photos of near-shore riverbed environment at the Hanford 300 Area



Figure 3.S4. Photos of near-shore riverbed environment at the Hanford 300 Area. (A) View from the north side of the Columbia River, illustrating the location of piezometer T3. (B) Close up of the riverbed gravel layer. (C) A large, periphyton-coated gravel stone. (D) Close up of the periphyton-coated gravel stone, illustrating the presence of abundant photosynthetic biomass.

The inset in (D) shows periphyton material loosely-associated with the gravel stone.

3.10.7 Figure (3.S5) Specific conductivity (SpC) of relevant groundwater and piezometer wells

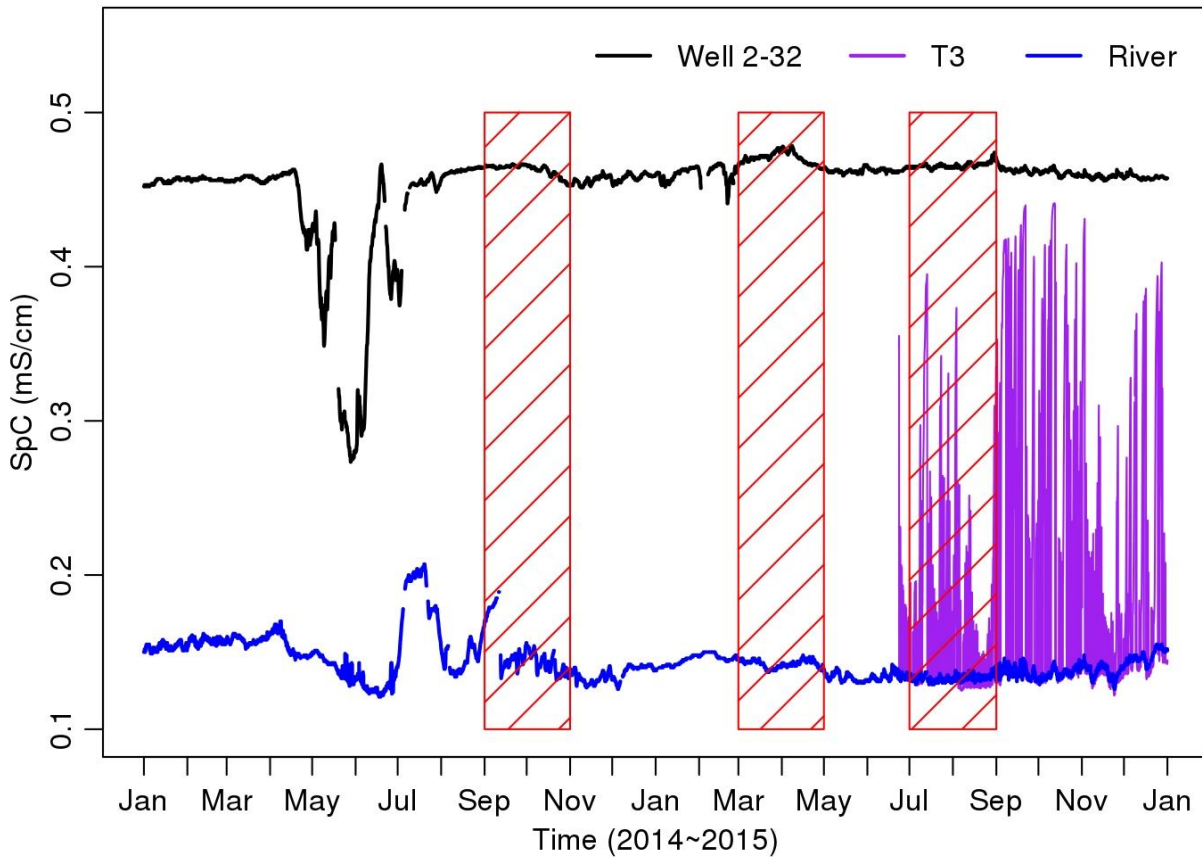


Figure 3.S5. Specific conductivity (SpC) of groundwater well 2-32, piezometer T3, and river water during 2014-2015 (data for piezometer T3 are available only the second half of 2015). Results are hourly measurements from a data logger connected to an *in situ* conductivity probe. The hatched orange rectangles indicate the times of *in situ* sand pack deployment. The frequent variation in piezometer fluid SpC indicates extensive river water-groundwater mixing.

3.10.8 Figure (3.S6) ATP and resazurin reduction data for Cross feed 2 experiment

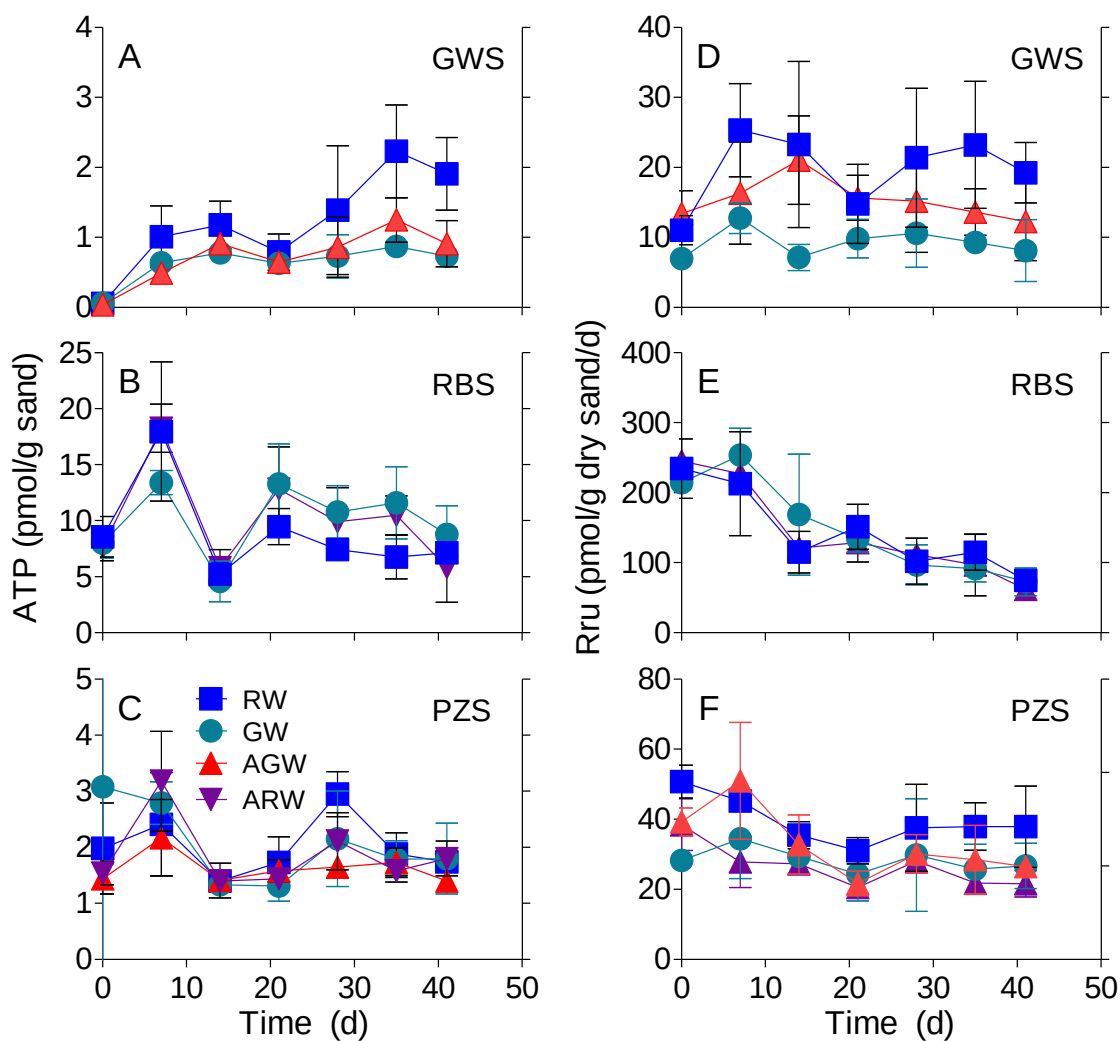


Figure 3.S6. ATP content and rates of resazurin transformation to resorufin for the groundwater (A, D), riverbed (B, E), and piezometer (C, F) sand colonized reactors for Cross feed experiment 2. Each data point shows the mean $\pm$ SD or range of triplicate or duplicate

3.10.9 Figure (3.S7) SEM images of diatom frustules in RBS material

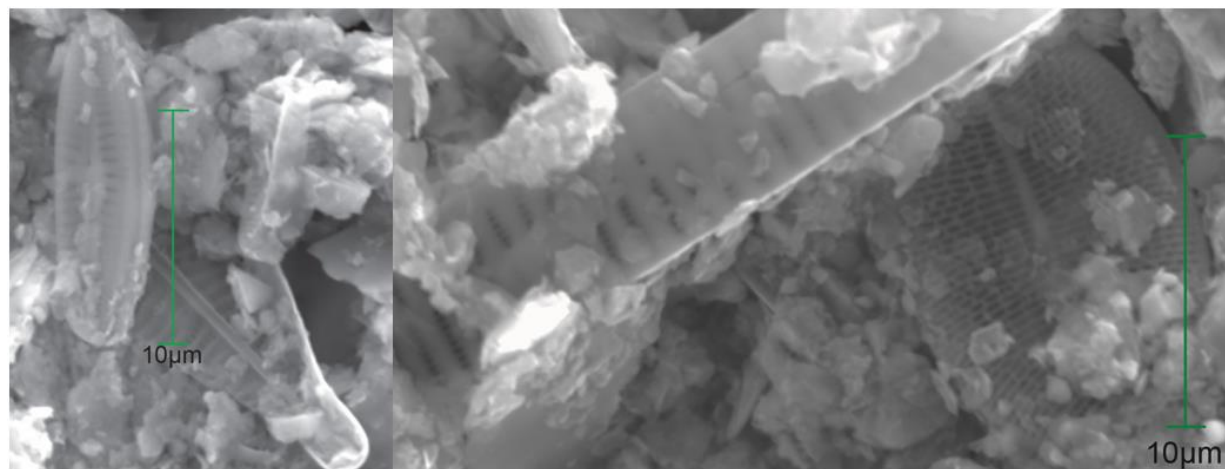


Figure 3.S7. SEM images of diatom frustules in RBS material.

3.10.10 Figure (3.S8) *Planctomycetes* and *Verrumicrobia* abundance for CF1 and CF2

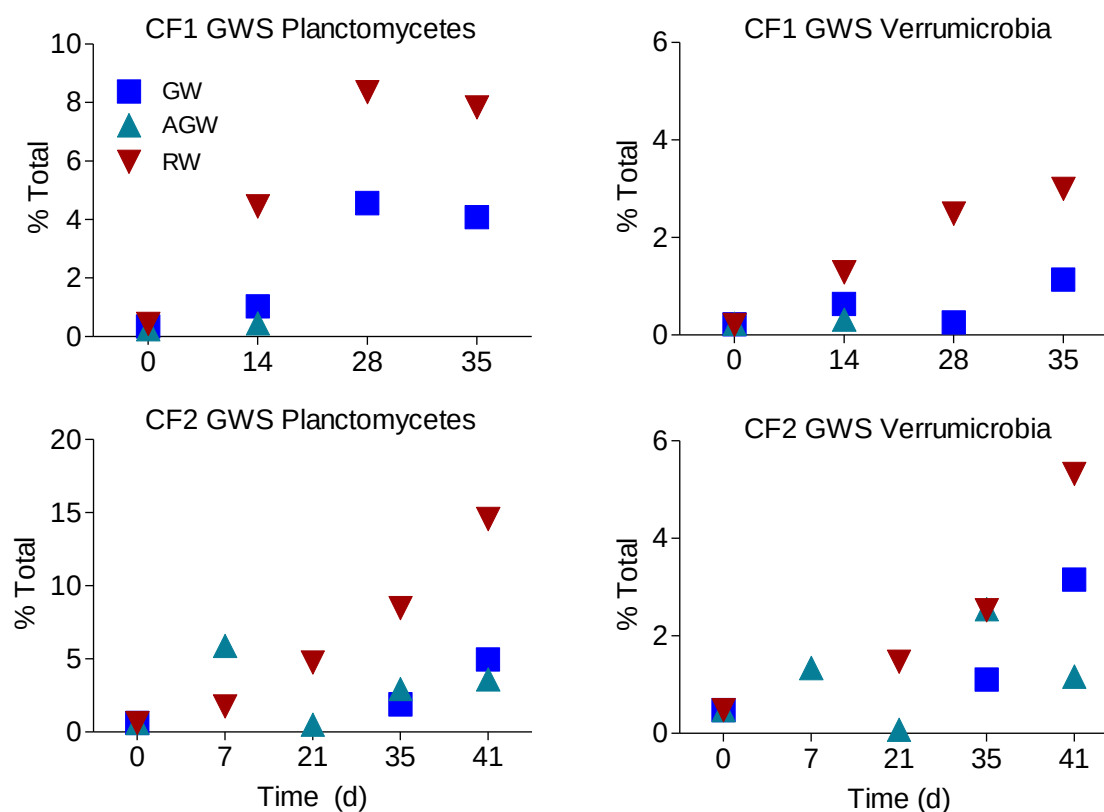


Figure 3.S8. Relative abundance of *Planctomycetes* and *Verrumicrobia* in the CF1 and CF2 GWS reactors exposed to different fluid types (GW, AGW, or RW). Each symbol represents results from a single 16S rRNA gene amplicon library.

