THESIS

IMPACT OF INOCULUM SOURCE AND PRIMARY CARBON SOURCE ON BIOTRANSFORMATION OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS

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ABSTRACT

IMPACT OF INOCULUM SOURCE AND PRIMARY CARBON SOURCE ON BIOTRANSFORMATION OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS

The production and use of pharmaceuticals and personal care products (PPCPs) has been on the rise and the occurrence of PPCPs has been continuously reported in effluents from wastewater treatment plants, indicating that PPCPs are not sufficiently removed by conventional treatment processes. Even at low concentrations, PPCPs may have harmful effects on ecosystems and human health. Biological treatment technologies such as activated sludge-based processes, membrane bioreactors, or soil-based treatment are typically regarded as a cost-effective, environmental-friendly, and can be less energy-intensive than physical-chemical treatment methods; thus, development of biologically-based treatment technologies to treat PPCPs is desirable. Although biotransformation has been demonstrated for various PPCPs, existing technologies still show incomplete and variable removals for many compounds. Appropriate process design and operation of biological treatment systems may enhance removal rates; however, the microorganisms required for efficient PPCP transformation and the operational factors that promote their growth and activity remain largely unknown. Several factors likely influence biotransformation including pH, temperature, hydraulic and biomass retention times, inoculum source, microorganisms present, substrate composition, as well as PPCP concentration. Knowledge of critical factors is needed to support development and design of biotreatment processes with improved efficiency.

Thus, the objective of this study was to investigate the hypotheses that PPCP biotransformation ability is dependent on (1) the microbial community, and (2) the primary carbon sources present. Six PPCPs (diclofenac, 5-fluorouracil, gabapentin, gemfibrozil, ibuprofen, and triclosan) were selected as model compounds in this study. The model PPCPs were selected because previously reported removal rates for these compounds in activated sludge processes and soil aquifer treatment (SAT) systems were generally low and variable, indicating that a better understanding of critical microorganisms and conditions is required to improve treatment efficiency.

To investigate the impact of microbial community composition, three types of inocula were tested: activated sludge (AS), sediment (Sd), and soil from a SAT system (SAT). Activated sludge was obtained from the Drake Water Reclamation Facility (DWRF) (Fort Collins, CO). Sediment was obtained from the Fossil Creek Ditch, which receives effluent from the DWRF. Soil, provided by Trussell Technologies Inc., was obtained from SAT column reactors, which were originally filled with soil gathered from near treated wastewater spreading grounds. The source water was tertiary treated effluent from the San Jose Creek Water Reclamation Plant (Whittier, CA). 100 µg/L of acetate was used as a primary carbon source. The microorganisms from each inoculum source were acclimated to all model PPCPs (50 µg/L) to generate the following inocula: AS-derived inoculum, Sd-derived inoculum, and SAT-derived inoculum. The acclimated biomass was used to seed reactors for biotransformation tests. The initial PPCP concentration used for acclimation and biotransformation tests was 50 µg/L. Gas chromatography coupled with mass spectrometry was used for monitoring PPCP biotransformation over 30 days. Of the inoculum sources tested, the AS- and Sd-derived inocula degraded PPCPs with higher removal efficiencies than the SAT-derived inoculum. Within 6

days, the AS-derived inoculum, the Sd-derived inoculum, and the SAT-derived inoculum removed >90% of 4, 3, and 2 out of 6 PPCPs, respectively. Gabapentin was not well degraded by any inoculum and approximately 80% remained at day 30.

To investigate the impact of primary carbon source, six types of primary carbon sources were tested: casamino acids (CA), a humic acids and peptone mixture (40:60 and 60:40 by molar concentration) (HP46 and HP64, respectively), molasses (ML), an organic acids mixture (including citric acid, lactic acid, and succinic acid) (OA), and phenol (PN). Each primary carbon source was provided at 1.2 μM. Separate microbial inocula for the carbon source tests were developed by pre-acclimation to all of the model PPCPs and carbon sources tested. The acclimation reactors were originally seeded with a mixed inoculum source comprised of the previously acclimated inocula. Acclimated inocula (CA-utilizing culture, HP46-utilizing culture, HP64-utilizing culture, ML-utilizing culture, OA-utilizing culture, and PN-utilizing culture) were used to seed the biotransformation tests. Of the primary carbon sources tested, CA-, OA-, and PN-utilizing cultures degraded PPCPs with relatively good removal. Within 6 days, the CA-utilizing culture removed >90% of 4 out of 6 PPCPs. In the same period, OA-utilizing culture and PN-utilizing culture removed >90% of 3 out of 6 PPCPs; ML-utilizing culture removed >90% of 2 out of 6 PPCPs; and both HP46- and HP64-utilzing culture removed >90% of 1 out of 6 PPCPs.

To identify microorganisms linked with PPCP biotransformation, microbial communities were characterized over time for all inocula and carbon sources tested. The results based on 16S rRNA gene amplicon sequencing analysis suggested that microbial community composition impacts PPCP biotransformation. Principal component analysis linked multiple specific phylotypes to PPCP biotransformation. By comparing different inoculum sources, we determined

that *Sphinogomonas*, unknown *Myxococcales*, *Beijerinckia*, *Methylophilus*, and unknown *Cytophagaceae* were linked with biotransformation of the model PPCPs. By comparing different primary carbon sources, unclassified *Myxococcales*, unknown *Methlyophilus*, unknown *Beijerinckia*, *Methlyovorus glucosotrophus*, unknown *Sphingomonas*, unknown *Bacteroidetes*, unknown *Planctomycetales*, unknown *Beijerinckiaceae*, and *Beijerinckia mobilis* were linked with degradation. Interestingly, reactor inoculated with AS-derived inocula and those inoculated with Sd-derived inocula both showed excellent capacity for PPCP biotransformation; however, their microbial community compositions were distinct. From this result, two hypotheses were formulated: PPCPs are biotransformed (1) by multiple phylotypes with redundant functions, or (2) by rare phylotypes present in the both biotransformation systems. Future work is needed to explore these hypotheses.

Overall, microbial community composition controlled by the inoculum sources and the primary carbon sources impacted biotransformation of the model PPCPs. This knowledge can be applied to support development of improved PPCP treatment technologies. Identification of phylotypes (i.e., by serial dilution and enrichment of less abundant phylotypes followed by new biotranformation tests) could be accomplished in future work. Additionally, elucidating biotransformation pathways and critical gene expression using metabolomics and metatranscriptomics would advance fundamental understanding of PPCP biotransformation.

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I thank my parents who just slightly nudged me a few years ago while I got lost in the middle of nowhere. They just had me just one step forward so that I could endure and even survive in the totally different environment of life. The research in this thesis would not exist without the support of my family. Yeona, my dear sister whom I trust the most, she never gave up to get my back. And Haein, as one of my friends, is the most wonderful person I have ever

known. She was my colleague in the past, she is my friend now, and she will be one of my life mentors. I got inspired by our trip to Europe where we shared many experiences and thoughts. That memory kept me to challenge hurdles. To me, Meg is my precious friend who I can lean on in Fort Collins. Although I do not have a religion, at this moment I thank God as I met her. I also thank to all of my friends that I could not list herein.

Lastly, I thank myself enduring all of those days in Fort Collins, Colorado, in the United States. At first, I was petrified with fear to do a thing. Nevertheless, the decision I made three years ago was the best one, and it made me matured from all of those experiences here. I firmly believe that I found a way to make things done more fantastically no matter what, no matter when, and no matter where I stuck. I will love me always, and hope you do so.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

1.1 Background

As the production and use of pharmaceuticals and personal care products (PPCPs) has been continuously increasing, PPCPs have been released into various water environments. Even at low concentrations (ng/L level), PPCPs may have adverse effects on ecosystems and human health. To deal with these concerns, methods for the degradation of PPCPs have been studied in engineered and controlled environments during wastewater treatment processes. Conventional activated sludge processes provide some PPCP degradation, but removal is minimal because these processes are not designed for PPCP removal. Many compounds are intractable with removal efficiencies well below 50%. Thus, new technologies are needed. Alternative biological treatment technologies such as membrane reactors or managed aquifer recharge systems have drawn attention.

However, to date, the rational design of efficacious biological treatment technologies has been hindered by limited knowledge of the types of microorganisms capable of PPCP biotransformation and the reactor conditions that promote their activity. Thus, studies of which phylotypes are critical and of optimal conditions (e.g., carbon source availability) for PPCP biotransformation need to be investigated to support development of advanced biological treatment technologies for PPCP treatment.

1.2 Research objective

The objective of this study was to investigate the hypotheses that (1) PPCP biotransformation depends on the microbial community, and (2) PPCP biotransformation

depends on the primary carbon source(s) available. To test these hypotheses, inocula first were obtained from three sources (activated sludge from a WWTP, sediment from the creek where effluents from WWTP are discharged, and soil from a laboratory-scale SAT column). In advance of biotransformation rate studies, microbial communities were pre-acclimated to build more stable and active communities. PPCP concentrations were monitored over time by gas chromatography coupled with mass spectrometry (GC-MS) methods developed as part of this work. Microbial community structures were analyzed over time for all inocula and reactor conditions tested by sequencing of 16S rRNA genes. The correlation between PPCP biotransformation and phylotypes present was studied by statistical analysis such as principle component analysis and Spearman's correlation.

1.3 Thesis structure

Chapter 2 is a literature review presenting previous research on PPCP degradation. It addresses existing concerns regarding PPCPs, worldwide PPCPs production and detection in various environments, and the potential harmful effects of PPCPs on ecosystems and human health. Abiotic and biotic treatment technologies for PPCP removal are also described, and the limited studies on biotransformation by PPCP-degrading organisms are reviewed. Analytical tools for measuring PPCPs in water and 16S rRNA gene analysis for microbial community characterization are described. Chapter 3 is structured as a manuscript for publication, and is about the impact of inoculum sources and primary carbon sources on PPCP biotransformation. In Chapter 4, a preliminary study about the impact of acclimation on PPCP biotransformation is described. Chapter 5 includes a summary and description of recommended future work. The

appendices include reactor operation details, analytical method development, and microbial community structure analysis at all taxonomic levels.

2. LITERATURE REVIEW

2.1 Existing concerns on PPCPs

2.1.1 Occurrence of PPCPs in water environment

PPCPs refers to a diverse group of products used to treat personal health or cosmetic reasons or to enhance growth or health of livestock in agribusiness (U.S. EPA. 2012). Also, PPCPs are defined as chemicals marketed for direct use by the consumer and having intended uses primarily on the human body (Daughton and Ternes. 1999). PPCPs are a diverse collection of thousands of chemical substances and include prescription and over-the-counter therapeutic drugs, veterinary drugs, fragrances, and cosmetics (Boxall et al, 2012; Spellman. 2014; Zhou *et al*. 2013). This broad term encompasses not only drugs and antiseptics, but also a wide range of compounds including veterinary and illicit drugs, fragrances, sunscreens, and PPCP human metabolites (Onesios and Bouwer. 2012).

PPCPs are of concern due to their occurrence in wastewater and surface water in metabolized and/or unmetabolized forms (Zhou *et al*. 2013; Radjenovic *et al*. 2009). PPCPs include hundreds of substances with widely varying physicochemical properties, environmental behaviors and biochemical activities (Caracciolo *et al*. 2015). Daughton and Ternes (1999) classified drugs as: hormones/mimics, antibiotics, blood lipid regulators, nonopioid analygesic/nonsteroidal anti-inflammatory drugs (NSAID), beta-blockers/beta2-sympathomimetics, antidepressants/obsessive-compulsive regulators, antiepileptic, antineoplastics, impotence drugs, tranquilizers, retinoids, and diagnostic contrast media. For example, antiepileptics are ubiquitous and prevalent due to insufficient removal by WWTPs.

Antineoplastics are highly toxic compounds, primarily from hospitals, which are poorly removed by WWTPs (Daughton and Ternes. 1999).

Worldwide production and consumption of PPCPs is substantial and varies by compound and location. It was estimated that 3.2×10^9 g tablets were consumed in the UK alone in 1998 (Wu *et al*. 2012). Regarding individual compounds, 3.6×10^9 g of paracetamol were produced in 2002 in the United States (Bedner and Maccrehan. 2006). In 2009, 6.7 tons of gabapentin was consumed Australia and 58.9 tons in Germany (Hermann *et al*. 2015). By 2012, consumption of gabapentin in Germany increased to 73.3 tons (Hermann *et al*. 2015). Worldwide annual production of triclosan in 1998 was approximately 1,500 tons, and its utilization in the United States was more than 450 tons (Dhillon *et al*. 2015).

Due to their massive production and utilization in our daily lives, PPCPs are released into wastewater and end up in wastewater treatment plants (WWTPs). The degradation of PPCPs in WWTPs depends on several factors—physico-chemical properties (e.g. solubility, vapor pressure, partitioning coefficient) and reactors conditions (e.g. temperature, pH) (Caracciolo *et al*. 2015). However, the degradation of PPCPs in WWTPs is incomplete. PPCPs are detected downstream of WWTPs, indicating they are being discharged from treatment processes (Onesios and Bouwer. 2012; Dhillon *et al*. 2015).

2.1.2 Impact of PPCPs on ecosystems and human health

PPCPs found in water supplies, groundwater, and the environment are of public concern because PPCPs are pharmacologically and physiologically active to affect homeostatic mechanisms in human body even at very low concentrations (Simazaki *et al*. 2015).

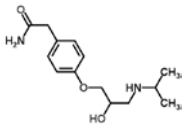
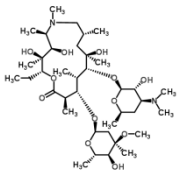
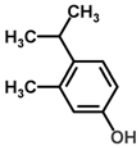
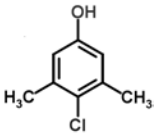
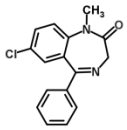
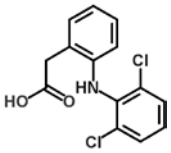
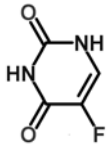
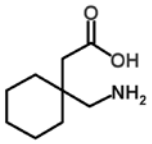
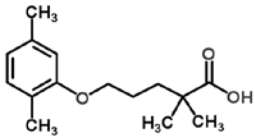
Additionally, Dhillon *et al.* (2015) found that triclosan may increase (~2-fold increase) production of chloroform (a possible carcinogen) in the drinking water.