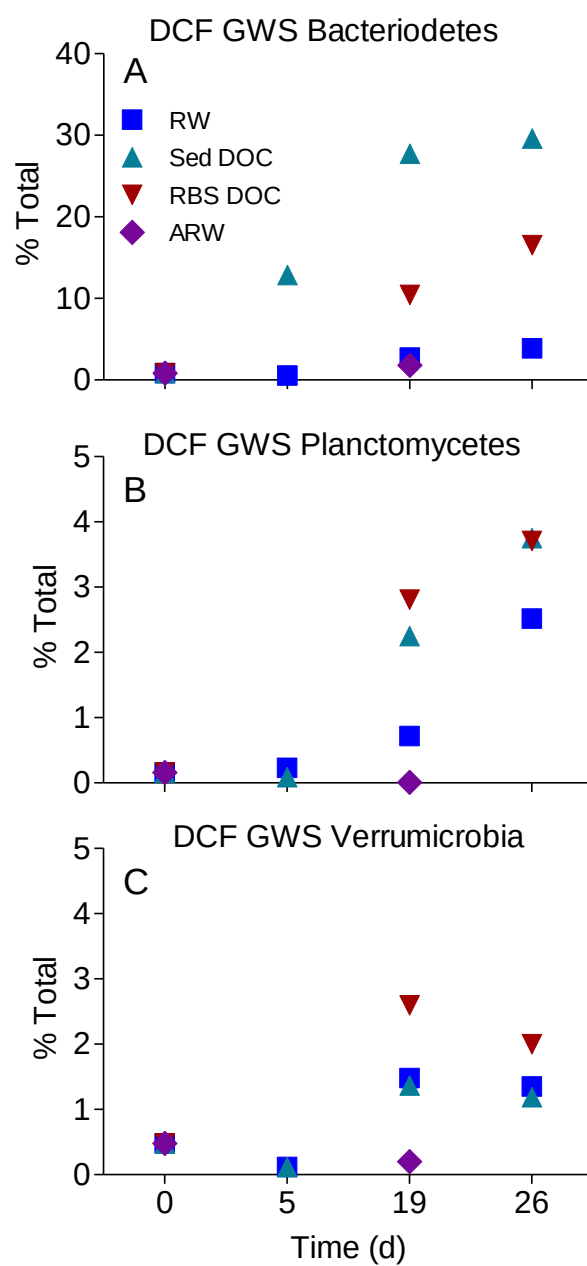
3.10.11 Figure (3.S9) *Planctomycetes* and *Verrumicrobia* abundance for DCF

Figure 3.S9. Relative abundance of *Bacteroidetes* (A), *Planctomycetes* (B), and *Verrucomicrobia* (C) in the DCF GWS reactors exposed to different fluid types (RW, Sed DOC, RBS DOC, or ARW). Each symbol represents results from a single 16S rRNA gene amplicon library.

3.10.12 Figure (3.S10) Image of reactors from CF1

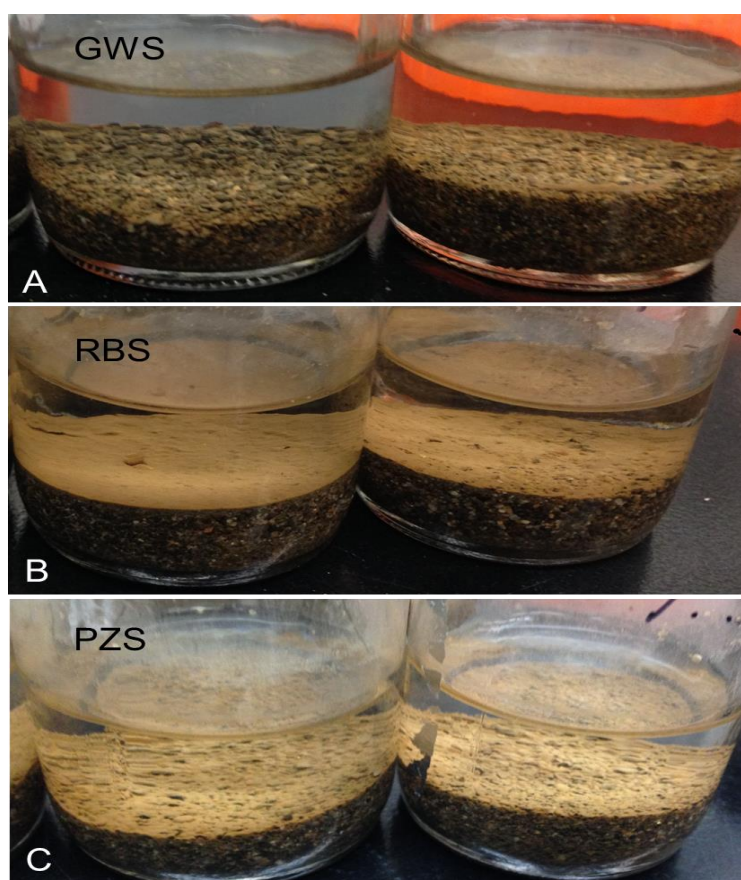


Figure 3.S10. Photos of GWS, RBS, and PZS reactors at the start of CF1. Note the presence of fine-grained materials on the surface the RBS, which are less abundant in the PZS and absent from the GWS.

3.10.13 Figure (3.S11) Conceptual diagram of hyporheic corridor

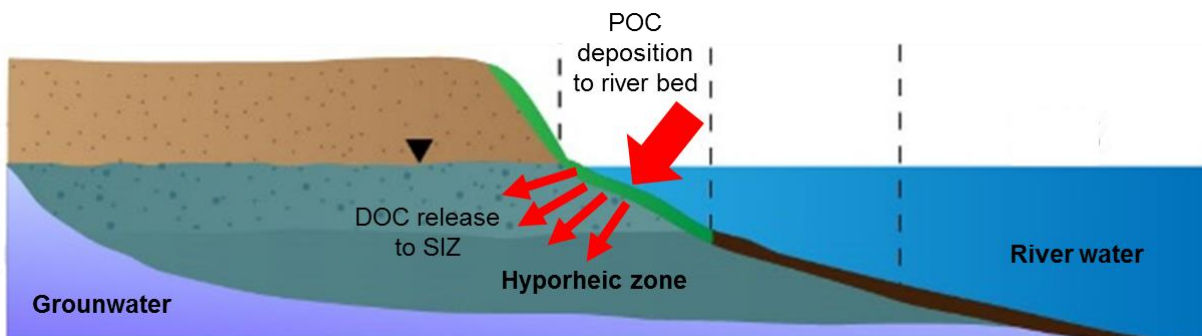


Figure 3.S11. Conceptual illustration of a key biogeochemical implication of this study, i.e. the potential for hydrologically-driven input of labile POC in the form of river water and/or periphyton photosynthetic biomass into the riverbed, and for subsequent flux of DOC derived from that biomass into the HC.

3.10.14 Table (3.S1) Statistical comparison of the cross feeding experiments

Table 3.S1. Pairwise reactor comparison for the cross feeding experiments. Only comparisons that were significant at $P < 0.05$ are shown (all other comparisons were not significant).

Treatment Comparison	Parameter	Value	Difference	p
CF1 GWS+AGW	Biomass ^a	0.53	0.20	0.0409
CF1 GWS+GW		0.33		
CF2 RWS+GW	Biomass	9.75	2.88	0.0307
CF2 RWS+RW		6.87		
CF2 GWS+RW	Biomass	1.62	0.87	2.99E-06
CF2 GWS+GW		0.75		
CF2 GWS+RW	Biomass	1.62	0.68	0.0009
CF2 GWS+AGW		0.94		
CF1 RWS+ARW	Respiration ^b	316.23	130.02	0.0374
CF1 RWS+RW		186.21		
CF2 GWS+RW	Respiration	38.02	21.04	5.36E-06
CF2 GWS+GW		16.98		
CF2 GWS+AGW	Respiration	26.30	9.32	0.0133
CF2 GWS+GW		16.98		
CF2 GWS+RW	Respiration	38.02	11.72	0.0326
CF2 GWS+AGW		26.30		
CF2 PZS+RW	Respiration	70.79	25.09	0.0127
CF2 PZS+ARW		45.71		

^a Units = pmol/g sand, ^b Units = pmol/g sand/48 hr

3.10.15 Table (3.S2) Statistical comparison of the DCF experiment

Table 3.S2. Pairwise comparisons for the desorption/cross feeding experiment. Only comparisons that were significant at $P < \sim 0.05$ are shown (all other comparisons were not significant).

Treatment Comparison	Parameter	Value	Difference	p
GWS+RW	Biomass ^a	0.66	0.37	0.0064
GWS+ARW		0.29		
GWS+Sed DOC	Biomass	0.60	0.32	0.0138
GWS+ARW		0.29		
GWS+RBS DOC	Biomass	0.51	0.22	0.0571
GWS+ARW		0.29		
GWS+ Sed DOC	Respiration ^b	89.13	51.97	0.0021
GWS+RW		37.15		
GWS+RBS DOC	Respiration	91.20	54.05	0.0015
GWS+RW		37.15		
PZS+ARW	Respiration	97.72	45.24	0.0253
PZS+RW		52.48		
PZS+Sed DOC	Respiration	109.65	57.17	0.0098
PZS+RW		52.48		
PZS+RBS DOC	Respiration	120.23	67.75	0.0038
PZS+RW		52.48		

^a Units = pmol/g sand, ^b Units = pmol/g sand/48 hr

3.10.16 Table (3.S3) Shannon and Simpson diversity for the cross feeding experiments

Table 3.S3. Shannon and Simpson diversity indices for GWS, RBS, and PZS in the cross feeding experiments.