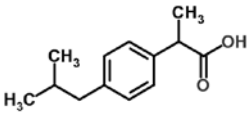
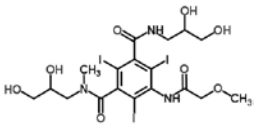
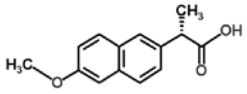
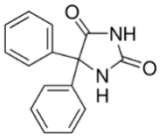
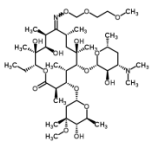
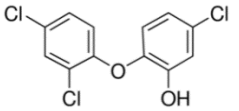
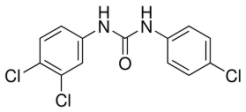
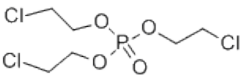
Furthermore, PPCPs may adversely affect ecosystems. PPCPs may change the growth, mortality, and community structure of algae and amphibians at low concentrations (Zhou *et al*. 2013; Hermann *et al*. 2015). Another example of potential adverse effects can be acute and chronic damage, bioaccumulation in tissues, damage to reproduction, and inhibition of cell proliferation (Wu *et al*. 2012). As an example of bioaccumulation, triclosan concentrations in fish are approximately three orders of magnitude higher compared to that in water (Dhillon *et al*. 2015).

2.1.3 Selected classes of PPCPs

For this study, we selected target compounds that show variable removal via WWTPs indicating that they could be removed by biological treatment, but research is still needed to enhance removal rates. Biosol (4-Isopropyl-3-methylphenol) and triclosan are antiseptic agents and have been found in surface waters and other aquatic systems. Ibuprofen and diclofenac are classified as nonsteroidal anti-inflammatory drugs (NSAID), and are frequently detected in surface waters (Caracciolo *et al*. 2015). The anticonvulsant drug gabapentin is used to treat various diseases (e.g. bipolar disorder, restless legs syndrome) and has increased in consumption (Herrmann *et al*. 2015). Gemfibrozil is classified as a blood lipid regulator (Caracciolo *et al*. 2015). Triclosan, a multi-purpose antiseptic agent, is of great concern due to its high consumption rate and association with altered endocrine function in humans (Caracciolo *et al*. 2015; Dhillon *et al*. 2015).

Table 1 PPCP compounds and removal efficiencies

Name ***Therapeutic use***	**CAS #**	**Structure**	**Removal Efficiency (%)**	**References**
Atenolol *Hypertension*	29122-68-7		NA	Benner *et al*. 2013
Azithromycin *Antibiotic*	117772-70-0		0.4-0.5	Onesios *et al*. 2009
Biosol *Antiseptic agent*	3228-02-2		4-28%; >99%; 80%	Onesios and Bouwer. 2012; Yu *et al*. 2006; Onesios *et al*. 2009
p-chloro-m-xylenol *Antiseptic agent*	88-04-0		18-47%	Onesios and Bouwer. 2012
Diazepam *Antidepressant*	439-14-5		38-60%	Onesios *et al*, 2009
Diclofenac *NSAID*	15307-86-5		0-6%; 18%; 30%	Onesios and Bouwer. 2012; Benner *et al*. 2013; Yu *et al*. 2006
5-Fluorouracil *Anticancer drug*	51-28-8		>99%; 2-50%	Onesios and Bouwer. 2012; Onesios *et al*. 2009
Gabapentin *Anticonvulsant*	60142-96-3		0-8%; >99%; 90%	Onesios and Bouwer. 2012; Yu *et al*. 2006; Onesios *et al*. 2009
Gemfibrozil *Lipid regulator*	25812-30-0		17->99%; 68%; >99%	Onesios and Bouwer. 2012; Yu *et al*. 2006; Onesios *et al*. 2009; Benner *et al*. 2013

Ibuprofen *NSAID*	15687-27-1		>99%; 87%	Onesios and Bouwer. 2012; Benner *et al*. 2013; Yu *et al*. 2006
Iopromide *X-ray contrast media*	73334-07-3		23-97%	Onesios *et al*. 2009
Naproxen *NSAID*	22204-53-1		23->99%; 88%	Onesios and Bouwer. 2012; Yu *et al*. 2006; Benner *et al*. 2013
Phenytoin *Anticonbulsant*	57-41-0		44%; 50%	Yu *et al*. 2006; Onesios *et al*. 2009; Benner *et al.* 2013
Roxithromycin *Antibiotic*	80214-83-1		85-95%	Onesios *et al*. 2009**;** Benner *et al*. 2013
Triclosan *Antiseptic agent*	3380-34-5		>99%; 69%; 38->99%	Onesios and Bouwer. 2012; Yu *et al*. 2006; Onesios *et al*. 2009
Triclocarban *Antiseptic agent*	101-20-2		21%-97%	Onesios *et al*. 2009
TCEP *Flame retardant*	15-96-8		0-15%	CEC4R08 WERF Report, and 12-12

2.2 PPCP removal technologies

2.2.1 Abiotic treatment technologies

Advanced treatment technologies involving physico-chemical processes, such as ultrafiltration and ozonation have been applied to eliminate some PPCPs. However, those technologies have high operating cost, high energy consumption, produce hazardous byproducts formation, or generate large amounts of sludge (Gupta and Thakur 2015). While ozonation was reported to efficiently degrade many micropollutants in wastewater (Margot *et al.* 2013), its disadvantages include incomplete oxidation, difficulty with destroying some recalcitrant compounds (e.g., TCEP) (Yoon *et al.* 2013), and formation of unknown by-products (Margot *et al.* 2013). Advanced oxidation processes (AOP) are a combination of ozone with other oxidant agents (e.g. UV radiation, hydrogen peroxide, TiO_2) and have been studied for degradation of polar pharmaceuticals (Petrovic *et al.* 2003). As well as other abiotic treatment technologies, however, byproducts can be formed during AOPs.

2.2.2 Biological treatment technology

2.2.2.1 Activated sludge processes

Activated sludge processes are widely used for general organic contaminant removal during wastewater treatments. Many studies investigate conventional activated sludge (CAS) processes for PPCP removal. Three mechanisms that are suggested to determine the fate of PPCPs during CAS process are biological degradation, sorption, and volatilization (Blair *et al.* 2015). Extended aeration activated sludge (EAAS), which employs an extended aeration period, was reported as one of the modifications of CAS process. Advantages of EAAS are low sludge generation and low ammonia concentration in sewage effluent. Disadvantages of EAAS process

include high hydraulic retention time (HRT) required, low organic loading rate, low active biomass, and low nutrient removal efficiency (Gupta and Thakur, 2015). And yet, fundamental knowledge to fully optimize EAAS processes for PPCP removal is required to be studied further.

2.2.2.2 Membrane biological reactor

Membrane biological reactor (MBR) is an efficient treatment technology for wastewater treatment and recycling (Wang *et al.* 2016). An advantage of using MBR for removal of PPCPs is that microorganisms such as antibiotic-resistant microorganisms can be retained in the system (Zhao *et al.* 2015). With high sludge retention time (SRT) and high mixed liquor suspended solids (MLSS) concentration as organic load, MBR is considered as useful system for PPCP biotransformation (Zhao *et al.* 2015). Zhao *et al.* (2015) showed that MBR seeded with aerobic granular sludge (GMBR) is able to remove some PPCPs, although degradation of the five drugs tested was variable (98.56% prednisolone, 84.02% naproxen, 87.85% norfloxacin, 77.83% sulfamethoxazole, and 63.32% ibuprofen). Wang *et al.* (2016) also reported removal of PPCPs by GMBR (removal efficiency 79.8% sulphamethoxazole and 64.4% ibuprofen).

2.2.2.3 Soil-based treatment

Managed aquifer recharge (MAR), a soil-based process, is capable of attenuating dissolved organic carbon (DOC) as well as some trace organic contaminants (Drewes *et al.* 2014) and shows significant removal efficiencies for several compounds (Li *et al.* 2014). Soil aquifer treatment (SAT), currently used as a water recycling approach in combination with other advanced treatment technologies, is a method of MAR in which WWTP effluent is applied

(Onesios-Barry *et al.* 2014). When soil passes through the SAT system, pollutants can be removed by biodegradation and sorption processes; however, existing technologies lead to incomplete and variable removals (Yu *et al.* 2006).

2.3 Microbiology of PPCP biodegradation

2.3.1 Pathways and metabolites

Knowledge about PPCP biodegradation pathways and metabolites is important to improve understanding of the environmental behavior of the parent compounds (Quintana *et al.* 2005). Mass spectrometry (MS)-based proteomics analyses can be applied to identify metabolites and to predict pathways (Kjeldal *et al.* 2016). Alidina *et al.* (2014a) suggested that different families of enzymes are involved in the biodegradation of the target compounds. To date, several studies have identified metabolites of PPCP biodegradation. Hydroxyibuprofen (OH-Ibu), carboxyibuprofen (CA-Ibu), and carboxylhydratropic acid (CA-HA) are metabolites from the biodegradation of ibuprofen (Zwiener *et al.* 2002). α-hydroxyisobutyric acid, lactic acid, and 4-chlorophenol are three main metabolites for the biodegradation of clofibric acid (Salgado *et al.* 2012).

One possible reaction for PPCP biotransformation is hydroxylation. Kjeldal *et al.* (2016) suggested that degradation of gemfibrozil by *Bacillus* sp. GeD10 is similar to the aerobic degradation of several compounds that have aromatic ring(s). Cytochrome P450 hydroxylase, alcohol dehydrogenase, and cathchol-2,3-dioxygenase were identified as enzymes involved in the biotransformation of gemfibrozil (Kjeldal *et al.* 2016). For naproxen biodegradation, enzymes including phenol monooxygenase, naphthalene monooxygenase, and hydroxyquinol 1,2-dioxygenase may be involved in the biodegradation process (Domaradzaka *et al.* 2015).

Ether cleavage was found to be the initial step for biodegradation of naproxen (Quintana *et al.* 2005), and opening of β-lactam ring was found to be one of the major steps for biodegradation of cefdinir (Selvi *et al.* 2014).

2.3.2 Factors influencing biodegradation during treatment

Understanding engineered and controlled microbial communities present would allow for efficient design and stable operation of PPCP degradation systems (Zhao *et al*. 2015). Changes in microbial community composition can reflect differences in microbial functions, which are connected to enhancing biotransformation capacity (Drewes *et al.* 2014; Hutalle-Schemelzer *et al.* 2010; Phan *et al.* 2016). Several factors may influence microbial communities present in treatment systems: indigenous community composition (e.g., in SAT systems), biomass, operational parameters, and composition and concentrations of substrates. Additional factors influencing the PPCP biotransformation during SAT that Onesios-Barry *et al.* (2014) suggested include effluent pretreatment, redox conditions, and wetting and drying cycles.

Operational parameters that have been shown to affect performance of the degradation include pH, temperature, and shaking speed (lab-scale reactors) (Selvi *et al.* 2014), fraction of heterotrophic and autotrophic biomass (Blair *et al.* 2015), SRT and HRT in a GMBR system (Xia *et al.* 2015), redox conditions in a SAT system (Alidina *et al.* 2014a), wetting and drying cycle in a SAT system (Onesios-Barry *et al.* 2014), and internal recirculation in anoxic-aerobic MBR system (Phan *et al.* 2016).

Initial concentration of substrate and initial composition of microbial community are also affect the microbial growth and degradation rate (Fortunato *et al.* 2016; Onesios-Barry *et al.* 2014; Selvi *et al.* 2014). Onesios-Barry *et al.* (2014) showed that PPCP removal efficiencies

were dependent on initial concentration of some PPCPs studied (biphenylol, *p*-chloro-*m*-cresol, chlorophene, diclofenac, 5-fluorouracil, ibuprofen, and valproic acid). Fortunato *et al.* (2016) pointed out that the selection of bacterial strains degrading target compounds may be an important strategy to boost process efficiency. Also, adaptation of the microbial community is an important factor for PPCP biodegradation (Wilson *et al*. 2013, Alidina *et al.* 2014a).

The presence of a primary carbon and energy source for microbial growth is also instrumental for PPCP biodegradation. Several studies found that removal efficiency of PPCPs was enhanced in the presence of primary substrates (Tran *et al.* 2009; Domaradzaka *et al.* 2015; Alidina *et al.* 2014b). For example, Domaradzaka *et al.* (2015) found that degradation efficiency of naproxen was significantly increased in the presence of glucose and phenol. The presence of primary substrate with proper conditions not only increases removal efficiency, but also reduces treatment time (Domaradzaka *et al.* 2015). Alidina *et al.* (2014b) indicated that the composition of the primary substrate could affect microbial community composition, diversity and gene expression, and may have a larger impact than the concentration on the biodegradation of PPCPs.

2.3.3 PPCP-degrading microorganisms

2.3.3.1 Studies on pure cultures

Pure cultures of bacterial strains degrading PPCPs have been reported in a few studies (Zhou *et al.*2013; Kjeldal *et al.* 2015; Domaradzaka *et al.* 2015; Gupta and Thakur. 2015; Selvi *et al*. 2014). Most of the strains were isolated from activated sludge in WWTPs. Isolates of *Sphingomonas* sp. are capable of degrading triclosan, ibuprofen, and gemfibrozil (Zhou *et al.* 2013). *Serratia* sp. ISTVKR1 is one of the isolates from sewage sludge during a biodegradation study of wastewater organic contaminants like phosphoric acid triphenyl ester and 4H-1-

benzopyran-4-one, 2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy- (Gupta and Thakur. 2015). *Ustilago* sp. SMN03, a fungus isolated from pharmaceutical wastewater, was identified as a novel strain able to utilize cefdinir as a sole carbon source (Selvi *et al.* 2014). *Bacillus* sp. GeD10 was isolated using gemfibrozil as a sole carbon and energy source, and degrades gemfibrozil to <60 ng/L (Kjeldal *et al.* 2015). *Planococcus* sp. S5 was found to degrade naproxen using glucose or phenol as a growth substrate (Domaradzaka *et al.* 2015).