Experiment	Shannon Index			Simpson Index		
	GWS	RBS	PZS	GWS	RBS	PZS
CF1	4.44	7.34	6.80	0.0407	0.0027	0.0076
CF2	4.97	7.47	6.83	0.0237	0.0024	0.0067
DCF	4.21	7.17	6.73	0.0545	0.033	0.0064

3.11 Chapter 3 Citations

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**Chapter 4 Influence of fresh particulate organic matter input on riverbed sediment
microbial metabolism**

4.1 Abstract

Most studies of riverine carbon cycling have focused on terrestrially derived organic carbon (OC) and ignored the contribution of fresh photosynthetic particulate organic matter (POM). Our previous studies demonstrated that deposition of fresh POM caused highly elevated rates of metabolism, increased activity of anaerobic heterotrophic organisms, and release of labile dissolved organic carbon (DOC) in aerobic riverbed sediment microcosms. Here, we investigated the release of DOC, total inorganic carbon (TIC) and methane (CH_4) from riverbed sediment microbial communities with or without the presence of fresh periphyton derived POM. Additionally, we amended native riverbed sediment with Fe(III) oxide minerals and removed the reactive Fe(III) oxide phase from native riverbed sediment using the citrate-dithionite-bicarbonate extraction (CBD) to test for the potential impact of OC-Fe(III) oxide complex formation on microbial activity. Results revealed that reactors amended with periphyton POM (addition equivalent to 15% of the endogenous POM pool) increased microbial activity ca. 2-fold (compared to unamended controls) under both aerobic and anaerobic conditions. Approximately twice as much of the added POM was metabolized under aerobic vs. anaerobic conditions. Fe(III) reduction was the dominant metabolic pathway in all reactors, with increased rates in Fe(III) oxide amended sediments. Sodium pyrophosphate extractions revealed that organo-metal complexes were highest in Fe(III) amended sediments and lowest in CBD treated sediments. Fe(III) amended reactors resulted in lower dissolved organic carbon (DOC), which agrees with current carbon models of Fe(III) oxide-OC complex formation. However, the Fe(III) amended reactors, which had the highest content of Fe(III) oxide-OC complexes, did not show significant decreases in TIC production. DOC concentrations were highest in anoxic reactors with added fresh POM showing the potential for DOC release during anaerobic metabolism.

4.2 Introduction

Rivers and other inland water systems are key sites of biogeochemical transformation and storage; they are also distinct ecosystems, geomorphological agents, and conduits for particulate organic carbon (POC) transport across continents to the oceans.(Trimmer et al., 2012b) Although biogeochemical activity in rivers is often divided between the water column and the bed, by far the greatest biogeochemical activity takes place in the riverbed, either at or just below the surface.(Findlay, 1995; Brunke and Gonser, 1997; Fellows et al., 2006; Oliver and Merrick, 2006; Clapcott and Barmuta, 2010) This is because the concentration and residence time of organic matter and associated microorganisms in sediments are typically several orders of magnitude greater than that in the overlying water.(Findlay, 2010; Tank et al., 2010) Although previous studies have directly examined the uptake and degradation of POM in permeable marine sediments (Kristensen et al., 1995; Huettel et al., 1996; Bühring et al., 2006; Huettel et al., 2007; Rocha, 2008), more research is required to address this issue in permeable riverbed sediments.(Hunter et al., 2016) The idea that significant depositional fluxes of POM and DOC can drive biogeochemical dynamics in hyporheic zones is consistent with previous studies (Moser et al., 2003; Stern et al., 2017) that demonstrated, in the Columbia River hyporheic corridor, the presence of relatively high numbers of viable aerobic and anaerobic heterotrophic bacteria, and freshly deposited autochthonous photosynthetic biomass was correlated with highly elevated rates of carbon metabolism.

Riverbed metabolism differs from lake bed metabolism because rivers are influenced by frequent high flow events, additional inputs of detritus and dissolved organic matter from surrounding watersheds, and a disconnect between seasonal variation in light and temperature. Because of these differences the seasonal timing of carbon, water, and energy that occurs in

lakes cannot be applied to rivers.(Bernhardt et al., 2018) The influence of freshly deposited autochthonous photosynthetic biomass on riverbed sediment microbial community metabolism rates is not fully understood. POM deposited into the sediment matrix can be divided into two categories; either derived from autochthonous photosynthetic biomass or from partially degraded terrestrial plant and animal biomass. (Giller et al., 1998) Although there are more forms of autochthonous carbon, this study focuses on aquatic algal/plant materials because of their possible significant influence on riverbed sediment biogeochemistry. One of the major contributors to ecosystem respiration in freshwater river sediments is degradation of POM from terrestrial sources (i.e., allochthonous organic carbon), hence most studies have focused on this aspect of the hyporheic corridor carbon cycle.(Romani et al., 1998; Battin and Sengschmitt, 1999; Bianchi et al., 2002; Battin et al., 2008; Shields et al., 2008; Tank et al., 2010; Gunina and Kuzyakov, 2014; Marwick et al., 2014; Bernhardt et al., 2018) A few previous studies have examined the influence of autochthonous photosynthetic biomass on sediment carbon metabolism in marine environments,(Kristensen et al., 1995; Bühring et al., 2006) as well as freshwater lakes.(Goedkoop et al., 1997; Schwarz et al., 2008; Yan et al., 2014) Several studies have identified that periphyton biomass plays a critical role in the riverbed sediment food web.(Peterson et al., 1993; Bunn et al., 1999; Hilton et al., 2006) In desert, glacial outwash, mountain, and tidal freshwater streams hyporheic zone metabolism rates have been shown to be correlated with autochthonous photosynthetic biomass production and hydrological exchange rates between the river and the hyporheic zone.(Sinsabaugh and Findlay, 1995; Jones Jr et al., 1997; Battin, 1999; McKnight et al., 1999) Experiments using mesh bags and litter bags as sediment and microbial community collectors in forest and desert streams revealed that deposition/metabolism of autochthonous periphyton POC and hydrological exchange in coarse

grained sediments was correlated with hyporheic zone microbial diversity and metabolism.(Boulton et al., 1991; Marmonier et al., 2010) The growth of periphyton species *Spirogyra* has been shown to be correlated with the production of methane in hyporheic zone sediments.(Liang et al., 2016) However, how riverbed sediment microbial community aerobic and anaerobic respiration rates as well as methanogenesis rates are directly affected by freshly deposited autochthonous photosynthetic biomass has not been extensively investigated.(Jones and Lock, 1993; Romaní and Sabater, 2001; Trimmer et al., 2012a; Hunter et al., 2016)

The Soil Continuum Model (Lehmann and Kleber, 2015) presents the currently most widely used biogeochemical model for the decomposition/preservation of soil organic matter (SOM) in natural soils and sediments. Previous theories ignored the crucial association of SOM with mineral phases, as well as the potential for all forms of OC to be decomposed by microbial communities.(Lehmann and Kleber, 2015) Because SOM is highly heterogenous in physiochemical structure and spatial distribution, it can be involved in a wide range of interactions with reactive minerals. Globally, on average 22% of sediment organic carbon is directly bound to a reactive iron oxide mineral phase.(Lalonde et al., 2012) Interestingly, this total amount of SOC quantified to be complexed with an iron oxide mineral phase was higher than the total surface area capacity calculated for the amount of iron oxide present. This excess sorbed SOC is due to the existence of SOC-Fe sediment aggregates or biofilm colloids that create additional surface area for SOM sorption to occur.(Thompson et al., 2006; Schmidt et al., 2011)

There is a wide variety of Fe oxide, hydroxides, or oxy-hydroxide phases in soils and sediments.(Cornell and Schwertmann, 2003) Their movement and chemical stability is primarily controlled by clay migration, lateral surface erosion, anaerobic microbial reduction by

dissimilatory iron reducing (DIR) bacteria or abiotic reduction by SOM.(Cornell and Schwertmann, 2003) Proposed mechanisms for the sorption of SOM to iron oxides include anion exchange, ligand exchange-surface complexation, hydrophobic interactions, hydrogen bonding and cation bridging.(Gu et al., 1994) Additionally, through van der Waals forces and hydrophobic interactions, the alkyl chains of SOM can sorb to iron hydroxides.(Gu et al., 1994) Molecular level electron donor-receptor interactions can occur between the aromatic portion of SOM and Fe(III) oxides minerals.(Keiluweit and Kleber, 2009) Other studies have shown inner-sphere and outer-sphere coordination interactions with the carboxyl groups found in SOM and the iron hydroxide mineral sediment phases.(Yoon et al., 2004; Yang et al., 2009) These mechanisms for SOM binding to Fe(III) oxides most likely only represent a few of the actual mechanisms that exist in natural sediments.

A previous study at the Columbia River Hanford 300 Area hyporheic corridor revealed that in oxic batch reactors containing sand that was previously incubated for 6-weeks in the riverbed of the Columbia River contained highly elevated rates of carbon metabolism, release of DOC, and presence of aerobic and anaerobic heterotrophic bacteria compared with reactors incubated in groundwater wells.(Stern et al., 2017) During in situ incubations, deposition of fresh POC from the river water column took place, and it was this source of OC that lead to the biogeochemical trends observed in the batch reactors. The goal of the current study is to explicitly evaluate the differential capacity of aerobic and anaerobic riverbed sediment microbial communities to metabolize OC derived in the form of either native river bed sediment POC or fresh photosynthetic (periphyton) biomass. Of further interest is the formation of Fe(III) oxide-OC aggregates which are hypothesized to protect labile OC from metabolic degradation.(Kleber, 2010) The two main hypothesis are that addition of fresh autochthonous POC will cause elevated

rates of aerobic and anaerobic respiration and that the presence of reactive Fe(III) oxide minerals will lead to the formation of OC-metal complexes that will protect carbon from biodegradation. Metabolic activity was monitored by measuring total inorganic carbon, methane, aqueous Fe(II), HCl- extractable Fe(II) production; as well as the consumption of DOC, nitrate, and sulfate. To determine the formation of Fe(III) oxide-OC aggregates we utilized the sodium pyrophosphate (Na-PPi) extraction at pH 7, known to target organo-metal complexes.(Claff et al., 2010; Bakina and Orlova, 2012; Heckman et al., 2018) Understanding how inputs of fresh POM, with or without the presence of a reactive Fe(III) oxide mineral phase, affects microbial metabolism and biogeochemistry in riverbed sediments provides new insights into freshwater C and Fe cycling processes.

4.3 Materials and methods

4.3.1 Hanford 300 Area hyporheic corridor sample collection

The Hanford 300 Area site is located on the north side of the Hanford Reach of the Columbia River in Richland, Washington, USA, a ca. 80-km free-flowing section of river below Priest Rapids Dam. Columbia Riverbed sediment, water, and fresh periphyton sample materials used for this experiment were collected on October 4th, 2017 at 46°22'21.553"N, 119°16'18.828"W at Hanford Area 300. Bulk nearshore riverbed sediment from just below the armored layer was collected and transferred into 1 L sterile Ball Jars. The natural Columbia River water that served as reactor fluid was collected and stored in sterile, acid-washed Nalgene 2 L Teflon FEP bottles (Thermo Fisher). Testing showed that the containers did not leach detectable amounts of DOC.

Freshwater periphyton algae samples were scraped directly from submerged rock surfaces into 50 mL Falcon tubes. All samples were stored immediately at 4° C.

4.3.2 Riverbed sediment sample preparation

Riverbed sediment was passed through a 106-micrometer sieve using DI water to isolate the fine-grained material. After a 24-hour settling period, the overlying water was removed and the remaining native OC and mineral mixture “Columbia Riverbed Fines” (CRF) material was freeze-dried. Two 100 g portions of the native CRF material were divided for further laboratory manipulation to create the citrate-bicarbonate-dithionite extracted Columbia riverbed fines (CBD-CRF), for removal the reactive Fe(III) oxide phase (Mehra and Jackson, 1960; Gleyzes et al., 2002; Poulton and Canfield, 2005); and Fe(III) oxide amended Columbia riverbed fines (Fe-CRF) to increase the overall surface area available for OC-Fe(III) oxide aggregate formation. The Fe-CRF material was created by rapid oxidation of 0.05 M anoxic FeCl₂ solution with the CRF in a 1 M NaHCO₃ buffer using an overhead stirrer. (Roden et al., 2000) The fresh POM used in this experiment was grown using the rock-scraped fresh POM sample collected as an inoculum in a media of 100 µM NH₄Cl, 10 µM KH₂PO₄ and 1 ml L⁻¹ trace element and vitamin solution for a 14-day growth period under full spectrum light growth conditions to promote optimal photosynthetic microbial community growth. Natural Columbia Riverbed rocks remaining from the CRF sieving procedure were used to promote growth of periphyton (Figure 4.S1). After the 14-day growth period, the POM suspension was concentrated by centrifugation. All prepared biological materials were freeze-dried before reactor assembly.

4.3.3 Experimental design

A batch reactor experiment was conducted using the CRF, Fe-CRF or CBD-CRF with or without the addition of freeze-dried POM under both oxic and anoxic conditions. Duplicate reactors containing 5 g of CRF, Fe-CRF or CBD-CRF were combined with 100 mL of Columbia River water with or without the addition of 40 mg of POM in 160 mL incubation bottles (Figure 4.S2). Anoxic reactors were flushed with pure N₂ for 30 minutes before sealing. All reactors were pasteurized at 80° C for 30 minutes, cooled to room temperature, then inoculated with 2 mL of Columbia Riverbed sediment slurry. Reactors were stored sideways shaking at 160 rpm (previous experiments showed that this was required to maintain O₂ diffusion throughout the oxic reactor sediments) at 25° C.

4.3.4 Analytical methods

CH₄ and total dissolved inorganic carbon measurements were made with a gas chromatograph (GC 14A, Shimadzu, Japan) equipped with a flame ionization detector as previously described.(Roden and Wetzel, 1996) TIC was calculated by summing the CO₂ produced in the headspace of the reactors with the dissolved inorganic carbon produced in the reactor fluid. Dissolved inorganic carbon was determined by combining 800 µL of centrifuged reactor sample with 800 µL of 0.5 M HCL in a 16 mL sealed reaction bottle, after 4 hours of reaction time the CO₂ released from solution by acidification was measured. Iron reduction was determined by monitoring the accumulation of aqueous and 0.5M HCl-extractable Fe(II) using the ferrozine assay.(Stookey, 1970) DOC was determined with Sievers M5310 C TOC analyzer with a GE autosampler. Dissolved organic carbon was removed in the instrument by acidification of the sample with 2 M HCl with a 5-minute sparging time. The sample was then injected into the

furnace set to 680 °C. Organic carbon standards were made from potassium hydrogen phthalate solid, (Acros Organics). The calibration range for the DOC determinations was from 0.10 to 10 mg C L⁻¹. Fractional C and N content of the CRF, Fe-CRF and CBD-CRF starting materials were determined by flash combustion on finely ground subsamples of dried material (20 mg) using a Flash EA 1112 CN Automatic Elemental Analyzer (Thermo Finnigan, Milan, Italy). Iron bound carbon was determined by Na-pyrophosphate extraction which targets carbon bound in organo-metal complexes.(Claff et al., 2010; Bakina and Orlova, 2012; Heckman et al., 2018) 13 mL of 0.1 M Na-pyrophosphate adjusted to pH 7 was combined with 2 mL of reactor sample in 15 mL Falcon tubes. Anoxic reactor extractions were performed inside the anaerobic glove bag. Samples were maintained at 25° C and vortexed every 15 minutes for 1 hour. After centrifugation at 5000 rpm for 10 minutes supernatant was measured for DOC content. Nitrate and sulfate concentrations were measured with a Dionex ICS-2100 ion chromatography system (Sunnyvale, CA, USA).

4.4 Results

4.4.1 Riverbed sediment properties

The native sieved, freeze-dried Columbia Riverbed sediment contained 1.38 % POC and 0.16 % PON (Table 4.S1). The iron-coated CRF (Fe-CRF) had 25 % less N and 11 % less total C overall. The CBD extracted CRF (CBD-CRF) had 43 % less PON and 47 % less POC percentage (Table S1). The total reactive Fe content of the CRF was 23.5 µmol/g; this value was 229 % higher in the Fe-CRF, and 89 % lower in the CBD-CRF sediment (Table 4.S1).

4.4.2 Total inorganic carbon and methane production

For both the anoxic and oxic reactors, the addition of 8.73 mmol L^{-1} of fresh POC resulted in an approximate 2-fold increase in TIC production (Figure 4.1). Anoxic CBD reactors showed much slower rates of TIC release compared to both the CRF and the Fe-CRF sediments. The Fe-CRF reactors did not show a decrease in TIC compared with the native CRF sediment (Figure 4.1). The largest amount of methane production occurred in the anoxic reactors with added fresh POM, with the highest values observed in the CRF+POM N_2 reactors with an average value of $0.5 \text{ mmol L}^{-1} \text{ CH}_4$ (Figure 4.2). The largest amount of methane produced in any reactor that was not amended with fresh POM was 10 times lower. Small amounts of methane production, up to $2.1 \text{ } \mu\text{mol L}^{-1}$, was observed in the oxic reactors at late timepoints possibly due to the development of anoxic patches within the sediment slurry. Anoxic utilization of added fresh POM was highest in the iron amended reactors with $27.7 \pm 3.0 \%$ oxidation of C to inorganic carbon (IC) and methane (Figure 4.3 and Table 4.S2).

4.4.3 Iron reduction trends

The increased Fe(III) content in the Fe-CRF+POM N_2 compared to the CRF+POM N_2 reactors led to a ca. 3-fold increase in Fe(III) reduction (Figure 4.4). Similar to the TIC trends, there was a ca. 2-fold increase in overall iron reduction with the addition of fresh POM. Fe-CRF+POM N_2 produced 12.5 mmol L^{-1} of 0.5M HCL-extractable Fe(II) while Fe-CRF N_2 produced 7.3 mmol L^{-1} . Anoxic CBD-CRF reactors, which had the reactive Fe(III) oxide phase removed prior to incubation, showed much lower Fe(III) reduction compared to the other reactor conditions and were overall similar to the oxic CBD-CRF reactors (Figure 4.4). Oxic reactors containing

reactive Fe(III) oxide phases (CRF and Fe-CRF) resulted in nearly undetectable amounts of aqueous Fe(II) (Figure 4.S3).

4.4.4 Dissolved and Na-PPi extractable OC trends

All oxic reactors except CBD-CRF O₂ without added fresh POM resulted in rapid depletion of the DOC carbon pool during initial time points (Figure 4.5). CBD-CRF O₂ reactors without added fresh POM resulted in slower metabolism of DOC over time. DOC concentrations in the anoxic reactors remained relatively stable over time with CBD-CRF reactors showing overall higher total DOC values. The addition of fresh POM increased the DOC concentrations in all anoxic reactors, and in contrast to the oxic reactors, depletion of DOC was not observed. In the anoxic reactors approximately 2 mmol L⁻¹ of the original added 8.73 mmol L⁻¹ fresh periphyton POC was observed as DOC (Figure 4.5). Fe-CRF reactors overall had the lowest DOC values while CBD-CRF reactors had the highest DOC values overall.

The Na-PPi extractions revealed that overall anoxic CBD-CRF sediments contained the lowest concentration of organo-metal complexes (Figure 4.6 and 4.S4). There was little difference between the Na-PPi extractable DOC values for the +POM and the -POM reactor conditions overall (Figure 4.6 and 4.S4). Na-PPi extractable DOC values remained relatively constant over time in all reactors, with anoxic Fe-CRF reactors showing the highest Na-PPi extractable DOC concentrations. In the oxic reactors overall approximately 6 % of the original carbon content of each reactor condition was observed as Na-ppi extractable DOC (Figure 4.S4). In the anoxic reactors this value increased to 12 % for Fe-CRF reactors and decreased to 2 % for the CBD-CRF reactors.

4.4.5 Nitrate and Sulfate trends

Nitrate was rapidly consumed in the anoxic reactors, whereas net accumulation of nitrate took place in the oxic reactors, with increased levels observed in the POM-amended slurries (Figure 4.7). Sulfate remained stable over time in the oxic reactors (Figures 4.8) but was consumed within the first 20 days for the anoxic reactors with added POM. Sulfate consumption took place more gradually in the anoxic reactors without added POM (Figure 4.8).

4.5 Discussion

4.5.1 Bulk OC metabolism

The addition of fresh POM stimulated a rapid increase in TIC production, methane production, overall DOC content, and iron reduction. $34.3 \pm 7.2\%$ of the total fresh POM carbon added to the oxic reactors was converted to IC and methane, compared to $18.1 \pm 3.4\%$ in the anoxic reactors (Figure 4.3 and Table 4.S2). The 2-fold difference in TIC between anoxic and oxic reactors with added fresh POM observed in this experiment agrees well with previous experiments. (Kristensen et al., 1995; Goedkoop et al., 1997), who showed that... Interestingly, metabolism increased 2-fold with just 15 % increase in overall carbon content. Comparing the TIC and methane production with the initial carbon content of the reactors the fresh POM was 5 times more labile than the native CRF material in the oxic reactors and 6 times more labile in the anoxic CRF reactors (Figure 4.3 and Table 4.S2). Although numerous previous studies in soil systems have demonstrated “priming” of endogenous POM metabolism through addition of fresh POM (cite references from Emily), a recent study using natural stream biofilms from the Oberer Seebach reach in Austria demonstrated that autochthonous carbon inputs did not show priming effects on

removal or incorporation of allochthonous organic matter (Bengtsson et al., 2014). Based on the similarity of our experiments to those of Bengtsson et al. (2014), we assume that the increase in carbon utilization rates in the +POM reactors resulted from direct metabolism of the added fresh POM. A similar methane stimulation by algae biomass additions was observed previously by Schwarz et al. (2008) and Liang et al. (2016).

4.5.2 Pathways of OC metabolism

The OC metabolism pathways investigated in this experiment were aerobic respiration, nitrate reduction, iron reduction, sulfate reduction, and methanogenesis. Although aerobic respiration was the dominant pathway of OC metabolism, in the anoxic reactors several different pathways of anaerobic respiration were active at different time points. In the oxic reactors there was small amounts of methane production and Fe(III) reduction hinting at the presence of anaerobic metabolism patches within the predominantly oxic sediment matrix. In the anoxic reactors the small amount of nitrate that were initially provided by the natural river water media solution was rapidly consumed during the initial time points, while sulfate reduction slower rate of reduction over time with -POM reactors showing sulfate reduction at late time points (Figure 4.8). Unlike all the other measured parameters nitrate trends were not affected by the addition of fresh POM. Fe(III) reduction was the most active metabolic pathway in both the +POM and -POM anoxic reactors (Figure 4.9, Table 4.S3 and 4.S4). Sulfate reduction had the highest percentage of total electron acceptor utilization in anoxic CBD-CRF reactors while methanogenesis had the highest percent of total electron acceptor utilization in anoxic +POM reactors (Figure 4.9, Table 4.S3 and 4.S4). The overall lower utilization of OC, consumption of DOC and Fe(III) reduction in the anoxic CBD-CRF reactors was most likely related to the elevated pH values observed in these

reactors (Figure 4.S5). These results indicate that fresh periphyton biomass is rapidly metabolized by riverbed sediment microbial communities and can stimulate production of methane, although Fe(III) reduction was the dominant metabolism pathway in the anoxic reactors with added fresh POC (Figure 4.9, Table 4.S3 and 4.S4). The native CRF material contained reactive Fe(III) oxide minerals that were not completely reduced after 74 days.

4.5.3 Production and turnover of DOC

The rapid consumption of DOC in the oxic reactors agrees well with previous results by Goedkoop et al. (1997). However, interestingly in the Goedkoop experiment, the DOC concentration in the porewater was much lower than the overlying fluid and remained relatively constant. Assuming that most of the microbial activity is happening within the sediment slurry and not in the overlying water, these results suggest that there is a rapid equilibrium between the aqueous DOC and the pore space DOC pools. Opposite of the oxic reactors, DOC was not consumed in the anoxic reactors and remained relatively stable over time. These results demonstrate that in riverbed sediments DOC could be released from anoxic zones, by advective flow and pore space diffusion, to potentially fuel further microbial activity in adjacent groundwater zones where DOC values are lower and in the aerobic riverbed sediments where DOC is rapidly consumed. (Stern et al., 2017)

4.5.4 Influence of Fe-bound OC on metabolism

The Na-PPi extraction revealed that all reactors contained pools of organo-metal complexes that remained relatively stable throughout the 74-day experiment. In the presence of oxygen, the

percent of the total carbon content bound in organo-metal complexes was similar for all reactor conditions and remained stable at approximately 6 %. However, in the anoxic reactors the percent of the total carbon content bound in organo-metal complexes varied widely depending on reactor condition (Figure 4.S4). The highest value observed was 12.8 % in the Fe-CRF -POM N₂ reactor, while the lowest value observed was in the CBD-CRF -POM N₂ reactor at 1.6 % (Figure 4.S4). These results show that in anoxic environments the formation of organo-metal complexes does depend on the reactive iron oxide content of the sediment. More investigation is needed to understand these dynamics in oxic sediments.