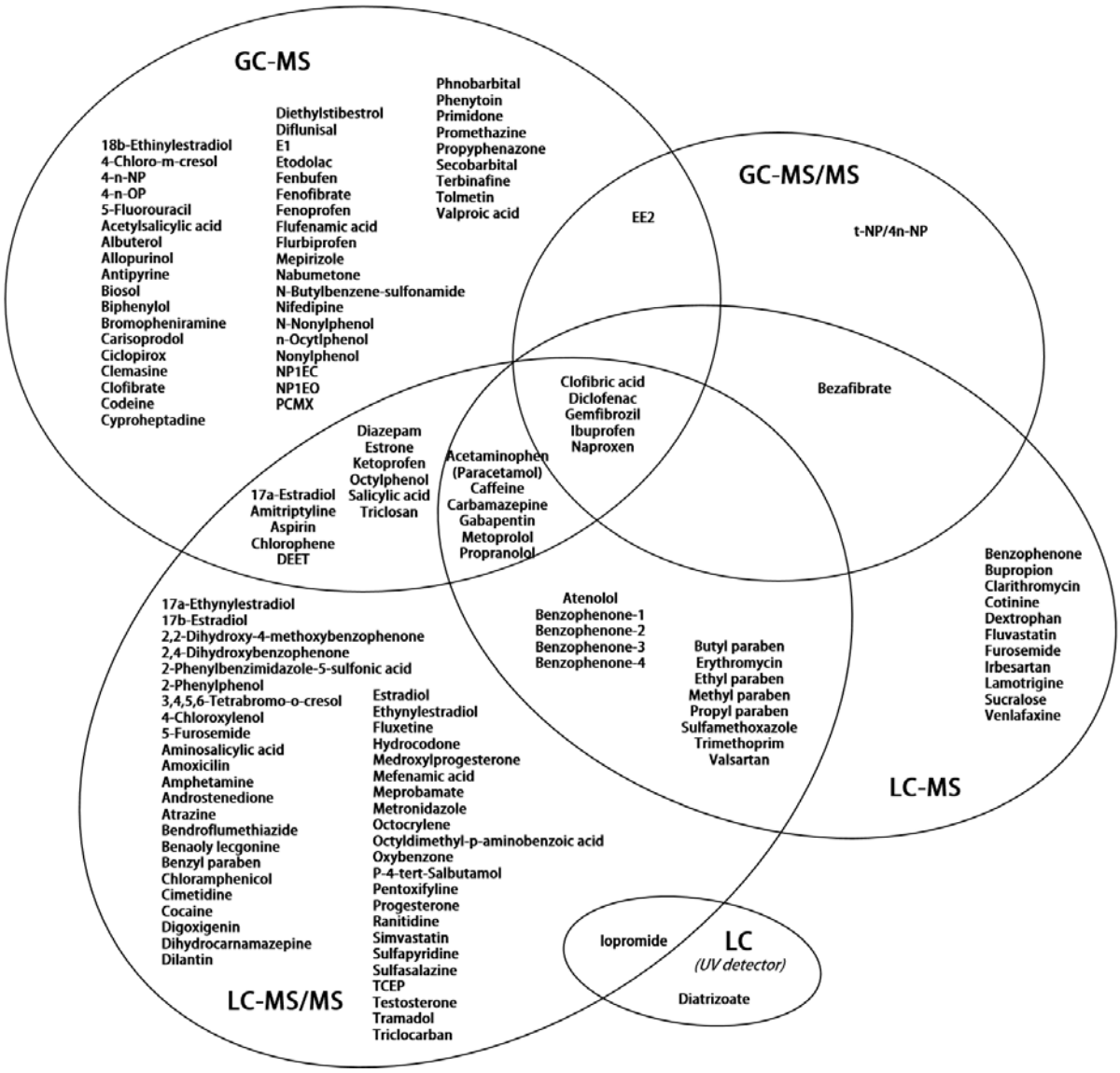
2.3.3.2 Studies on mixed communities

Little is known about the key microbial players in PPCP biodegradation, because many studies have investigated uncharacterized or poorly characterized mixed communities (e.g. indigenous microbial communities or enriched cultures). Complex microbial populations likely have synergistic effects on PPCP biodegradation (Zhao *et al.* 2015). Using mixed microbial cultures, 51% of clofibric acid was removed (initial concentration was 2 mg/L) in aerobic sequencing batch reactors (SBRs) (Salgado *et al.* 2012), and ibuprofen was removed in to below detection limits within 33 h (initial concentration was 100 mg/L) (Fortunato *et al.* 2016). *Zooglea, Tolumonas, Arcobacter, Terrimonas,* and *Singulisphaera* were found in a GMBR system that could degrade antibacterial and anti-inflammatory organic compounds (Wang *et al.* 2016). *Zooglea* was also found in a microbial community from a granular sludge sequencing bioreactor (GSBR) treating PPCPs (Zhao *et al.* 2015). However, the specific microbial phylotypes involved in PPCP biotransformation within mixed microbial communities in reactor systems are generally unknown. Lack of knowledge regarding critical species is a barrier to rational design and optimization of energy-efficient, low-cost, biological treatment technologies for PPCPs.

2.4 Detection and analysis

2.4.1 Analytic tools

In general, analyses using gas chromatography (GC) and liquid chromatography (LC) have been employed for detection of PPCPs in many environmental samples. In light of their selectivity and sensitivity, GC and LC enable the detection of PPCPs even at very low concentrations. Both analyses are commonly coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) for qualitative and quantitative analysis (Lindholm *et al*. 2014). Environmental samples make method development more challenging due to their complex matrices (e.g. sediment, sludge, soil, surface water, drinking water, or wastewater) (Yu *et al*. 2012). Furthermore, the large number of compounds means that there are numerous functional groups to be analyzed. Multiple functional groups make method development more complicated (Yu *et al*. 2012). Depending on factors including physico-chemical properties and matrices for analytes, either GC-MS or LC-MS can be selected as an advantageous detection method. The instruments used for detection of PPCPs in environmental samples are summarized in Fig. 1.



This figures is developed based on published studies: Bisceglia *et al*. 2010; Eichhorn *et al*. 2005; Espejo *et al*. 2014; Gracia-Lor *et al*. 2012; Kalsch. 1999; Kasprzyk-Hordern *et al*. 2008; Kim *et al*. 2007; Koutsouba *et al*. 2003; Kumirska *et al*. 2015; Lahti and Oikari. 2011; Langenhoff *et al*. 2013; Moder *et al*. 2007; Nieto *et al*. 2009; Onesios and Bouwer. 2012; Pavlovic *et al*. 2010; Quintana *et al*. 2005; Vanderford *et al*. 2003; Wabaidur *et al*. 2014; Xia *et al*. 2015; Yu *et al*. 2006; Yu *et al*. 2007; Yu *et al*. 2011; Yu *et al*. 2012; Yu and Wu. 2012; Simazaki *et al*. 2015; Zhou *et al*. 2013.

Figure 1 Methods used for detection of PPCPs in environmental samples

2.4.1.1 GC-MS

High efficiency of organic molecule separation, high sensitivity, very little waste production, and low cost are the advantages of using GC. For GC analysis, target compounds must be thermally stable and volatile. Therefore, for GC many compounds need derivatization to convert the compounds to be more thermally stable and volatile.

To detect low concentrations of analytes in environmental samples, extraction and concentration of target analytes is often required (Lindholm *et al*. 2014; Terzopoulou *et al*. 2015). Solid phase extraction (SPE) is widely employed for sample clean-up (Yu and Wu. 2012), concentration of analytes, and exchanging a sorbent to a more polar compound. Solid phase sorbent in SPE cartridges can be selected to be a non-polar (e.g. C18, C8) or polar (e.g. SI, NH2) sorbent bed depending on polarity of the analytes. Likewise, eluent solvent can be selected depending on physico-chemical properties of the analytes (Radjenovic *et al*. 2007). SPE is a highly selective, rapid, and efficient process (Lindholm *et al*. 2014). However, SPE cartridges are costly, making SPE-based detection methods expensive.

Derivatization is required especially in GC analysis for the compounds that are not easily volatilized (Radjenovic *et al*. 2007). Derivatization decreases polarity, increases thermal stability, and increases volatility (Dettmer-Wilde and Engewald. 2014). Also with derivatization, peaks get sharper, peak resolution increases, and sensitivity increases. Silaytion is the most popular derivatization reaction and N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), and N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamid (MTBSTFA) are widely employed as derivatizing agents (Bisceglia *et al*. 2010) (Table 2). Yet, a few derivatizing agents have proven suitable for pharmaceuticals and the utility of specific derivatizing agents depends on the functional groups

present (Yu *et al*. 2012). For instance, derivatization of compounds having R-OH or R-COOH groups showed can be derivatized using pentafluorobenzyl briomide (PFBBr) (Moder *et al*. 2007).

Table 2 Derivatization reactions and reagents by functional groups

Derivatization	Description	Functional groups	Reagent
Silylation	Silyl derivatives are formed by displacement of active hydrogen on the functional groups	Alcohols	BSA, BSA+TMCS, BSTFA,BSTFA+TMCS, HMDS, PFBBr
		Amides	BSA, BSTFA, MTBSTFA
		Amines	BSA, BSA+TMCS, BSTFA,BSTFA+TMCS
		Carboxylic Acid	BSA, BSA+TMCS, BSTFA,BSTFA+TMCS, PFBBr
Acylation	Conversion of compounds with active hydrogen on the functional groups into esters, thioesters and amides.	Amines	Acetic Anhydride, HFBA, PFPA, TFAA
		Amides	HFBA, PFPA, TFAA
Alkylation	Replacement of an active hydrogen by an aliphatic or aliphatic-aromatic (benzyl) group.	Amines	DMF-DBA, DMF-DEA, DMF-DMA, DMF-DPA, PFBBr, TMAH
		Amides	DMF-DBA, DMF-DEA, DMF-DMA, DMF-DPA,

BSA: N,O-Bis(trimethylsilyl)-acetamide
BSTFA: N,O-Bis(trimethylsilyl)trifluoroacetamide
DMF-DBA: N,N-Dimethylformamide/ Di-tert-butyl acetal
DMF-DEA: N,N-Dimethylformamide/ Diethyl acetal
DMF-DMA: N,N-Dimethylformamide/ Dimethyl acetal
DMF-DPA: N ,N-Dimethylformamide/ Dipropyl acetal
HFBA: Heptafluorobutyric acid anhydride
HMDS: Hexamethyldisilazane
MSTFA: N-Methyl-N-trimethylsilyltrifluoroacetamide
MTBSTFA: N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamid
PFBBr: Pentafluorobenzylbromide
TFAA: Trifluoroacetic acid anhydride
TMAH: Trimethylanilinium hydroxide
TMCS: Trimethylchlorosilane

For better sensitivity and selectivity of the analysis, determination of the proper ionization technique and scan technique is crucial for method development (Dettermer-Wilde and Engewald. 2014). There are two types of ionization techniques: electron impact ionization (EI) and chemical ionization (CI). In electron impact ionization, an electron is stripped away from molecules leaving M+ (cation radical), which tends to fragment to give smaller ions. The

fragmented ions in the same compound have the same ratio. Operation in EI mode mostly runs at a fixed energy of 70 eV, promising reproducible high ionization efficiency (Dettmer-Wilde and Engewald. 2014). EI is the most widely used technique to produce ions from neutral molecules entering the mass spectrometer via the GC capillary (Dettmer-Wilde and Engewald. 2014). Ionization before entering MS involves removing or adding electrons to a molecule creating an ion with a specific mass per charge ratio (m/z). Ions are accelerated through MS by an electromagnetic field. In chemical ionization, excess reagent gas (such as methane) present in source becomes ionized. Ions are produced by collision of the analyte and reagent gas. The CI spectrum depends on the type of reagent gas used for ionization and the type of ions produced by positive CI or negative CI mode (Dettmer-Wilde and Engewald. 2014).

Selection of a mass analyzer depends on which analysis method is needed, target or non-target analysis. Single quadrupole is used for fast scanning in full scan mode and nominal mass resolution in single ion monitoring (SIM) mode. Tandem MS (MS/MS) or a time-of-flight (ToF)-MS is appropriate for structure elucidation. ToF analyzers measure the flight time of ions through the ion flight paths so that it is able to calculate mass accurately. An ion trap mass analyzer is applied to detect multiple product ion scans in one run.

- In full scan mode, a range of masses passing through the mass filter is monitored over a period of time. All ions within a defined mass range (50-500 m/z) are detected with ~4 scans per second.
- In SIM mode, specific masses instead of a wide range are monitored and this makes sensitivity higher.
- In multiple reaction monitoring (MRM) mode, a collision cell placed between quadrupole mass filters breaks parent (or precursor) ions into several daughter (or

product) ions. The parent ions and the daughter ions for one compound are called transition. MRM is to monitor the fragmentation of parent to daughter ion in specific retention time window.

The limited number of non-polar and volatile compounds restrict successful application of GC-MS analysis for PPCPs and makes the analysis time-consuming due to the need for the derivatization process (Radjenovic *et al*. 2007; Gros *et al*. 2008). The main challenge with GC-MS for analysis of PPCP is based on the volatility and functional groups within the compounds. Typically, PPCP have low volatility and possess polar functional groups with active hydrogens (e.g. –OH, -NH, -NH_2), which requires derivatization before analysis to reduce polarity and increase volatility (Yu *et al*. 2012; Yu and Wu. 2012).

2.4.1.2 LC-MS

While compound determination by GC has one further step of derivatization which LC does not have, LC can be a useful option. A few LC analyses require a derivatization step, but most methods using LC do not. LC has been widely used because of its high sensitivity and selectivity, especially with LC-MS/MS. Many trace determination analyses of pharmaceuticals and wastewater-derived micropollutants have utilized multi-residue methods with LC-MS/MS (Richardson, 2007). LC is highly applicable for the detection of numerous polar and amphiphilic compounds (Lindholm *et al*. 2014). Usually polar and non-volatile pharmaceuticals can be easily analyzed by LC without derivatization. Reversed-phase high performance liquid chromatography (HPLC) is preferred for separation of polar organic pollutants from silica-bonded columns (Gros *et al*. 2008). Although LC analysis is favorable to many researches for its advantages,

considerable costs compared to GC analysis often makes many researchers avoid using this method (Bisceglia *et al*. 2010).

When LC-MS is applied to PPCP analyses, an ionization step is required in order to force droplets into the charged gas phase before entering the mass spectrometer. Types of ionization used in LC-MS are electrospray ionization (ESI), atmospheric pressure chemical ionization, and atmospheric pressure photoionization. ESI is the most used ionization method in LC analysis (Lindholm *et al*. 2014).