Because DOC was consumed in the oxic reactors while the Na-PPi extractable carbon pool remained constant, it can be assumed that there is little or no exchange between the organo-metal complexed carbon pool and the pore space or overlying water DOC pool. The Fe-CRF material had the largest organo-metal carbon pool, however this small increase was not proportional to the overall increase in reactive Fe(III) oxide present. Interestingly, in all reactors all of the forms of metabolism mentioned above were occurring while the organo-metal carbon pools remained unchanged. The lack of exchange between the DOC carbon pool and the organo-metal complexed carbon pool shows that the organo-metal complexed carbon pool was not metabolized. Because of the presence of other more labile pools of OC, such as fresh periphyton POM and DOC, were available for microbial metabolism in this experiment, it was not explicitly tested if organo-metal complexed carbon is protected from microbial metabolism.

4.5.5 Environmental Implications

The hyporheic corridor has been identified as a microbial “hotspot” and a critical part of the freshwater carbon cycle.(McClain et al., 2003; Trimmer et al., 2012a) Previous studies at the

Columbia River Hanford Area 300 demonstrated that POM was more influential on microbial activity than the DOC.(Stern et al., 2017) This study further elaborates our understanding of the influence of POM on sediment microbial community activity. In zones where autochthonous photosynthetic biomass is deposited, aerobic respiration, nitrate reduction, sulfate reduction, methanogenesis, and Fe(III) oxide reduction can be expected (Figure 4.S6). The formation of organo-metal complexes that protect OC from microbial metabolism can be expected to vary based on the amount of reactive Fe(III) oxide phase present for complex formation, however further studies are needed to understand if this pool of protected OC is susceptible to biodegradation.

The partitioning between oxic and anoxic zones in riverbeds is highly dynamic and depends on the oxygen penetration depth, which varies depending on both microbial activity and the water flow/diffusion properties of the sediment matrix.(Greig et al., 2007) The presence of small patches of methanogenesis and Fe(III) reduction within the aerobic reactor sediments showed that microbial metabolism within the hyporheic zone is highly heterogenous with zones of anaerobic respiration and methanogenesis existing within the predominantly oxic layer.(Boulton et al., 1991; Brune et al., 2000) In the natural environment, sulfate and nitrate reduction, which occurred only in the first few time points, is likely occurring more continuously as movement of fluid through water flow and pore space diffusion would provide additional sources. Depending on river stage, water can move from the hyporheic zone into the groundwater zone or from the hyporheic zone into the river. Although DOC is not the main driver of metabolic activity, water movement through riverbed sediment anoxic zones will release accumulated DOC (Figure 4.S6) that can be quickly metabolized in zones of aerobic respiration, as observed in this experiment. The fact that in the anoxic reactors methanogenesis,

Fe(III) reduction, nitrate reduction and sulfate reduction all occurred throughout the experiment shows that microbial metabolism in river bed sediments are highly dynamic, metabolically flexible, and interconnected.

4.6 Figures

4.6.1 Figure (4.1) Total inorganic carbon production

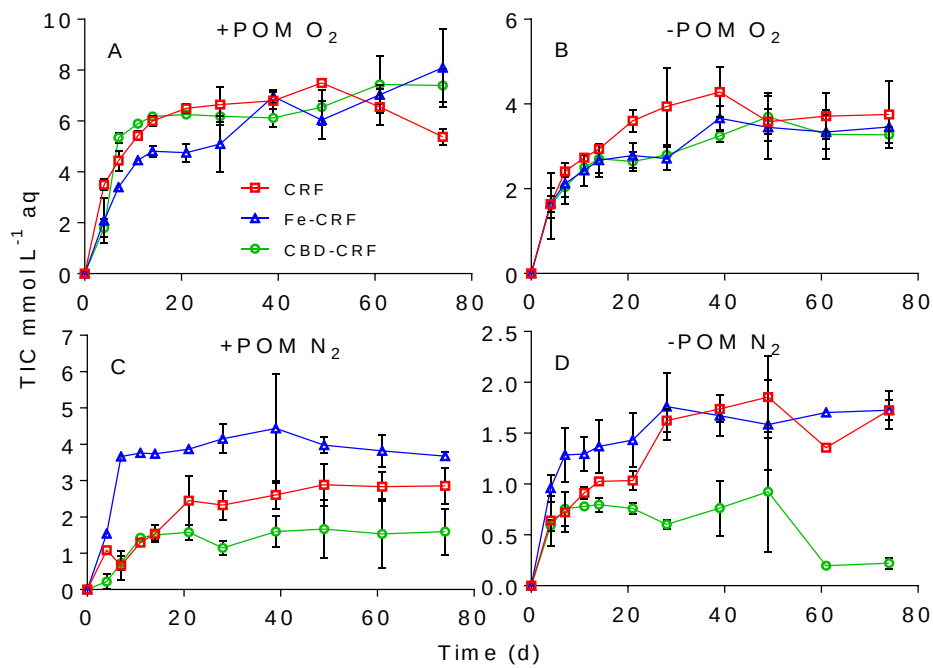


Figure 4.1. Total Inorganic Carbon Production for reactor conditions: (A) Aerobic with algae/periphyton POM added, (B) Aerobic without algae/periphyton POM added, (C) Anoxic with algae/periphyton POM added, (D) Anoxic without algae/periphyton POM added. Values represent averages of duplicate reactors with error bars representing range.

4.6.2 Figure (4.2) Total methane production

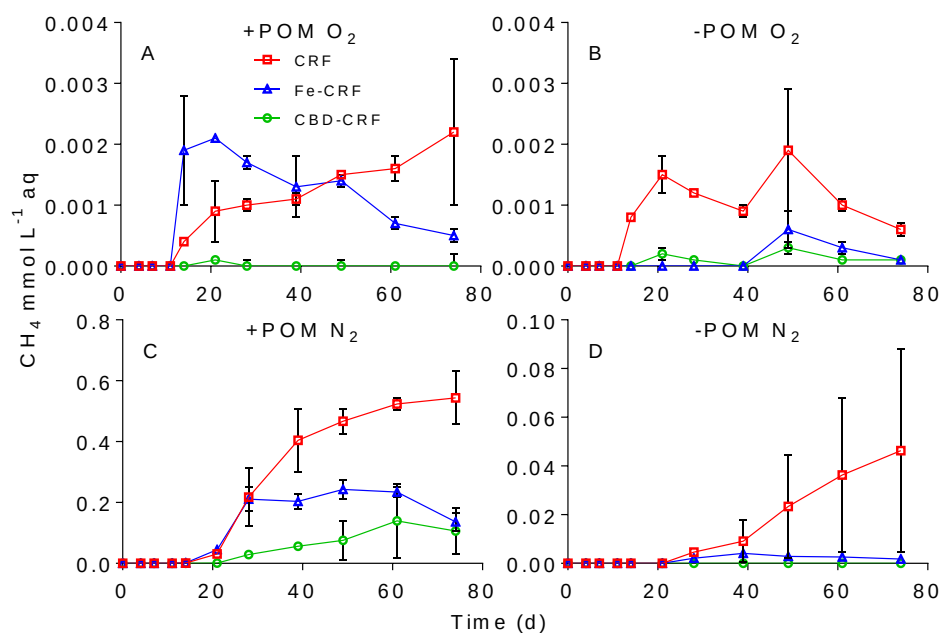


Figure 4.2. Total Methane Production for reactor conditions: (A) Aerobic with algae/periphyton POM added, (B) Aerobic without algae/periphyton POM added, (C) Anoxic with algae/periphyton POM added, (D) Anoxic without algae/periphyton POM added. Values represent averages of duplicate reactors with error bars representing range.

4.6.3 Figure (4.3) Percent total carbon content utilization

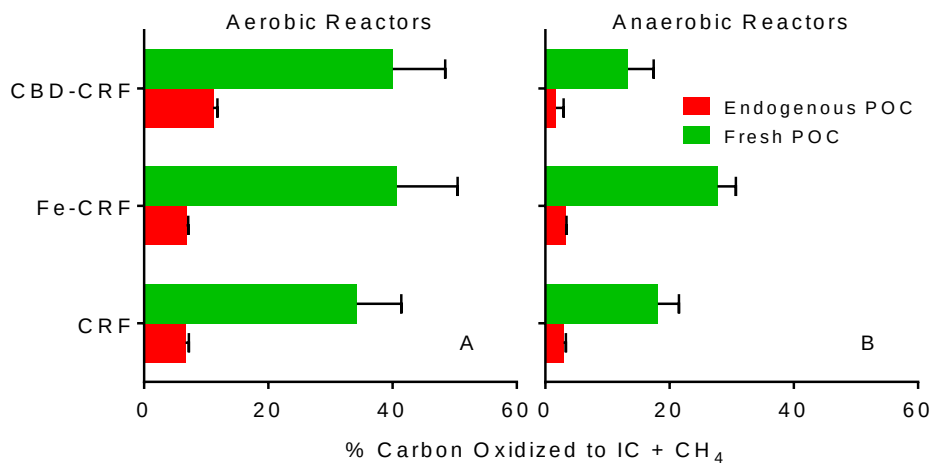


Figure 4.3. Percent total carbon content converted to inorganic carbon (IC) and methane (CH₄) for aerobic (A) and anaerobic (B) reactors. Endogenous POC represents the CRF/Fe-CRF/CBD-CRF material without the addition of fresh POC. Values represent the average of the final four timepoints in the experiment.

4.6.4 Figure (4.4) 0.5 M HCl extractable Fe(II)

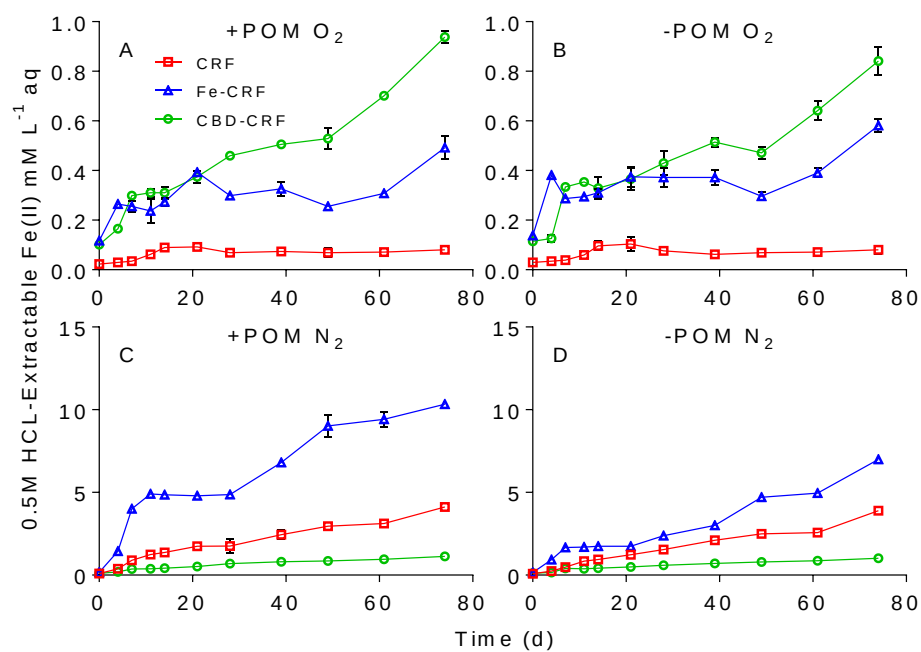


Figure 4.4. 0.5 M HCL extractable Fe(II) production for reactor conditions: (A) Aerobic with algae/periphyton POM added, (B) Aerobic without algae/periphyton POM added, (C) Anoxic with algae/periphyton POM added, (D) Anoxic without algae/periphyton POM added. Values represent averages of duplicate reactors with error bars representing range.

4.6.5 Figure (4.5) Dissolved organic carbon content

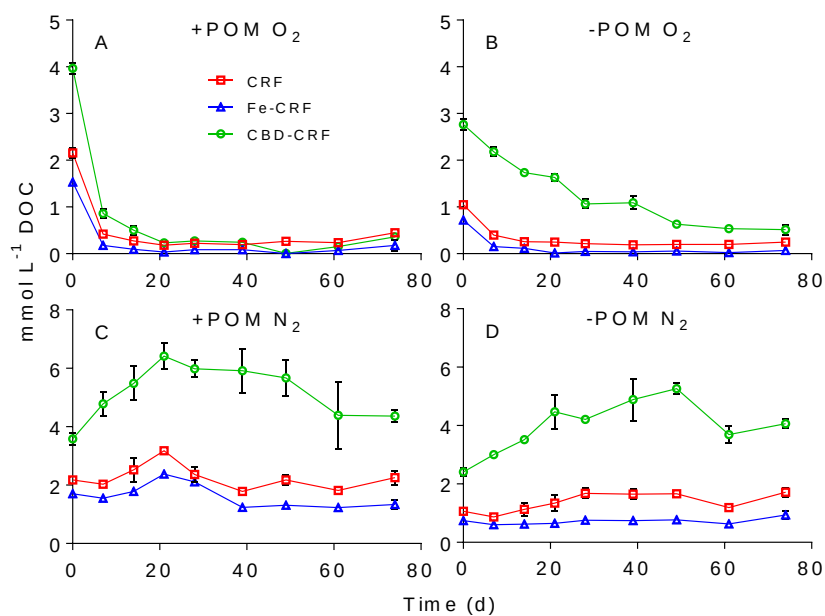


Figure 4.5. Dissolved organic carbon content for reactor conditions: (A) Aerobic with algae/periphyton POM added, (B) Aerobic without algae/periphyton POM added, (C) Anoxic with algae/periphyton POM added, (D) Anoxic without algae/periphyton POM added. Values represent averages of duplicate reactors with error bars representing range.

4.6.6 Figure (4.6) Organo-metal complexed organic carbon

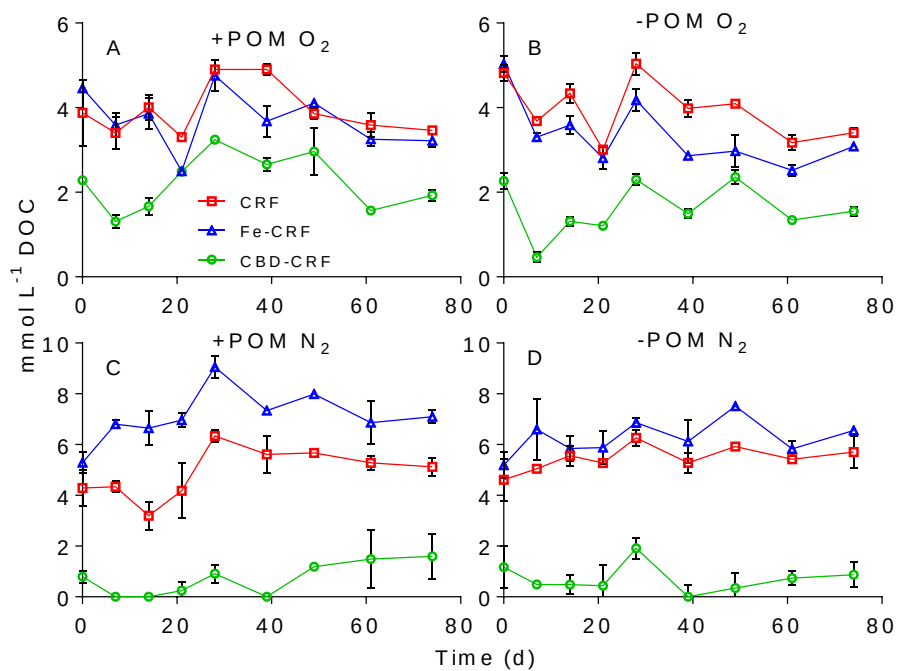


Figure 4.6. Organo-metal complexed carbon content by sodium pyrophosphate extraction for reactor conditions: (A) Aerobic with algae/periphyton POM added, (B) Aerobic without algae/periphyton POM added, (C) Anoxic with algae/periphyton POM added, (D) Anoxic without algae/periphyton POM added. Values represent averages of duplicate reactors with error bars representing range.

4.6.7 Figure (4.7) Nitrate Content

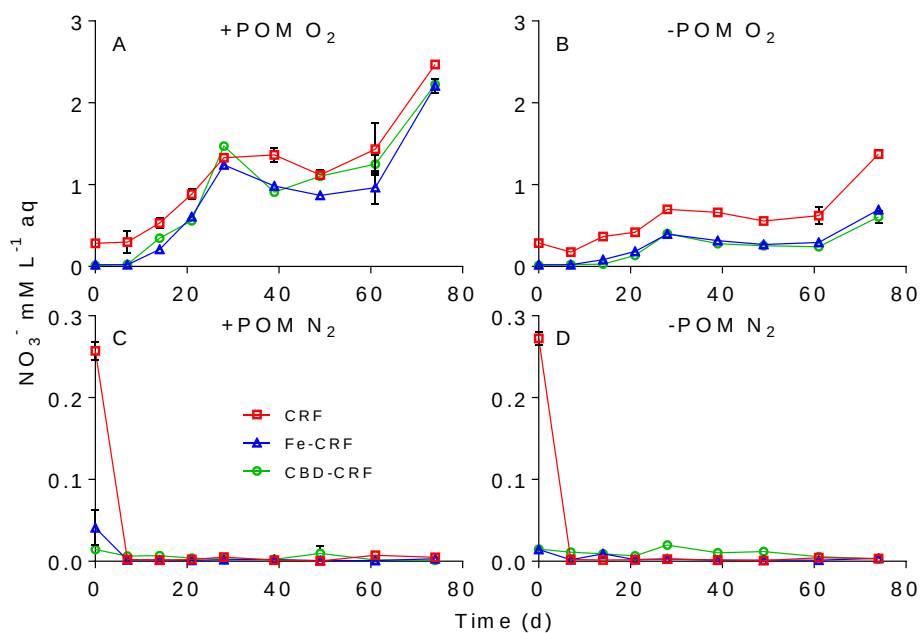


Figure 4.7. Nitrate concentrations for reactor conditions: (A) Aerobic with algae/periphyton POM added, (B) Aerobic without algae/periphyton POM added, (C) Anoxic with algae/periphyton POM added, (D) Anoxic without algae/periphyton POM added. Values represent averages of duplicate reactors with error bars representing range.

4.6.8 Figure (4.8) Sulfate content

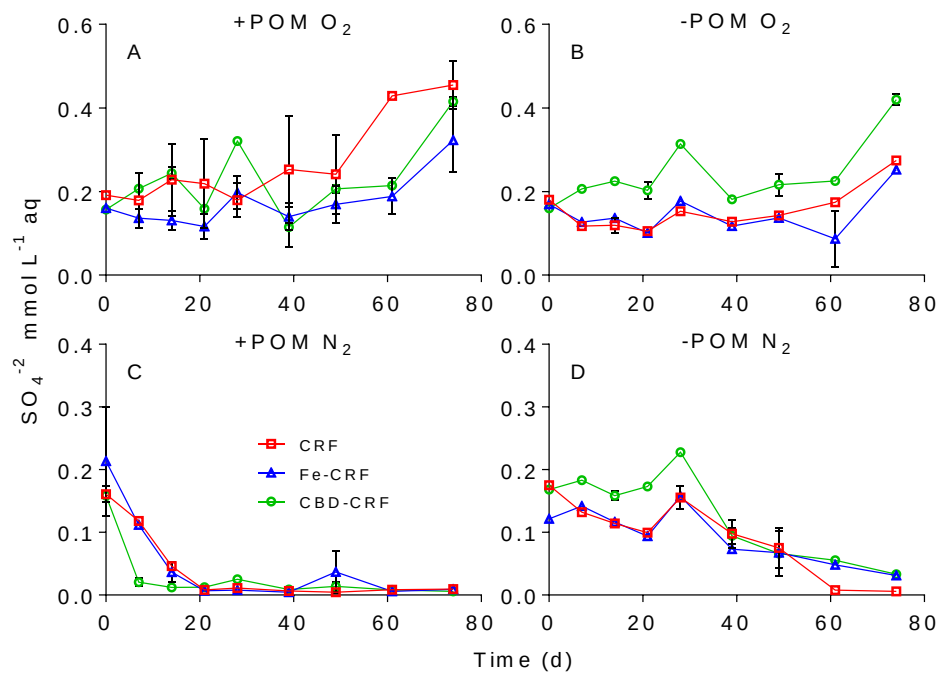


Figure 4.8. Sulfate concentrations for reactor conditions: (A) Aerobic with algae/periphyton POM added, (B) Aerobic without algae/periphyton POM added, (C) Anoxic with algae/periphyton POM added, (D) Anoxic without algae/periphyton POM added. Values represent averages of duplicate reactors with error bars representing range.

4.6.9 Figure (4.9) Percent total respiration and percent total electron acceptor utilization

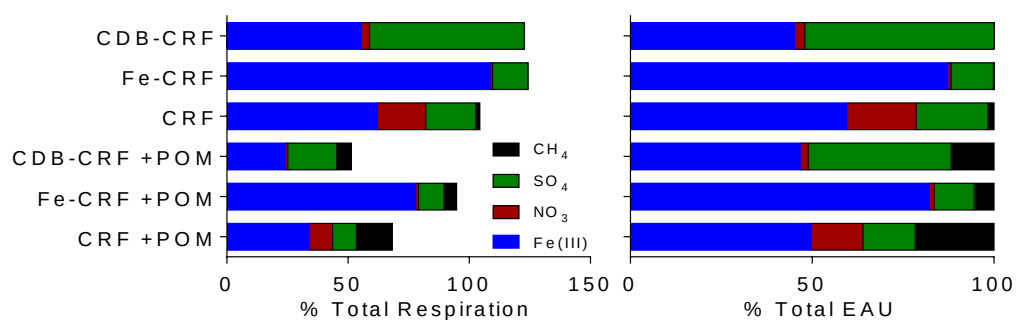


Figure 4.9. % Total respiration and % total electron acceptor utilization for all anoxic reactor conditions. % total respiration is calculated based on the average TIC and methane production at the final four time points. % electron acceptor utilization was calculated based on the sum of the average methane production, and Fe(III), SO₄, and NO₃ reduction for the final four timepoints.

4.7 Supplemental material

4.7.1 Figure (S.41) Image of periphyton growth

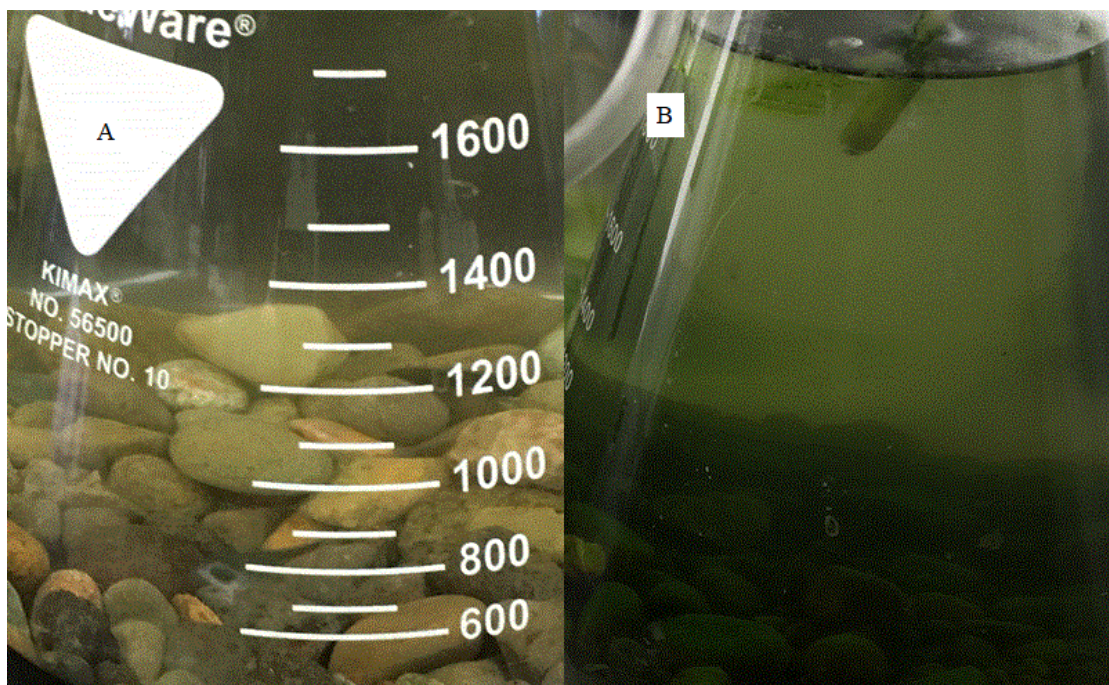


Figure 4.S1. Images of (A) pre and (B) post growth period algae/periphyton growth conditions.