2.4.1.3 Method validation

Since there are large deviations in accuracy for trace analyses, an isotope-labeled compound or a structurally analogous compound is used as an internal standard (Bowers *et al*. 1993; Lindholm *et al*. 2014). Employing a stable isotope-labeled internal standard is considered to be an accurate method (Bowers *et al*. 1993) for minimizing errors of quantification (Stokvis *et al*. 2005). Ideally, it is best to have an internal standard for each target compound.

Precision of a certain method can be determined by estimating a relative standard deviation (RSD), which is a percentage of the standard deviation for an average value of data points from the analyses. Limit of detection (LOD) refers to the lowest concentration that can be detected, while limit of quantification (LOQ) refers to the lowest concentration that can be accurately and reliably quantified with the method. One way, LOD and LOQ are determined by using a signal to noise ratio (S/N) from the analysis. LOD and LOQ are calculated by dividing by 3 and 10 of S/N, respectively. Another way to calculate LOD and LOQ values is to use the results of the standard calibration curve. For the calculation, the theoretical concentration and area ratio of each point injection, regression line for the calibration curve, and standard deviation

of the regression line are needed. The theoretical concentration is the concentration calculated from the standard calibration curve, determined by regression analysis. Area ratio is the ratio of internal standard area to analyte area. Standard deviation of the regression line can be obtained by using STEYX function in Excel. LOD and LOQ are calculated by multiplying 3 and 10, respectively, the ratio of the standard deviation to the slope.

$$LOD = 3 \times \frac{standard\ deviation\ of\ the\ curve}{slope}$$

$$LOQ = 10 \times \frac{standard\ deviation\ of\ the\ curve}{slope}$$

Table 3 shows reported detection limits for compounds of interest with GC and LC. A detection limit for biosol and 5-fluorouracil was only reported with GC in the references found. Most of the method development studies finding detection limits have used pure water in the calibration standards so the values listed are lower than what can achieve for real samples with complex matrices. Despite difficulties of detecting analytes due to the complex environmental samples—drinking water, surface water, sediment, wastewater, and sludge - the published detection limits are quite low. As shown in Table 3, biosol has a detection limit of 2 ng/L in pure water. Diclofenac has a detection limit of 0.06 - 2 ng/L in pure water, 1.9 – 11 ng/g in soil, 2.3 – 17 ng/L in wastewater, 1.2 ng/L in drinking water, and 0.3 ng/L in surface water. 5-Fluorouracil has a detection limit of 17 ng/L in pure water. Detection limits for gabapentin were 15 – 300 ng/L in pure water, 0.6 ng/L in surface water, and 2 ng/L in wastewater. Detection limits for gemfibrozil were 0.04-25 ng/L in pure water, 5 ng/g in sludge, 0.9 ng/L in wastewater and 2.4 ng/L in drinking water. Detection limits of ibuprofen were 0.01-150 ng/L in pure water, 0.3 ng/L in surface water, 0.3 ng/g in soil, 2 ng/g in sludge, and 2 - 4 ng/L in wastewater. Detection limits

of triclosan were 1 - 100 ng/L in pure water, 2.4 ng/L in drinking water, 7 – 97 ng/L in wastewater, 4 ng/L in surface water, 0.06 ng/g in sediment, and 0.2 ng/g in sludge.

Table 3 Detection limits of selected PPCPs on GC and LC analyses

Compound	Instrument	Detection limit	Sample matrix	Reference
Biosol	GC	2 ng/L	Pure water	Yu *et al*. 2012
Diclofenac	GC	38 ng/L	Pure water	Koutsouba *et al*. 2003
	GC	1.9 ng/g	Soil	Kumirska *et al*. 2015
	GC	0.06 ng/L	Pure water	Moder *et al*. 2007
	GC	11 ng/g	Soil	Yu and Wu. 2012
	GC	2.3 ng/L	Wastewater	Yu *et al*. 2007
	GC	1.2 ng/L	Drinking water	Yu *et al*. 2007
	GC	1 ng/L	Pure water	Yu *et al*. 2012
	LC	9.6 pg	Pure water	Gracia-Lor *et al*. 2012
	LC	0.15 ug/L	Pure water	Kasprzyk-Hordem *et al*. 2008
	LC	0.3 ng/L	Surface water	Kasprzyk-Hordem *et al*. 2008
	LC	5 ng/L	Wastewater (effluent)	Kasprzyk-Hordem *et al*. 2008
	LC	17 ng/L	Wastewater (influent)	Kasprzyk-Hordem *et al*. 2008
	LC	2.5 ng/L	Wastewater	Simazaki *et al*. 2015
	LC	0.12 ug/L	Pure water	Lahti and Oikari. 2011
5-Fluorouracil	GC	17 ng/L	Pure water	Yu *et al*. 2012
Gabapentin	GC	15 ng/L	Pure water	Yu *et al*. 2012
	LC	1.4 pg	Pure water	Gracia-Lor *et al*. 2012
	LC	0.3 ug/L	Pure water	Kasprzyk-Hordem *et al*. 2008
	LC	0.6 ng/L	Surface water	Kasprzyk-Hordem *et al*. 2008
	LC	2 ng/L	Wastewater (effluent)	Kasprzyk-Hordem *et al*. 2008
	LC	2 ng/L	Wastewater (influent)	Kasprzyk-Hordem *et al*. 2008
Gemfibrozil	GC	0.04 ng/L	Pure water	Moder *et al*. 2007
	GC	3 ng/L	Pure water	Yu *et al*. 2012
	GC	5.0 ng/g	Sludge	Yu and Wu. 2012
	GC	0.9 ng/L	Wastewater	Yu *et al*. 2007
	GC	2.4 ng/L	Drinking water	Yu *et al*. 2007
	LC	25 ng/L	Pure water	Zhou *et al*. 2013
Ibuprofen	GC	36 ng/L	Pure water	Koutsouba *et al*. 2003
	GC	0.01 ng/L	Pure water	Moder *et al*. 2007
	GC	1 ng/L	Pure water	Yu *et al*. 2012
	GC	0.3 ng/g	Soil	Kumirska *et al*. 2015
	GC	2.0 ng/g	Sludge	Yu and Wu. 2012
	GC	3.6 ng/L	Wastewater	Yu *et al*. 2007
	GC	1.0 ng/L	Drinking water	Yu *et al*. 2007

	LC	107 pg	Pure water	Gracia-Lor *et al*. 2012
	LC	0.1 ug/L	Pure water	Kasprzyk-Hordem *et al*. 2008
	LC	150 ng/L	Pure water	Zhou *et al*. 2013
	LC	0.3 ng/L	Surface water	Kasprzyk-Hordem *et al*. 2008
	LC	2 ng/L	Wastewater (effluent)	Kasprzyk-Hordem *et al*. 2008
	LC	4 ng/L	Wastewater (influent)	Kasprzyk-Hordem *et al*. 2008
	LC	1.1 ng/L	Wastewater	Simazaki *et al*. 2015
Triclosan	GC	2 ng/L	Pure water	Yu *et al*. 2012
	GC	7.0 ng/L	Wastewater	Yu *et al*. 2007
	GC	2.4 ng/L	Drinking water	Yu *et al*. 2007
	LC	1 ug/L	Pure water	Kasprzyk-Hordem *et al*. 2008
	LC	100 ng/L	Pure water	Zhou *et al* 2013
	LC	4 ng/L	Surface water	Kasprzyk-Hordem *et al*. 2008
	LC	72 ng/L	Wastewater (effluent)	Kasprzyk-Hordem *et al*. 2008
	LC	97 ng/L	Wastewater (influent)	Kasprzyk-Hordem *et al*. 2008
	LC	60 ng/L	Wastewater	Simazaki *et al*. 2015
	LC	0.06 ng/g	Sediment	Yu *et al*. 2011
	LC	0.2 ng/g	Sludge	Yu *et al*. 2011

2.4.2 Microbial analysis

Because microbial community structure determines the specific functions of the community (e.g. bacterial growth and metabolic potential), identifying the microbial members present in treatment systems can provide insights into which phylotypes are critical for achieving efficient biodegradation. To investigate microbial groups that may be responsible for PPCP biodegradation, conventional molecular biological techniques including denaturing gradient gel electrophoresis (DGGE), fluorescence *in situ* hybridization (FISH), and terminal restriction fragment length polymorphisms (T-RFLP) have used. Reversible terminator sequencing based on 16S rRNA gene sequencing is a high-throughput method to provide a phylogenetic profile of microbial community present and has been successfully applied in diverse environmental studies (Li *et al.* 2012; Zhao *et al.* 2015).

2.5 Summary

To date, concerns regarding PPCPs as emerging contaminants have significantly increased as production and use of the compounds has been on the rise. Treatment of PPCPs in advance of discharge from WWTPs is necessary since exposure at even low levels (e.g. ng/L concentrations) poses a health threat both to ecosystems and humans. Numerous physico-chemical treatment technologies, as well as biological treatment technologies, have been applied to removal PPCPs, although with mixed success. Biological treatment technology is considered more desirable because it is environmentally sound, energy efficient, and cost effective. Various biological treatment technologies such as CAS, MBR, and SAT have been studied for PPCP biodegradation, and laboratory studies have examined biodegradation with pure cultures and mixed communities. Microbial community composition was expected to impact PPCP biodegradation. There are several factors that likely affect the composition of microbial communities involved in biodegradation, such as system operational parameters (e.g. SRT), primary substrate composition, and PPCP concentration. However, the key microbial phylotypes involved in PPCP biodegradation remain largely unknown, and the reactor conditions that promote their growth and the expression of required enzymes have yet to be identified.

3. PPCP BIOTRANSFORMATION: THE IMPACT OF INOCULUM SOURCES AND PRIMARY CARBON SOURCES

3.1 Introduction

The production and use of PPCPs has been continuously increasing. In 1998, an estimated 3,200 tons of PPCPs were consumed in the United Kingdom (Wu *et al*. 2012). Worldwide annual production of triclosan in the same year was approximately 1,500 tons, and its utilization in the United States was more than 450 tons (Dhillon *et al*. 2015). In Germany, the consumption of gabapentin increased from 58.9 tons in 2009 to 73.3 tons in 2012 (Herrmann *et al*. 2015). Many PPCPs are recalcitrant, leading to incomplete or minimal removal by wastewater treatment systems; thus, PPCPs are routinely discharged into surface water or sediment (Caracciolo *et al*. 2015). PPCPs are present in very low concentrations, ng/L to µg/L level, in various aquatic environmental systems such as drinking water supplies, surface water, and groundwater (Blair *et al*. 2015; Kwon and Rodriguez. 2014; Onesios-Barry *et al*. 2014; Quintana *et al*. 2005; Tran *et al*. 2009; Yu *et al*. 2006; Zhao *et al*. 2015).

To date, various methods for the degradation of PPCPs have been studied in engineered and controlled environments. Compared to physico-chemical treatments, biological treatment processes are promising for PPCP degradation due to reduced cost and energy use, increased operational efficiency and sustainability, and ability to treat a wide range of compounds (Domaradzka *et al*. 2015; Zhao *et al*. 2015; Selvi *et al*. 2014). Conventional activated sludge (CAS) is one biological treatment technology that has been studied for the degradation of PPCPs (Tran *et al*. 2009; Blair *et al*. 2015). It was shown that PPCPs could be removed or transformed in CAS systems by biological degradation, sorption, and volatilization (Blair *et al*. 2015; Lahti and Oikari. 2011). However, removal efficiency was low, variable between treatment plants, and

unpredictable due to lack of knowledge regarding critical microorganisms and reactor operating conditions. Soil aquifer treatment (SAT), another biological degradation method, utilizes biofiltration processes in soil (Onesios-Barry *et al*. 2014). PPCP removal efficiencies using SAT vary widely (0% to 99%) depending on the system type and specific compound (Onesios and Bouwer, 2012).

Although biodegradation has been demonstrated for many PPCPs, others are recalcitrant, and for others the potential for biodegradation is not fully known. For example, in CAS systems, nearly complete degradation of ibuprofen (94%) was reported (Tran *et al*. 2009), while 5-fluorouracil was much more recalcitrant (2-50%) (Onesios *et al*. 2009). Different studies in CAS systems reported highly variable degradation of gemfibrozil from batch reactor (>99%) to full scale WWTPs (<10%) (Kwon and Rodriguez, 2014; Tran *et al*. 2009; Onesios *et al.* 2009). In SAT systems, triclosan was nearly completely degraded or removed in most of the studies. For instance, greater than 99% removal during passage through a SAT column system (Yu *et al*. 2006), and 97% removal in activated sludge system (Stasinakis *et al*. 2007) were reported. Ibuprofen was removed greater than 80%, whereas 5-fluorouracil and diclofenac were removed less than 60% in a SAT column system (Yu *et al*. 2006).

Removal of PPCPs in biological systems is likely linked to the microorganisms present in the treatment systems. Therefore, various factors that could impact microbial community composition could also impact PPCP removal efficiencies. Although primary substrates are known to influence microbial community structure in treatment systems, little is known about which microorganism(s) are important and which primary substrates support the growth and desired activity of critical microorganisms (Onesios and Bouwer. 2012; Li *et al.* 2014). The presence of a primary carbon source has been shown to increase PPCP removal efficiencies

(Lahti and Oikari. 2011). For example, naproxen degradation was improved in the presence of glucose (Domaradzka *et al*. 2015; Marchlewicz *et al*. 2016) and phenol (Domaradzka *et al*. 2015). Biodegradation with microorganisms from different inoculum sources is also not well-studied. For example, studies about which microorganisms are linked with PPCP biodegradation, or even possibly interfere with PPCP biodegradation, are still limited.

Several studies have isolated pure cultures capable of PPCP biodegradation. Isolates of *Sphingomonas* sp. are capable of degrading triclosan, ibuprofen, and gemfibrozil (Zhou *et al*. 2013). *Bacillus* sp. GeD10, a gemfibrozil-degrading bacterium, was isolated from activated sludge (Kjeldal *et al*. 2016). *Pautlibacter* sp. I11, also isolated from activated sludge, can degrade ibuprofen (Almeida *et al*. 2013). Domaradzka *et al*. (2015) indicated that *Planococcus* sp. strain S5 showed cometabolic degradation of naproxen. *Bacillus thuringiensis* B1 (2015b) isolated from soil is able to degrade ibuprofen and naproxen (Marchlewicz *et al*. 2016). Using mixed communities for PPCP biodegradation is more representative of real environments and treatment systems. Many studies used mixed communities from environmental sources, such as activated sludge or sediment, and observed high removal efficiency in laboratory batch reactors (Yu *et al*. 2006; Tran *et al*. 2009; Lahti and Oikari. 2011), wastewater treatment system (Kwon and Rodriguez. 2014), and SAT systems (Onesios-Barry *et al*. 2014). However, PPCP removal is complicated and achieving stable removal remains difficult, and further removal varies by compound. Adaptation of the microbial community could be one of the important factors (Drewes *et al*. 2006) to improve microbial biodegradation. For example, it has been shown that acclimation may help to stabilize and even increase microbial activity in biodegradation process (Rauch-Williams *et al*. 2010; Wilson *et al*. 2013; Alidina *et al*. 2014a). However, the identity of key microbes within mixed microbial communities in PPCP treatment processes and the reactor

conditions that promote their growth and expression of required enzymes are still largely unknown.

Thus, the objective of this study was to investigate hypotheses that PPCP biotransformation ability is dependent on (1) the microbial community, and (2) the primary carbon sources present. We focused on six PPCP compounds (diclofenac, 5-fluorouracil, gabapentin, gemfibrozil, ibuprofen, and triclosan) for which reported removal rates in activated sludge processes and SAT systems are generally low and variable, indicating a lack of knowledge of critical microorganisms and conditions and a potential opportunity to improve treatment. We evaluated degradation of these PPCPs using microbial communities derived from three initial inoculum sources (activated sludge, sediment historically impacted by WWTP effluent, and SAT material), and analyzed the structure of the microbial communities over time during degradation to identify microbial phylotypes linked with biodegradation. Additionally, we evaluated degradation of these PPCPs in the context of six primary carbon sources (casamino acids, molasses, humic acids and peptone mixtures, organic acids, and phenol) and examined resulting changes in microbial communities. This study was based on two experimental approaches: bioacclimation and batch treatment. In advance of actual biodegradation, microbial communities were pre-acclimated based on the expectation of generating a more stable and active community. We applied molecular methods (16S rRNA gene amplicon sequencing via Illumina next-generation sequencing) for the phylogenetic identification of microorganisms in the reactors. Correlations between PPCP biotransformation and microbial community structure were investigated to link phylotypes with biodegradation of each compound.

3.2 Material and methods

3.2.1 Inoculum sources

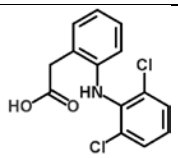
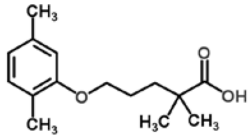
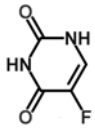
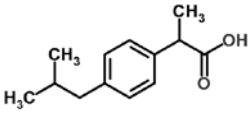
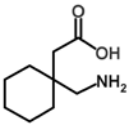
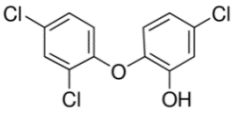
Activated sludge (AS) was taken from Drake Water Reclamation Facility (DWRF) (Fort Collins, Colorado, United States). Sediment (Sd) was taken from the Fossil Creek Ditch, which receives effluent flow from DWRF. SAT material was taken from columns treating trace organic contaminants, including PPCPs, in tertiary treated wastewater from San Jose Creek Water Reclamation Plant (Whittier, CA, United States); SAT material was provided courtesy of Trussell Technologies (Trussell *et al*. 2015). These columns had been fed with wastewater treated by either chlorination or ozonation. The soil from both types of SAT systems (SAT) was mixed and used for the inoculum source.

3.2.2 Reactor setup and operation

To investigate the impact of inoculum source on PPCP biotransformation, 2 L-flask batch reactors for acclimation were setup for each inoculum. Trace mineral solution including KH_2PO_4, K_2HPO_4, Na_2HPO_4, $FeCl_3 \cdot 6H_2O$, NH_4Cl, $MgSO_4$, and $CaCl_2 \cdot 2H_2O$ was prepared according to Yu *et al*. (2006) and autoclaved for sterilization. Composition and concentration of each chemicals are shown in Table 8 (Appendix A1). 6 model PPCPs were selected for investigation (Table 4). Solvent for the PPCP stock solutions were water, ethanol, and methanol (Table 10 in Appendix A1). These filter-sterilized PPCPs were provided to the reactors at a concentration of 50 µg/L. This concentration represents the high end of what might be observed in contaminated wastewater; however, it was selected to allow for accurate quantification of biotransformation over at least one order of magnitude. The primary carbon sources were casamino acid, two mixtures of humic acids with peptone (at a molar ratio of 60:40 and 40:60

humic acid: peptone), molasses, organic acids (mixture of citric acid, lactic acid, and succinic acid), and phenol (Table 9 in Appendix A1). All carbon sources were provided at a concentration of 1.2 µM. Microbial inoculum consisted of 1 mL of 1:100 diluted inoculum sources from AS, Sd, and SAT that were acclimated to all of the target PPCPs. The original microbial inoculum used to develop the acclimated inocula for the primary carbon source test consisted of a mixed inoculum source using the previously acclimated inocula (AS: Sd: SAT=49:49:2, by volume). Biomass growth of the acclimation reactor systems were monitored approximately two months.

Table 4 Chemical structure and therapeutic class of the target compounds

Compound (CAS#) Therapeutic class	Structure	Compound (CAS#) Therapeutic class	Structure
Diclofenac (15307-86-5) NSAID		Gemfibrozil (25812-30-3) Lipid regulator	
5-Fluorouracil (51-28-8) Anticancer drug		Ibuprofen (15687-27-1) NSAID	
Gabapentin (60142-96-3) Anticonvulsant		Triclosan (3380-34-5) Antiseptic agent	

2 L-flasks containing 700 mL system were used as acclimation reactors. The reactors consisted of inoculum, trace mineral solution, primary carbon source, PPCPs, and sand. The mineral solution was a growth media containing trace nutrients (described in Yu *et al*. 2006), and

acetate was provided as a primary carbon source at 100 µg/L. Sands were prepared in 2 L-flasks for the bioacclimation tests and in 150 ml-flasks for the biotransformation tests (10% by volume). The test was maintained at 25°C with constant mixing at 120 rpm. Optical density at 600 nm and protein concentration using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) were measured weekly to monitor microbial growth (from Fig.14 to Fig.17 in Appendix A2). Biomass was calculated from protein measurement results, and the protein fraction used for the calculation was assumed approximately 55%. Every two weeks, systems reached a certain amount of biomass (e.g. $OD_{600} > 0.5$) were taken out its half by volume and refilled with fresh medium including primary carbon source and PPCPs, in order to maintain a consistent biomass during the acclimation period. Equivalent quantities (based on biomass measurements) of acclimated biomass from each inocula were transferred from each acclimation system to each batch biotransformation test.

3.2.3 Biotransformation tests

250-mL flasks containing 100 mL of media were used as biotransformation test reactors. Reactors had different inoculum sources derived from either AS, Sd, or SAT and had acetate as a primary carbon source. Biological triplicates were run for each inoculum source. Before transferred to biotransformation system, biomass of the acclimated inoculum was normalized regarding OD_{600} and protein measurement results to ensure that all reactor systems conduct biotransformation with the same biomass. The biomass was adjusted its concentration by using medium. Inocula for biotransformation were washed to avoid carrying over any PPCPs or primary carbon sources remained in the biomass transferred. The system consisted of inoculum, media, 100 µg/L of acetate as a primary carbon source, 50 µg/L of PPCPs, and sand (10% by

volume) (Fig. 2). Killed control reactors were autoclaved before adding PPCPs. The tests were maintained at 25°C. All reactors were kept on a shaker with mixing speed at 120 rpm to maintain aerobic conditions.

To investigate the impact of primary carbon source on PPCP biotransformation, acclimation of mixed inocula with different primary carbon sources were performed. AS, Sd, and SAT were mixed to generate the mixed inoculum. Flask reactor systems and operational conditions were the same as described above. Biological triplicates were run for each carbon source. Reactors contained different primary carbon source, casamino acids (CA-utilizing culture), humic acids: peptone (40:60) (HP46-utilizing culture), humic acid: peptone (60:40) (HP64-utilizing culture), molasses (ML-utilizing culture), organic acids (OA-utilizing culture), and phenol (PN-utilizing culture) (chemicals were purchased from Sigma Aldrich, Thermo Fisher Scientific, and B&G foods). Composition and concentrations are shown in Table 9 (Appendix A1). The primary carbon sources were provided with the same molar concentration (1.2 μM) to the biotransformation systems. Batch biotransformation tests were inoculated with equivalent biomasses of the different acclimated inocula, based on total protein measurement; OD_{600} measurements were unreliable due to the use of sand, and biofilm biomass, and biomass aggregates.

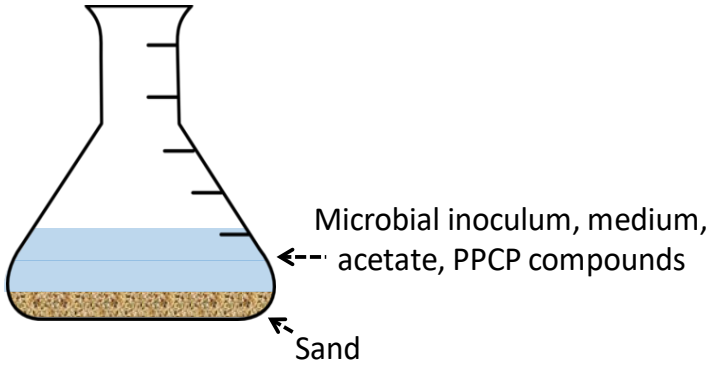


Figure 2 Flask reactor system used for biotransformation test.

3.2.4 Chemical analysis

Compounds analyzed by gas chromatography coupled with mass spectrometry (GC/MS) analysis included: diclofenac, gabapentin, gemfibrozil, 5-fluorouracil, ibuprofen, and triclosan (Sigma Aldrich, St. Louis, MO). LC/MS grade water (Fisher Scientific, Fair Lawn, NJ), LC/MS grade acetonitrile (Fisher Scientific, Fair Lawn, NJ), LC/MS grade methanol (Fisher Scientific, Fair Lawn, NJ), pyridine (Sigma Aldrich, St. Louis, MO), and N-Methyl-N-trimethylsilyltrifluoroacetamide (MTBSTFA) + 1% Tertbutyldimetheylchlorosilane (TBDMCS) (Thermo Fisher Scientific, Waltham, MA) were used for GC-MS analysis. 1 mL of each sample was taken from biotransformation reactors and stored at -80°C until sample preparation. Samples were thawed and centrifuged at 3,750 rpm for 5 minutes at 4°C. 100 µL of supernatant from each sample was taken and added to 100 µL of 10 µg/mL internal standards mixed solution in a glass vial. All samples were dried down under nitrogen. 50 µL of pyridine was added, and the samples were vortexed for 10 minutes. Then 50 µL of MTBSTFA+1% TBDMCS were added. Samples were incubated for 1 hour at 60°C and sonicated for 10 minutes. After another 1 hour of

incubation 60°C, samples were moved to 4°C. Samples were centrifuged at 3,750 rpm for 5 minutes at 4°C. The supernatant was transferred, avoiding precipitated salts.
Theses derivatized samples were stored in 4°C until GC-MS analysis.

GC-MS analyses were performed using TRACE GC ULTRA equipped with ISQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Methods were developed at the Proteomics and Metabolomics Core Facility (Colorado State University, Fort Collins, CO). 1 µL aliquots of the samples were injected in split mode with split flow set to 12 mL/min, onto a programmed temperature vaporization injector maintained at 285°C. Chromatographic separations were carried out on a TG-5MS column (30 m × 0.25 mm × 0.25 µm). The temperature gradient program was started at 140°C and held for 0.5 min, ramped at 15°C/min to 330°C, with 3-min hold at 330°C. Helium was used as the carrier gas with 1.2 mL/min of constant flowrate. The MS was operated under electron ionization (EI) mode. The MS transfer line and the ion source temperature was 300°C and 260°C, respectively. For quantification, data was acquired using selected ion monitoring (SIM). Confirmation of compound identify was achieved through acquisition of full scan spectra (m/z 50-650 with 0.2 s/scan). Data was processed using Chromeleon^{TM} 7.2 SR4 (Thermo Fisher Scientific, Waltham, MA).

Quality control (QC) samples, which consist of aliquots of the mixture from each biotransformation sample, were included in the sequences to confirm that the instrument was properly and stably ran during the analysis time. Nine-point calibration curves were generated for concentrations from 0.5 ng/L to 100,000 ng/L. Curves for all target compounds were linear over this range ($R^2 \geq 0.99$). Limits of detection (LOD) and limits of quantitation (LOQ) were calculated by multiplying by 3 and 10, respectively, by the ratio of the signal to noise ratio (S/N) to the average of theoretical concentration of the 3 lowest curve points detected. All curve

samples were prepared in duplicate. GC-MS method retention times and qualifying ion masses are shown in Table 5. LODs and LOQs for each PPCP in media with each carbon source are shown in Table 6. Because carbon source is not expected to have a major impact on LOQ, and for simplicity, we used average LODs across the media in calculating estimated % removals, when PPCP concentrations were below detection limits (i.e., we assumed concentrations equal to the average LOD to conservatively estimate removals when no values were obtained). Isotope-labeled internal standards (diclofenac-$_d$4, 5-fluorouracil-15$_n$2, gabapentin-$_d$4, gemfibrozil-$_d$6, ibuprofen-$_d$3, and triclosan-$_d$3) were purchased from Medical Isotopes and CDN Isotopes and used for quantitation and validation of the analysis. Internal standards were spiked at a concentration of 0.5 ng/mL to the samples before derivatization.

Table 5 Analysis information of selected PPCP compounds.

Compound	Mass (g/mol)	Retention time (min)	Qualifying ions
Diclofenac	296.2	10.29	214, 352
Diclofenac-d4	300.2	10.28	218, 356
5-Fluorouracil	130.0	5.31	301, 343
5-Fluorouracil-15n2	132.1	5.32	304, 346
Gabapentin	171.2	5.94	210, 211
Gabapentin-d4	175.3	5.91	214, 215
Gemfibrozil	250.0	8.11	243, 307
Gemfibrozil-d6	256.4	8.11	249, 313
Ibuprofen	206.0	6.02	160, 263
Ibuprofen-d3	209.3	5.99	165, 267
Triclosan	287.0	9.28	345
Triclosan-d3	290.0	9.27	352, 353

Table 6 Detection limits of selected PPCPs. Standard curve samples were tested in background of the medium with the individual primary carbon source. Samples on the curves were prepared in duplicate. All units are µg/L.