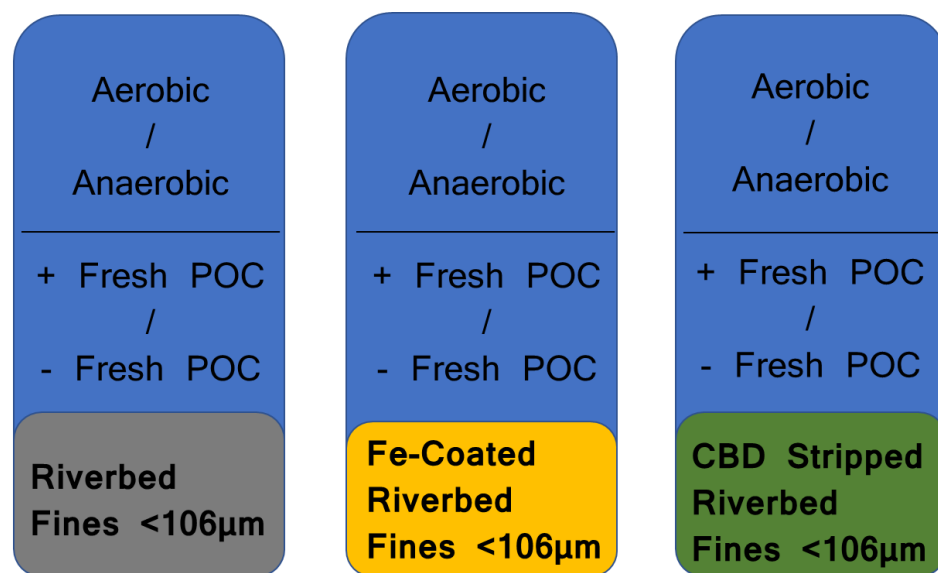
4.7.2 Figure (4.S2) Schematic diagram of reactor setup

Figure 4.S2. Schematic diagram of reactor setup.

4.7.3 Figure (4.S3) Aqueous Fe(II) content

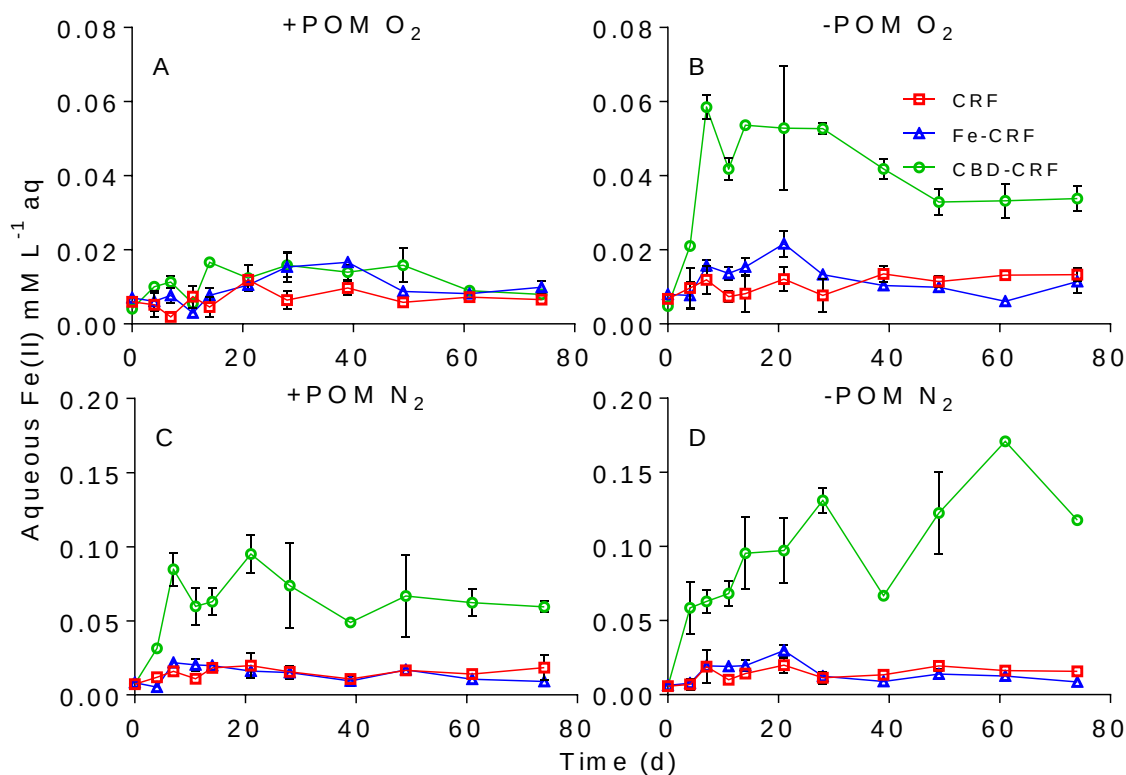


Figure 4.S3. Aqueous Fe(II) Production for reactor conditions: (A) Aerobic with algae/periphyton POM added, (B) Aerobic without algae/periphyton POM added, (C) Anoxic with algae/periphyton POM added, (D) Anoxic without algae/periphyton POM added. Values represent averages of duplicate reactors with error bars representing range.

4.7.4 Figure (4.S4) Organo-metal complexed carbon content

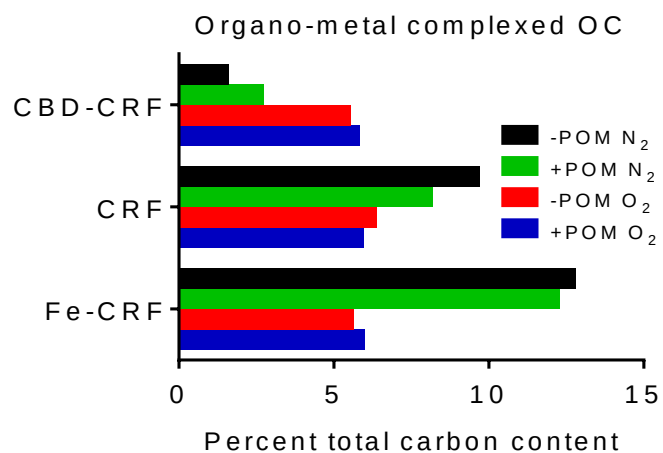


Figure 4.S4. Percent of total carbon content observed complexed in organo-metal aggregates.

Values represent the average Na-ppi extractable DOC for the final four time points normalized to the original carbon content at the start of the experiment for each reactor condition.

4.7.5 Figure (4.S5) pH values

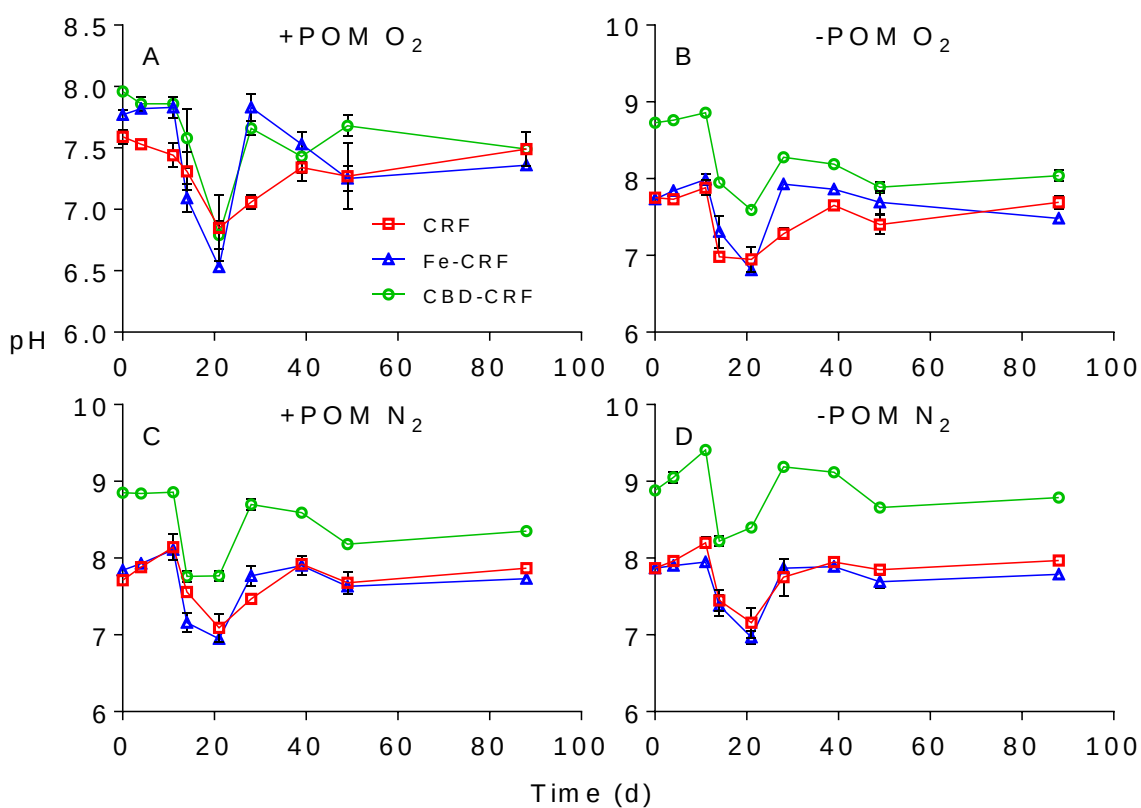


Figure 4.S5. pH values for reactor conditions: (A) Aerobic with algae/periphyton POM added, (B) Aerobic without algae/periphyton POM added, (C) Anoxic with algae/periphyton POM added, (D) Anoxic without algae/periphyton POM added. Values represent averages of duplicate reactors with error bars representing range.