Primary carbon source / PPCP	Acetate		Casamino acid		Molasses		Organic acids		Humic acid: Peptone 60:40		Humic acid: Peptone 40:60		Phenol		Average	
	LOD (ug/L)	LOQ (ug/L)	LOD (ug/L)	LOQ (ug/L)	LOD (ug/L)	LOQ (ug/L)	LOD (ug/L)	LOQ (ug/L)	LOD (ug/L)	LOQ (ug/L)	LOD (ug/L)	LOQ (ug/L)	LOD (ug/L)	LOQ (ug/L)	LOD (ug/L)	LOQ (ug/L)
Diclofenac	0.28	0.95	2.72	9.06	7.10	23.65	5.43	18.10	7.19	23.98	5.21	17.37	8.79	29.29	4.86	17.48
5-fluorouracil	0.37	1.22	0.03	0.10	0.03	0.09	0.18	0.60	0.02	0.08	0.07	0.25	0.06	0.21	0.11	0.36
Gabapentin	0.03	0.11	0.02	0.08	0.12	0.39	0.08	0.25	0.05	0.17	0.13	0.43	0.09	0.29	0.07	0.25
Gemfibrozil	0.23	0.75	1.99	6.63	0.31	1.03	0.32	1.08	2.42	8.05	2.96	9.87	2.56	8.52	1.54	5.13
Ibuprofen	9.80	32.65	0.95	3.15	0.16	0.54	0.11	0.37	0.34	1.12	0.23	0.75	17.07*	56.90*	4.09	13.64
Triclosan	0.90	2.99	2.78	9.25	5.18	17.27	5.26	17.54	4.32	14.41	4.45	14.84	5.74	19.13	4.09	13.63

* LOD and LOQ for ibuprofen-phenol were obtained by using standard deviation of the calibration curve.

3.2.5 Microbial community analysis

Microbial samples (1 mL) were taken from the reactor operation at day 0, day 14, and day 30. Reactor systems were vigorously shaken before taking liquid and sand samples. All samples were centrifuged at 10,000g for 1 min and stored at -20°C before extraction. DNA was extracted using PowerSoil DNA Isolation kits (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. Extracted DNA were preserved at -20°C. 16S rRNA gene amplicon sequencing was performed with Illumina MiSeq (Illumina, Inc. San Diego, CA) by Research and Testing Laboratories (Lubbock, TX) using 28F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3'). The raw sequences were quality filtered and trimmed in mothur v.1.37 (Schloss *et al*. 2009) and classified using the RDP database (Cole *et al*. 2013). To investigate correlations between microbial composition and PPCP biotransformation by model compounds, statistical analyses were performed using the PAST 3.0 software (Hammer *et al*. 2001). Principal component analyses (PCA) were conducted to visualize patterns in community structure and compound degradation as related to acclimation and primary carbon source. Spearman's correlations analyses were conducted to identify correlations of phylotype abundances with degradation of PPCPs.

3.3 Results

3.3.1 PPCP biotransformation with different inoculum sources

Although some compounds (5-fluorouracil and triclosan) were quickly biotransformed regardless of inoculum source, for others (diclofenac, gemfibrozil, and ibuprofen) degradation was strongly dependent on inoculum source. No significant degradation was observed in the killed control reactors indicating that observed losses were due to biotransformation and not

sorption or volatilization (Fig. 4). Diclofenac and gemfibrozil were degraded rapidly by AS-derived inoculum and Sd-derived inoculum, but not by SAT-derived inoculum. Ibuprofen was generally degraded immediately by the AS-derived inoculum and the Sd-derived inoculum, but with the SAT-derived inoculum there was a lag before degradation initiated. Further, the AS-derived inoculum removed diclofenac, gemfibrozil and ibuprofen to >90% within 4, 6 and 12 days, respectively (Fig. 3C, 3D, and 3E). The Sd-derived inoculum removed 5-fluorouracil, triclosan, and diclofenac to greater than 90% within 2 days (Fig. 3A, 3B, and 3C). Gemfibrozil and ibuprofen were removed to greater than 90% within 4 and 12 days (Fig. 3D, 3E and 3F). SAT-derived inoculum removed ibuprofen to greater than 90% by 16 days (Fig. 3E). Diclofenac, gemfibrozil, and gabapentin were hardly removed by the SAT-derived inoculum, with approximately 99%, 66%, and 87% of these compounds remaining at day 30, respectively (Fig. 3C, 3D, and 3F). Gabapentin was not well degraded by any of the inoculum with approximately 80% remaining at day 30 across all samples (Fig. 3F). However, for this compound, standard deviations were higher and the data was more variable, indicating potential analytical problems that could have obscured some degradation trends. All reactors were initially seeded with the same biomass, and the nominal biomass concentration was similar between all inocula over time. Thus, the higher degradation rates observed for the AS-derived and Sd-derived inocula were not due to differences in cell concentrations, but rather to differences in specific microbial phylotypes present.

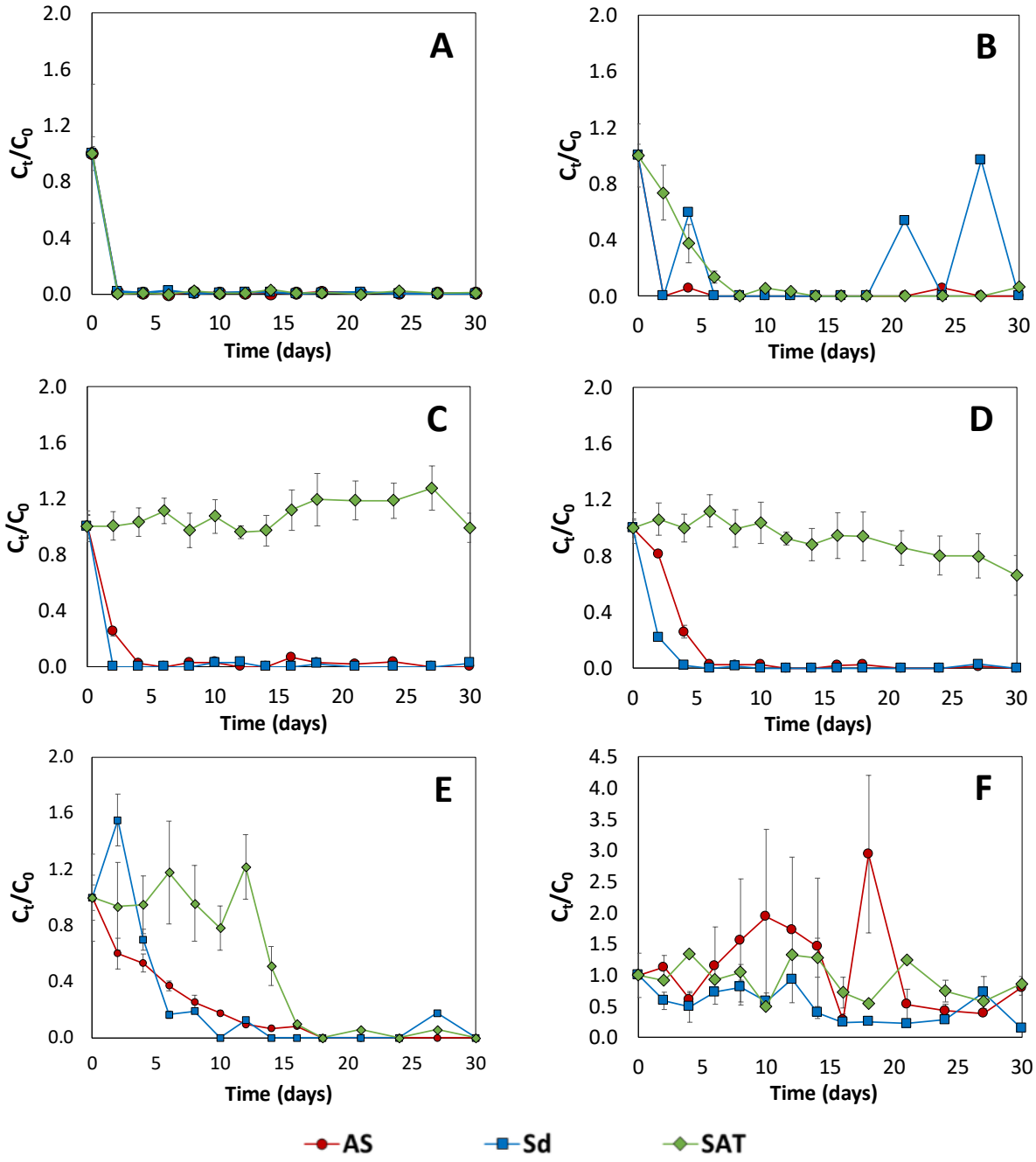


Figure 3 Fraction remaining of PPCPs for different inoculum sources. Inoculum sources used were acclimated cultures originally seeded with activated sludge (AS), sediment (Sd), and soil from SAT reactor (SAT). Compounds monitored are (A) 5-fluorouracil, (B) triclosan, (C) diclofenac, (D) gemfibrozil, (E) ibuprofen, and (F) gabapentin. Stable concentrations were observed in all killed controls (Fig. 4). Error bars represent standard deviations for biological triplicates.

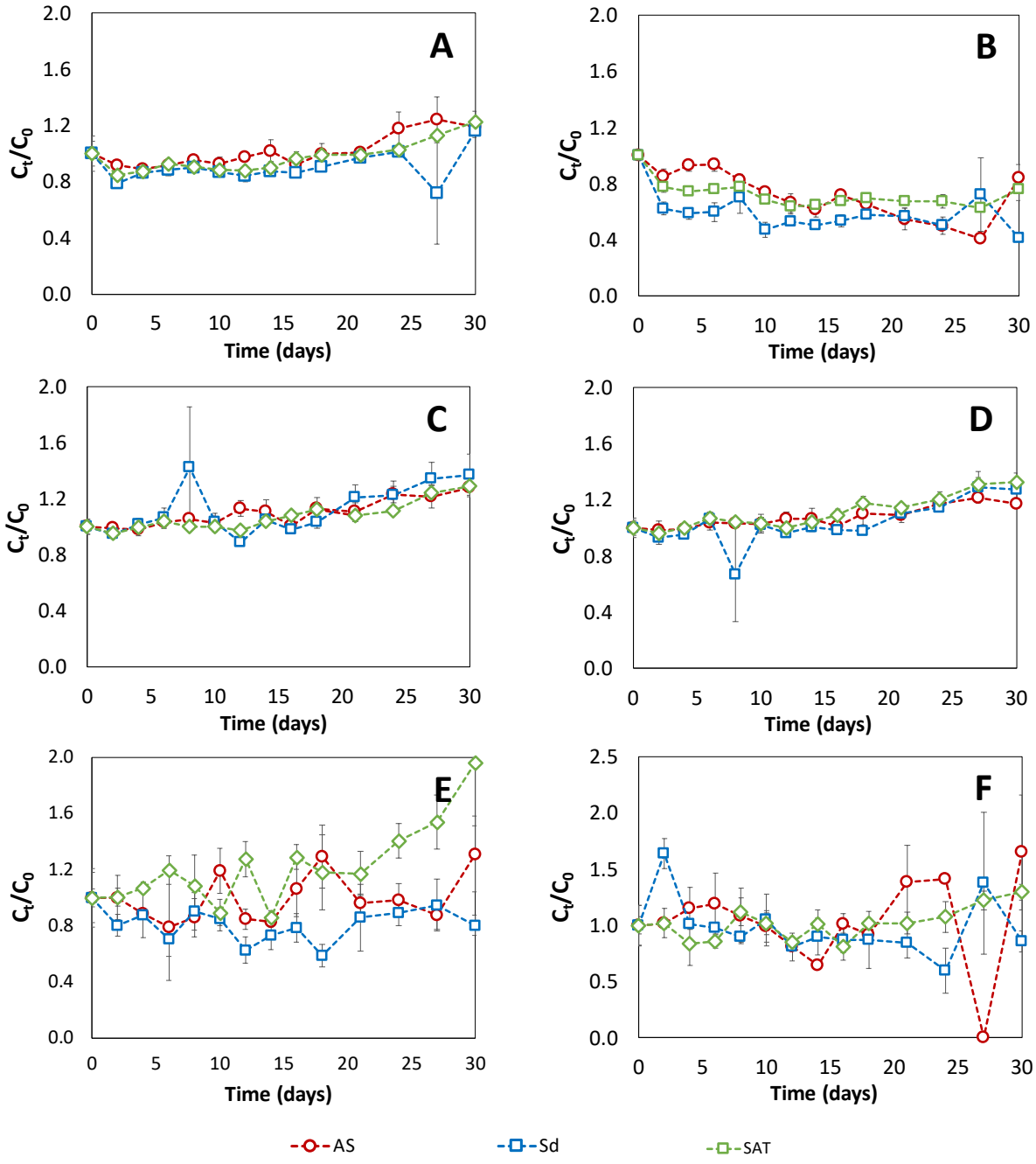


Figure 4 Fraction remaining of PPCPs for different inoculum sources in killed controls. Inoculum sources used were activated sludge (AS), sediment (Sd), and soil from SAT reactor (SAT). Compounds monitored are (A) 5-fluorouracil, (B) triclosan, (C) diclofenac, (D) gemfibrozil, (E) ibuprofen, and (F) gabapentin. Error bars represent standard deviations for biological triplicates.

3.3.2 Composition of the microbial community enriched from different inoculum sources

The major phylotypes in the AS-derived inoculum seeded reactors were *Beijerinckia*, unknown *Cytophagaceae*, and unknown *Myxococcales* (Fig. 5). *Beijerinckia* increased over time (5.93% at day 0, 9.84% at day 14, and 14.21% at day 30). Unknown *Cytophagaceae* increased first (8.94% at day 0 and 28.89% at day 14) but decreased after day 14 (21.19% at day 30). Unknown *Myxococcales* also increased initially (5.01% at day 0 and 13.58% at day 14), then decreased after day 14 (5.17% at day 30). Also, unclassified *Planctomycetales* (12.14% at day 0 and 6.60% at day 30) and *Gloebacter* (7.17% at day 0 and 5.86% at day 14) were less abundant. *Sphingomonas* was present at day 0 (8.72%), but not present afterwards.