4.7.6 Figure (4.S6) Conceptual model of hyporheic corridor biogeochemical interactions

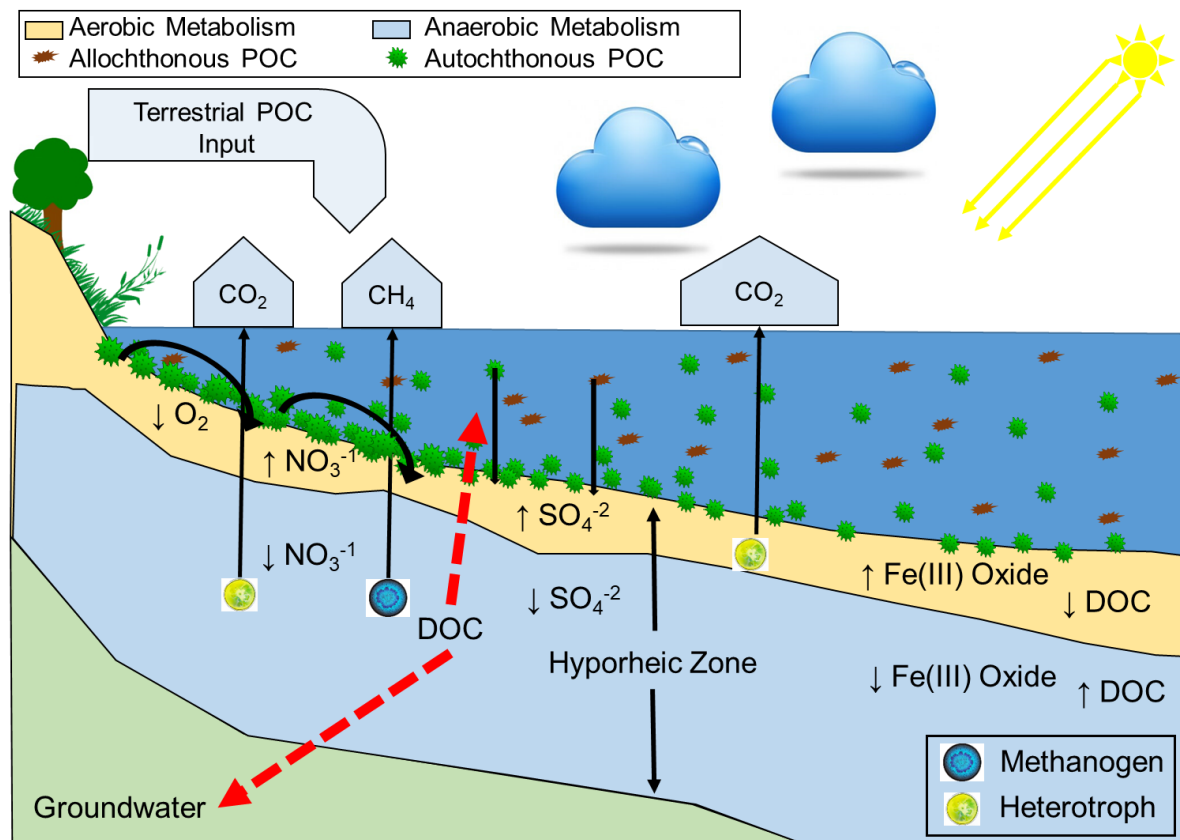


Figure 4.S6. Conceptual model of the biogeochemical processes related to fresh algae/periphyton POC metabolism in the hyporheic corridor.

4.6.8 Table (4.S1) Nitrogen, carbon and iron contents for the riverbed sediment materials

Table 4.S1. Nitrogen, Carbon, and Iron contents for CRF, Fe-CRF and CBD-CRF materials.

Nitrogen and Carbon contents for the freeze-dried fresh POM.

	% N	% C	$\mu\text{M/g Fe(III)}$
CRF	0.16	1.38	23.53
Fe-CRF	0.12	1.22	77.47
CBD-CRF	0.09	0.73	2.43
Fresh POM	3.8	26.24	

4.6.9 Table (4.S2) Percent oxidation of varying carbon sources to inorganic carbon and methane

Table 4.S2. Percent oxidation of endogenous or added POC to inorganic carbon (IC), methane (CH₄), or inorganic carbon and methane combined (IC+CH₄).

System	Endogenous POC (mmol/L) ^a	% Endogenous POC Oxidized to IC ^c	% Endogenous POC Oxidized to CH ₄ ^c	% Endogenous POC Oxidized to IC+CH ₄ ^c
CR Fines, O ₂	57.50	6.67 ± 0.54	0.00 ± 0.00	6.67 ± 0.54
CR Fines, N ₂	57.50	2.90 ± 0.38	0.05 ± 0.03	2.95 ± 0.37
Fe-CR Fines, O ₂	50.83	6.84 ± 0.27	0.00 ± 0.00	6.84 ± 0.27
Fe-CR Fines, N ₂	50.83	3.29 ± 0.12	0.01 ± 0.00	3.29 ± 0.12
CDB-CR Fines, O ₂	30.42	11.10 ± 0.71	0.00 ± 0.00	11.10 ± 0.71
CDB-CR Fines, N ₂	30.42	1.73 ± 1.22	0.00 ± 0.00	1.73 ± 1.22
	Added POC (mmol/L) ^b	% Added POC Oxidized to IC ^d	% Added POC Oxidized to CH ₄ ^d	% Added POC Oxidized to IC+CH ₄ ^d
CR Fines, O ₂	8.73	31.18 ± 10.79	0.01 ± 0.01	34.26 ± 7.16
CR Fines, N ₂	8.73	12.89 ± 2.95	5.22 ± 0.53	18.11 ± 3.39
Fe-CR Fines, O ₂	8.73	40.70 ± 9.78	0.01 ± 0.00	40.70 ± 9.78
Fe-CR Fines, N ₂	8.73	25.31 ± 2.50	2.40 ± 0.57	27.71 ± 2.99
CDB-CR Fines, O ₂	8.73	40.06 ± 8.43	0.00 ± 0.00	40.06 ± 8.43
CDB-CR Fines, N ₂	8.73	12.25 ± 3.77	1.08 ± 0.42	13.33 ± 4.13

^a Based on the measured POC content of the materials (see Table S1) and the mass of material added to each reactor.

^b Based on the POC content of the algae/periphyton and the mass of material added to each reactor.

^c Computed from the average of IC and CH₄ values for the final four time points (days 39-74) in the -POM reactors.

^d Computed from the average of the difference between IC and CH₄ in the +POM vs. -POM reactors for the final four time points (days 39-74).

4.6.10 Table (4.S3) Percent total respiration and total electron acceptor utilization in the anoxic reactors

Table 4.S3. Percent of total respiration or total electron acceptor utilization accounted for by different terminal electron-accepting pathways in the anoxic reactors. Percent total respiration represents the total electrons donated to anaerobic metabolism pathways divided by the average electrons donated to TIC production for the final four timepoints Percent total electron acceptor utilization represents the total electrons donated to all anaerobic metabolism pathways divided by the total electrons donated for each individual anaerobic metabolism pathway for the final four time points.

Treatment	Pathway	% Total Respiration	% Total Electron Acceptor Utilization
CRF +POM	Fe(III) reduction	33.9	49.6
	Nitrate reduction	9.8	14.4
	Sulfate reduction	9.8	14.4
	CH ₄ production	14.8	21.7
	Total	68.3	
CRF -POM	Fe(III) reduction	62.0	59.4
	Nitrate reduction	20.1	19.2
	Sulfate reduction	20.6	19.8
	CH ₄ production	1.7	1.6
	Total	104.4	
Fe-CRF +POM	Fe(III) reduction	77.9	82.2
	Nitrate reduction	1.2	1.3
	Sulfate reduction	10.4	11.0
	CH ₄ production	5.2	5.5
	Total	94.8	
Fe-CRF -POM	Fe(III) reduction	108.5	87.3
	Nitrate reduction	1.1	0.9
	Sulfate reduction	14.5	11.7
	CH ₄ production	0.2	0.1
	Total	124.3	

CDB-CRF +POM	Fe(III) reduction	24.0	46.7
	Nitrate reduction	1.1	2.2
	Sulfate reduction	20.2	39.3
	CH ₄ production	6.0	11.7
	Total	51.4	
CDB-CRF - POM	Fe(III) reduction	55.4	45.1
	Nitrate reduction	3.5	2.9
	Sulfate reduction	63.9	52.0
	CH ₄ production	0.0	0.0
	Total	122.8	
+POM Mean		71.5	
+POM SD		21.86	
-POM Mean		117.1	
-POM SD		11.06	
Grand Mean		94.3	
Grand SD		29.4	

4.6.11 Table (4.S4) Mean percent of total respiration and total electron acceptor utilization for the +POM and -POM reactor conditions

Table 4.S4. Mean percent of total respiration or total electron acceptor utilization accounted for by different terminal electron-accepting pathways in the anoxic reactors. Percent total respiration represents the total electrons donated to anaerobic metabolism pathways divided by the average electrons donated to TIC production for the final four timepoints Percent total electron acceptor utilization represents the total electrons donated to all anaerobic metabolism pathways divided by the total electrons donated for each individual anaerobic metabolism pathway for the final four time points.

Pathway	% Total Respiration					
	+POM		-POM		Grand Mean	
	Mean	SD	Mean	SD	Mean	SD
Fe(III) reduction	45.3	28.7	75.3	29.0	60.3	30.6
Nitrate reduction	4.1	5.0	8.2	10.3	6.1	7.6
Sulfate reduction	13.5	5.8	33.0	26.9	23.2	20.4
CH ₄ production	8.7	5.3	0.6	0.9	4.6	5.6

Pathway	% Total Electron Acceptor Utilization					
	+POM		-POM		Grand Mean	
	Mean	SD	Mean	SD	Mean	SD
Fe(III) reduction	59.5	19.7	63.9	21.5	61.7	18.6
Nitrate reduction	6.0	7.3	7.7	10.1	6.8	7.9
Sulfate reduction	21.6	15.5	27.8	21.4	24.7	17.0
CH ₄ production	13.0	8.2	0.6	0.9	6.8	8.5

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5.0 Conclusion

As atmospheric concentrations of carbon dioxide continue to climb past 410 ppm due to anthropogenic inputs it is critical to study and understand how to control global carbon emissions. The global soil and sediment OC pool is at least three times larger than the pool of all living plants and the atmosphere combined. Fluxes of carbon to the atmosphere from soils and sediments globally are greater than all anthropogenic carbon emissions combined. There for understanding what influences carbon emissions from soil and sediment microbial communities is important for the global carbon cycle. The research in this document has focused on freshwater hyporheic zone and adjacent groundwater zone microbial community metabolism because it represents a hotspot in microbial activity where elevated rates of metabolism are observed. This increased microbial activity is a product of constant river and groundwater mixing and also because of constant carbon inputs from the river water column. This research has focused on what forms of organic carbon inputs to the riverbed sediment matrix cause the observed elevated rates of microbial metabolism.

In chapter two it was demonstrated that humic acids, a previously thought to be recalcitrant form of organic carbon, can be anaerobically metabolized producing labile byproducts that can contribute to dissimilatory iron reduction. Based on the metagenomic analysis the microorganisms that were involved in the humic acid degradation were predominantly not dissimilatory reducing bacteria. This shows that in complex microbial communities organisms are involved in complex networks of metabolism utilizing products from one metabolism pathway as substrates for another. The dissimilatory iron reducing bacteria community structure was dependent on the carbon source showing that microbial communities are flexible and will adapt quickly to changes in electron donor availability.

In chapter 3 it was demonstrated that in hyporheic zone sediments DOC can not only be consumed but also be accumulated and released. Inputs of POM from allochthonous and autochthonous sources to the river bed sediment matrix caused this release of DOC and were a major factor that contributed to the elevated rates of metabolism observed in this environment. In reactors that contained sand previously incubated in the Columbia River bed, inputs of allochthonous and autochthonous POM were correlated with presence of aerobic and anaerobic heterotrophic metabolizing organisms. The presence of photosynthetic cyanobacteria (i.e. autochthonous POM) was also observed in the riverbed sand pack microbial communities. Therefore, the deposition of POM will influence the microbial community structure, function as well as the local biogeochemistry in the hyporheic zone.

In chapter 4 it was demonstrated that the deposition of fresh periphyton biomass into the sediment matrix causes organic carbon utilization to increase significantly. Preferential metabolism of the fresh periphyton biomass was also correlated with elevated rates of iron reduction and methanogenesis. The presence of iron reduction and methanogenesis in the oxic reactors showed that within a sediment matrix there are patches of variable metabolism when oxygen gets depleted by microbial activity. DOC was not consumed in the anoxic reactors showing that hyporheic zone sediments can accumulate DOC which will be released with advective water flow and be available for microbial metabolism in zones where oxygen is present. Overall chapters 3 and 4 both provide evidence that POC, specifically periphyton biomass, not DOC is the main contributor to the elevated rates of metabolism observed in hyporheic zone sediments.

Further experiments are required to understand which heterotrophic metabolizing organisms are responsible for the preferential metabolism of periphyton biomass. These

organisms could be large contributors to fluxes carbon dioxide and methane from sediments to the atmosphere globally. Having a better understanding of the metabolic processes involved in freshwater sediment metabolism of periphyton biomass would also contribute to our understanding of the breakdown processes involved in the transformation of POC to DOC. As nitrogen and phosphorous inputs to the natural environment from anthropogenic sources continue to increase global issues with eutrophication of rivers will continue to be a problem. Due to the critical role that periphyton biomass plays in river system gross primary production and now based on this research also in overall ecosystem respiration more research is required to address this issue.