Major phylotypes in the Sd-derived inoculum seeded reactors were *Nevskia*, *Sphingomonas*, and *Hyphomicrobium* (Fig. 5). *Nevskia* were highly abundant at day 0 (71.38%), but substantially decreased during the incubation period (15.43% at day 14, 0.92% at day 30). *Sphingomonas* increased initially (15.74% at day 0, 65.29% at day 14), but decreased after day 14 (9.51%). *Hyphomicrobium* increased by day 14 (5.28% at day 0, 8.06% at day 14), then decreased (5.07% at day 30). Unclassified *Planctomycetales* and unknown *Bradyrhizobiaceae* were also observed. Unclassified *Planctomycetales* was scarcely present during the initial period of incubation (0.36% at day 0 and 0.02 % at day 14) but increased in abundance by the end of the study (14.79%). Unknown *Bradyrhizobiaceae* increased during biotransformation period (1.13% at day 0, 5.87% at day 14, and 12.11% at day 30). At day 30, *Prosthecomicrobium* (6.59%), unknown *Armatimonadetes* (6.44%), and unclassified *Bacteroidetes* (5.20%) were also detected.

The most abundant phylotype in the SAT-derived inoculum was unknown *Myxococcales*, however, after day 0, *Ideonella*, *Ohtaekwangia*, and unknown *Planctomycetales* also were observed (Fig. 5). Unknown *Myxococcales* was the most abundant genus, but decreased through

the incubation period (77.78% at day 0, 69.92% at day 14, and 46.58% at day 30). *Ideonella* presented as the second most abundant phylotype at day 14 (6.99%) but were rare at other times (0.03% at day 0, 2.48% at day 30). *Ohtaekwangia* was not detected at day 0, but increased during the period (0.25% at day 14 and 7.53% at day 30). Similarly, unknown *Planctomycetales* was hardly present at day 0 (0.02%), but increased during the period (0.09% at day 14 and 9.90 at day 30).

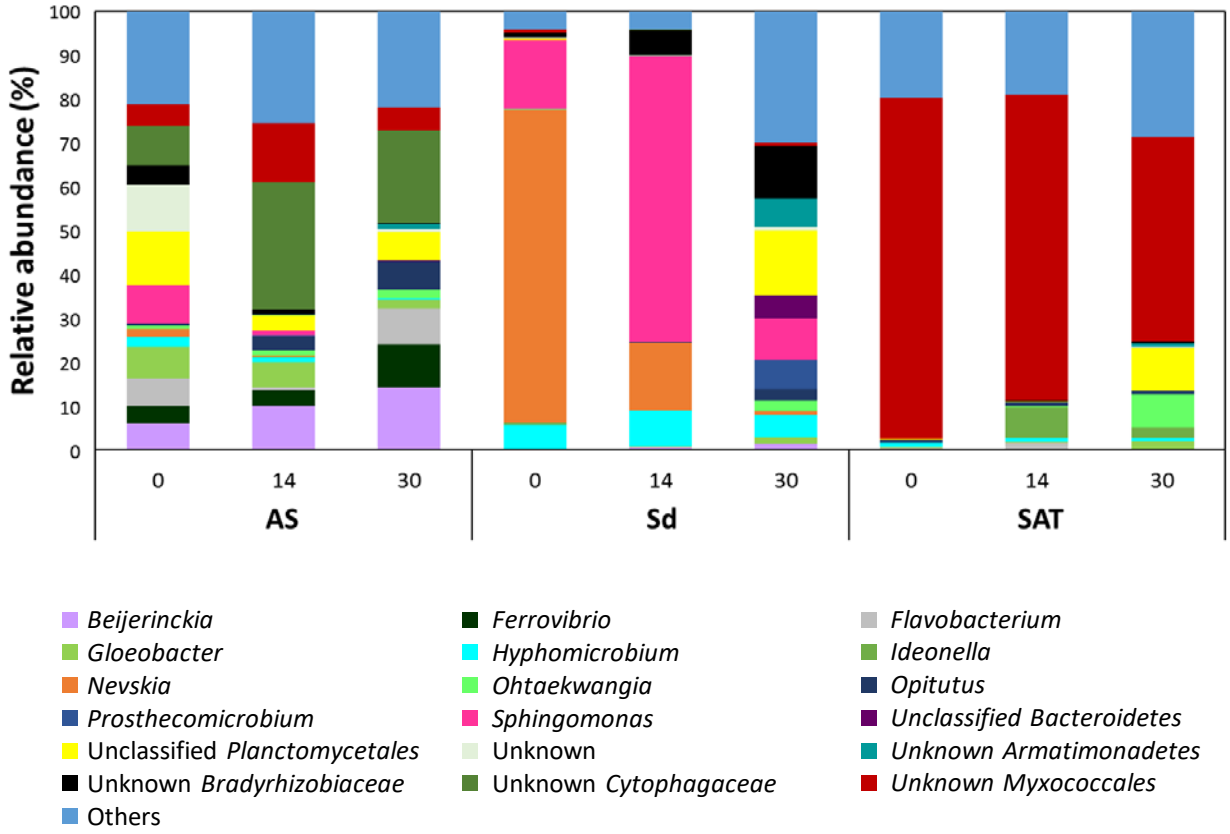


Figure 5 Microbial community structures analyzed at the genus level. Microbial communities were pre-acclimated with different inoculum sources—activated sludge (AS), sediment (Sd), and soil from a SAT reactor (SAT). The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

3.3.3 PPCP biotransformation with different primary carbon sources

For most PPCPs tested (except for 5-fluorouracil and gabapentin), biotransformation rates depended on primary carbon source. In this biotransformation test, no significant degradation was observed in the killed control reactors (Fig. 7). Neither of the humic acids and peptone mixtures worked well for biotransformation of the model compounds. Additionally, gabapentin was difficult to detect accurately with the analytical method developed for this study, and thus it is not possible to draw robust conclusions regarding the impact of carbon source on gabapentin degradation. Some compounds were detected certain amounts of concentration above 10% in the later day of biotransformation even though they removed >90% within relatively short period (e.g. 5-fluorouracil in the CA-utilizing culture showed >90% removal within 0.75 day, but it showed its remaining fraction above 0.1 at day 1.25, day 6, and day 16), indicating there might be reversible transformations.

The CA-utilizing culture removed 5-fluorouracil and triclosan to greater than 90% within 0.75 and 2 days, respectively (Fig. 6A and 6E). Conversely, diclofenac was removed close to 90% within 6 days but was not removed further during the period (Fig. 6B), gemfibrozil was not removed well with approximately 39% remaining at day 30 (Fig. 6C), and ibuprofen was removed to greater than 90% within 14 days (Fig. 6D). Gabapentin was scarcely removed with approximately 83% remaining at the end of the incubation period (Fig. 6F). Similar to the CA-utilizing culture, the OA-utilizing culture removed 5-fluorouracil and triclosan to greater than 90% within 0.75 and 2 days, respectively (Fig. 6A and 6E) and diclofenac by 4 days (Fig. 6B). Furthermore, greater than 90% of the gemfibrozil and ibuprofen were removed within 8 and 18 days, respectively (Fig. 6C and 6D). Gabapentin was not significantly removed over 30 days (Fig. 6F). The PN-utilizing culture removed 5-fluorouracil greater than 90% within 0.25 days

(Fig. 6A). By this culture, diclofenac, gemfibrozil, and triclosan were removed greater than 90% within 6, 12, and 4 days, respectively (Fig 6B, 6C and 6E). Ibuprofen was gradually removed up to approximately 86% during 30 days (Fig. 6D). Gabapentin was also slowly removed with approximately 37% remaining at day 30 (Fig. 6F). The ML-utilizing culture removed 5-fluorouracil and triclosan greater than 90% within 0.75 and 2 days, respectively (Fig. 6A and 6E). This culture slowly removed gemfibrozil during 30 days with less than 10% remaining at the end of the period (Fig. 6C). Ibuprofen was removed greater than 90% within 16 days (Fig. 6D). Diclofenac and gabapentin was scarcely removed (Fig. 6B and 6F).

The HP46-utilizing culture and the HP64-utilizing culture only removed one of the six PPCPs by day 6 (Fig. 6). 5-fluorouracil showed >90% removal in the reactors of HP46- and HP-64-utilizing culture within 6 days (Fig. 6A). For the other compounds, PPCP biotransformation was poor, regardless of ratio of humic acid to peptone. Diclofenac was not removed well with approximately 78% (HP46-utilizing culture) and 65% (HP64-utilizing culture) remaining after 30 days (Fig. 6B). Degradation of gemfibrozil was observed for 10 days, but then the concentrations of the compound remained steady (Fig. 6C). Ibuprofen also showed >90% removal by those cultures, at 14 days and 18 days, respectively (Fig. 6D). Triclosan showed >90% removal after 27 days from both systems (Fig. 6E).

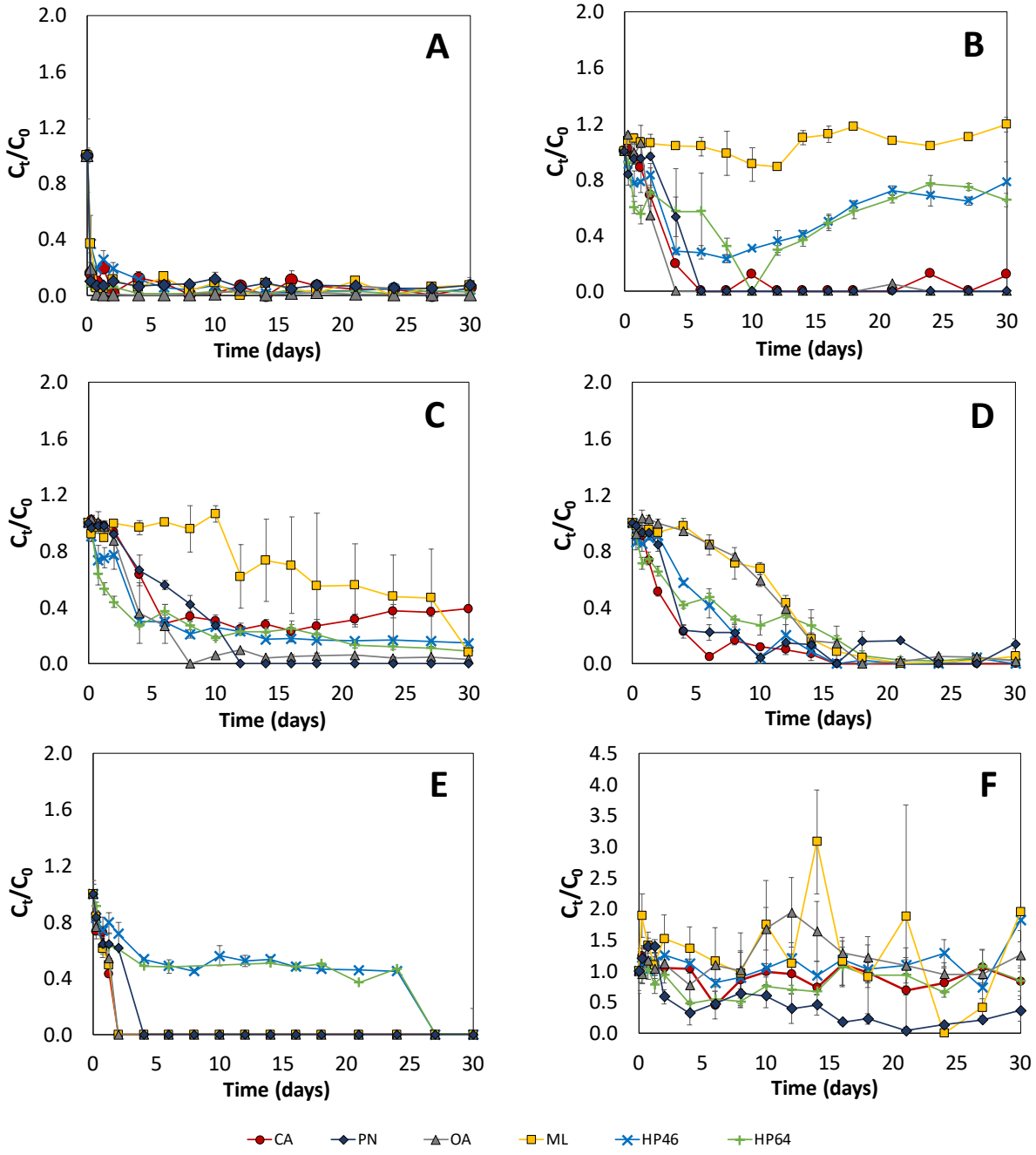


Figure 6 Fraction of remaining PPCPs from different carbon sources. Carbon sources used were casamino acid (CA), phenol (PN) organic acids (OA), molasses (ML), and humic acid and peptone mixture (HP46, HP64). Compounds monitored are (A) 5-fluorouracil, (B) diclofenac, (C) gemfibrozil, (D) ibuprofen, (E) triclosan, and (F) gabapentin. Stable concentrations were observed in all killed controls (Fig. 7). Error bars represent standard deviations for biological triplicates.

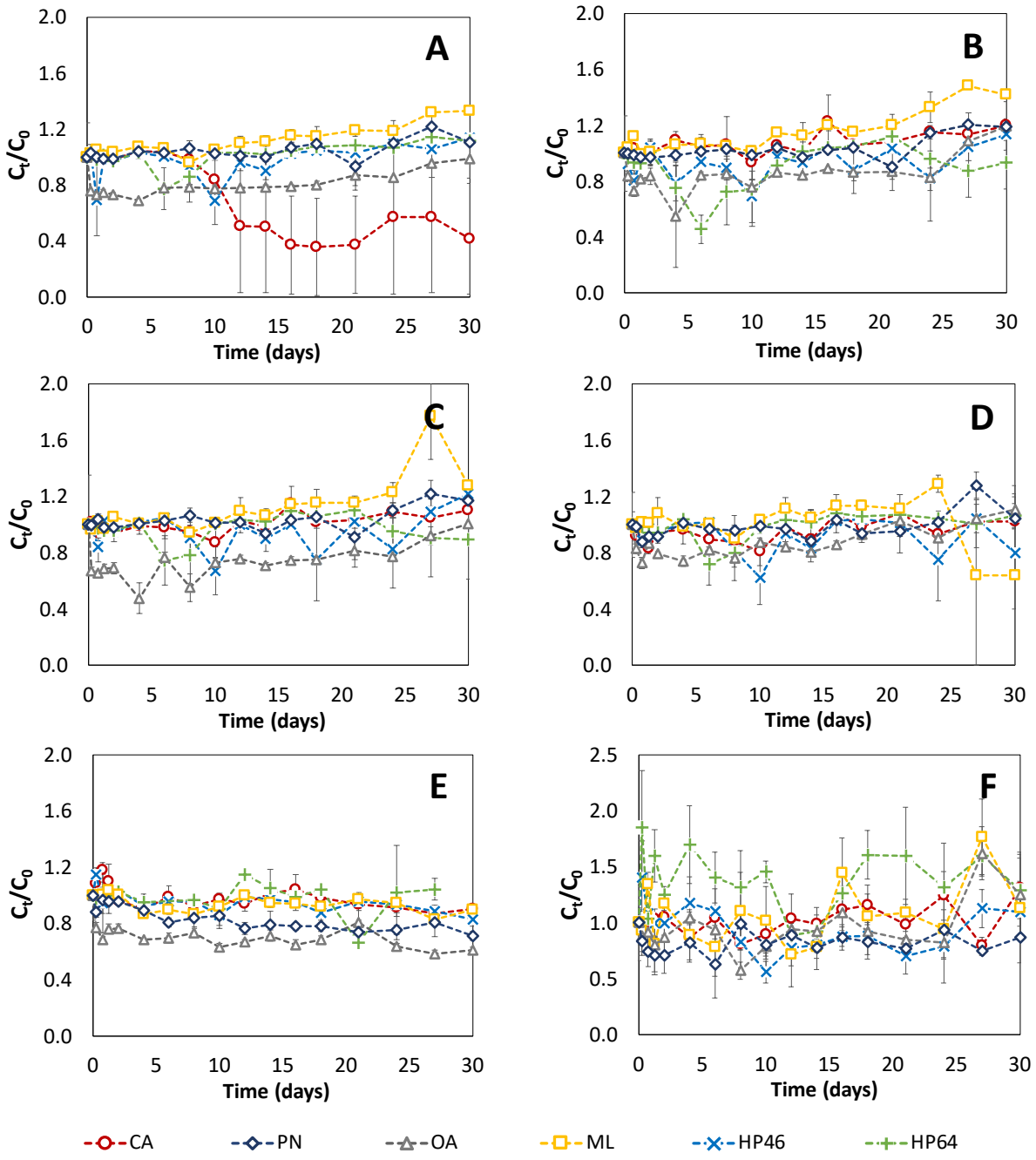


Figure 7 Fraction of remaining PPCPs from different carbon sources in killed controls. Carbon sources used were casamino acid (CA), phenol (PN) organic acids (OA), molasses (ML), and humic acid and peptone mixture (HP46, HP64). Compounds monitored are (A) 5-fluorouracil, (B) diclofenac, (C) gemfibrozil, (D) ibuprofen, (E) triclosan, and (F) gabapentin. Error bars represent standard deviations for biological triplicates.

3.3.4 Composition of microbial communities utilizing different primary carbon sources

The major phylotypes in the CA-utilizing culture were *Beijerinckia*, unknown *Methylophilaceae*, and unknown *Myxococcales* (Fig. 8). *Beijerinckia* was the most abundant genus by day 14 (15.10% at day 0 and 13.80% at day 14) but then decreased to less than 5% by day 30. Unknown *Methylophilaceae* was hardly present at day 0, but continued to increase over time (6.24% at day 14 and 17.53% at day 30). Unknown *Myxococcales* was not observed at day 0, but was present thereafter (9.12% at day 14 and 5.90% at day 30). *Hassallia*, *Methylophilus*, unclassified *Planctomycetales*, and unknown *Bacteroidetes* also were observed. *Hassallia* was the most dominant genus at day 0 (23.90%) but was not observed afterwards. *Methylophilus* was the most abundant genus at day 14 (20.86%) but not in the other periods. Unclassified *Planctomycetales* and unknown *Bacteroidetes* were hardly present by day 14 but increased by day 30 (24.30% and 23.23%, respectively).

Major phylotypes in the OA-utilizing culture were unknown *Bacteroidetes*, unknown *Beijerinckiaceae*, and unknown *Planctomycetales* (Fig. 8). Unknown *Bacteroidetes* increased initially (7.43% at day 0 and 12.76% at day 14), but decreased after 14 days to 0.18% at day 30. Unknown *Beijerinckiaceae* was present as the most dominant genus along the incubation period, but slightly decreased at day 14 (37.16% at day 0, 25.26% at day 14, and 38.14% at day 30). Unknown *Planctomycetales* was hardly present initially but substantially increased by day 30 (31.85%). *Beijerinckia* and *Sphingomonas* were also observed as abundant only at day 0 (11.21% and 19.03%, respectively), then continued to decrease to less than 5% during the incubation period.

Major phylotypes in the PN-utilizing culture were *Beijerinckia*, *Ideonella*, and *Sphingomonas* (Fig. 8). *Beijerinckia* was the only genus present consistently over the incubation

period (17.80% at day 0, 19.61% at day 14and 10.46% at day 30). *Ideonella* increased by day 14 (5.31% at day 0 and 18.06% at day 14), but then decreased to 1.66% by day 30. *Sphingomonas* was hardly present at day 0 (0.11%) but increased over time (5.93% at day 14 and 15.70% at day 30). Unclassified *Planctomycetales*, unknown *Chitinophagaceae* were also observed and they were detected only at day 30 (18.51%, and 17.15% respectively).

Major phylotypes in the ML-utilizing culture were *Methylophilus, Ohtaekwangia,* unknown *Myxococcales,* and *Beijerinckia* (Fig. 8). *Methylophilus* was the most dominant genus initially, but decreased over time (33.80% at day 0, 17.53% at day 14, and 0.44% at day 30). *Ohtaekwangia* was hardly present by day 14, but greatly increased by 30 days (26.25%). Unknown *Myxococcales* was abundant throughout the period, increased during 14 days (10.98% at day 0 and 41.77% at day 14), then decreased by day 30 (27.86%). *Asticcacaulis* and *Methylovorus* were also observed in the culture. *Asticcacaulis* was abundant at day 0 (17.88%), but hardly present in other periods. *Methylovorus* increased by day (5.81% at day 0, 13.67% at day 14), but was hardly present at day 30 (0.73%).

Major phylotypes in the HP46-utilizing culture and the HP64-utilizing culture were *Beijerinckia* (Fig. 8). Regardless of mixture ratio (40:60 and 60:40), the most dominant genus throughout the incubation period was *Beijerinckia*. In both culture, *Beijerinckia* continued to increase by day 30 and reached approximately 99% of the microbial community composition. In the HP46-utiliizng culture, *Sphingomonas* was abundant at day 0 (23.82%) but after then was less than 5% of the community. In the HP64-utillizing culture, *Nevskia* was abundant at day 0 (7.00%) but after was detected less than 5%.

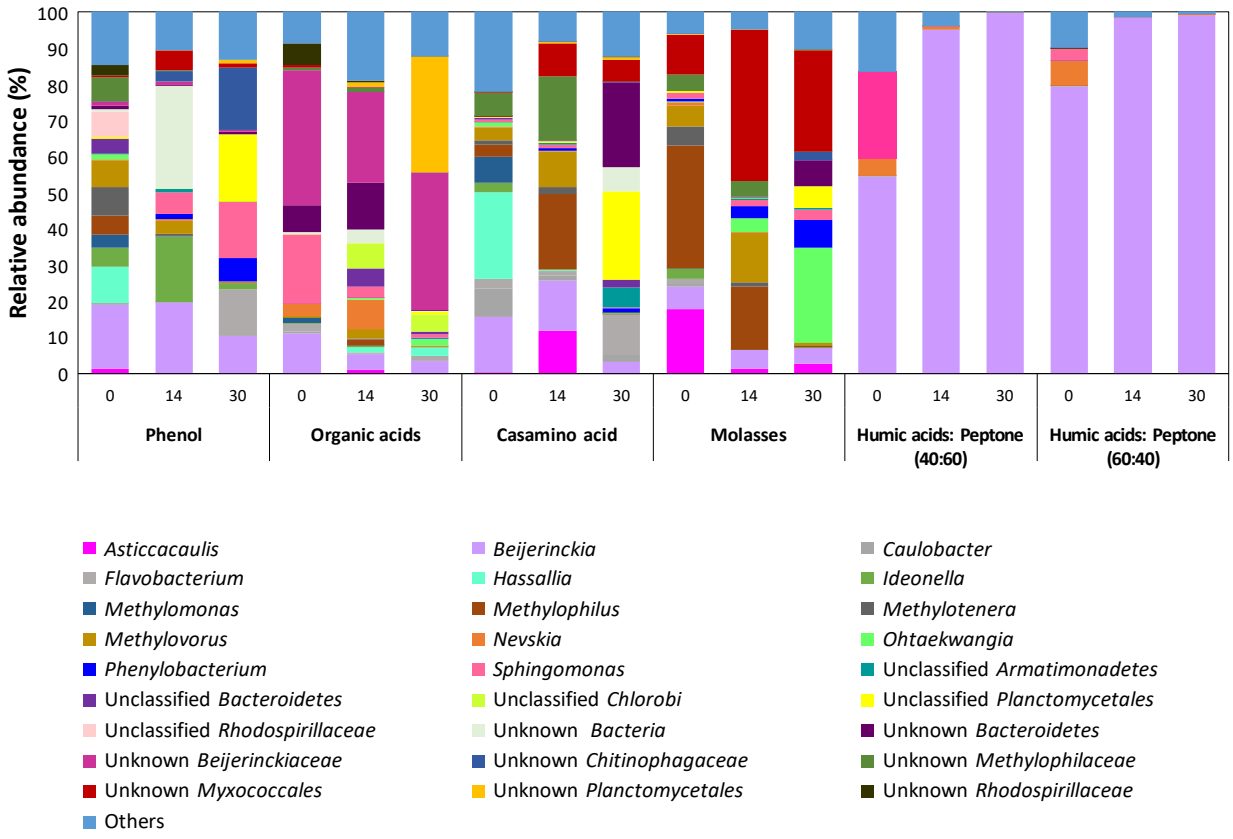


Figure 8 Microbial community structures analyzed at genus level. Microbial communities were pre-acclimated with different carbon sources. The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

3.4. Discussion

3.4.1 Impact of inoculum source on PPCP biotransformation

PPCP biotransformation was observed to depend on microbial community composition for several model compounds. The AS-derived inoculum removed four out of six PPCPs within six days. The Sd-derived inoculum removed three out of six PPCPs within six days. The SAT enriched culture removed only two out of six PPCPs within six days. Despite comparable culture conditions including the same primary carbon source (acetate), cultures inoculated with SAT-

derived inoculum showed relatively poor degradation capabilities, while those inoculated with AS- and Sd-derived inoculum showed rapid degradation of diclofenac, gemfibrozil, ibuprofen, and triclosan. Thus, data support the hypothesis that key microbes are required for PPCP removal. Variable removal rates of the compounds examined in this study have been documented in the literature. However, the factors that determine removal rates have remained largely unknown.

A previous study examining PPCP degradation under varying ammonium concentrations found that systems based on activated sludge are generally good at degrading ibuprofen regardless of culturing conditions, consistent with our findings of the lability of this compound (Tran *et al.* 2016). Similarly, in another study, cultures from sediment showed near-complete removal of ibuprofen regardless of previous exposure to ibuprofen (Alidina *et al.* 2014a). However, a study using aerobic granular sludge as an inoculum source determined that only 63.3% of the ibuprofen was degraded in GMBR system with 4 h of HRT, and the authors hypothesized that sorption of the ibuprofen onto the granules make the remaining ibuprofen biologically inaccessible (Wang *et al.* 2016). By contrast, others have reported that sorption of ibuprofen to standard activated sludge is minimal (Phan *et al.* 2016), which would theoretically lead to high bioavailability. Conversely, one study using SAT-derived inocula found that degradation was more variable, and depended on the initial concentration of ibuprofen (Onesios-Barry *et al.* 2014).

AS- and Sd-derived inocula were efficient at degrading gemfibrozil (>90% removal by day 6 and day 4, respectively). Other studies have reported variations in the degradation efficiency of gemfibrozil; a batch system inoculated with activated sludge degraded gemfibrozil approximately 30-80%, depending on initial ammonium concentrations (Tran *et al.* 2009).

Furthermore, a membrane bioreactor inoculated with activated sludge showed gemfibrozil removals of approximately 48% with 25 days of sludge retention time, and approximately 87% degradation without sludge removal (Phan *et al.* 2016). Taken together, these results indicate that the degradation of gemfibrozil with activated sludge may be more strongly dependent on specific microbial populations that require specific conditions, and our system achieved these conditions and therefore these populations. Interestingly in another study, cultures from sediments removed more gemfibrozil with high amounts of humic acid than with low amounts (Alidina *et al.* 2014b). By contrast, Sd-derived inoculum from a related study showed partial removal of gemfibrozil (~37%), and removal was consistent under pre-exposed and non-exposed conditions (Alidina *et al.* 2014b). Like the AS-derived inoculum, the microbial populations that are responsible for gemfibrozil degradation may depend on the culturing conditions such as the primary carbon sources. As in our study, a SAT-derived inoculum was unable to remove gemfibrozil (66.3% remaining), indicating that those microbial populations are not amenable to gemfibrozil degradation (Onesios-Barry *et al.* 2014).

In our study, diclofenac was quickly removed by AS- and Sd-derived inoculum (>90% within 4 days). In contrast, other studies have found diclofenac to be much more recalcitrant, depending on the inoculum source and culturing conditions. In addition, Phan *et al.* (2016) reported only 6% removal of diclofenac in a membrane bioreactor at a 25 day of sludge retention. Tran *et al.* (2009) reported approximately 40% removal of diclofenac after 6 days of incubation in a 100 ml-batch system by a culture based on activated sludge, although increasing the ammonium increased the diclofenac removal to greater than 75% over the same period. Similarly, Sd-derived inoculum removed a maximum of approximately 55% under high humic acid conditions, but as low as approximately 3% under low humic acid conditions (Alidina *et al.*

2014b). Interestingly, SAT-derived inocula showed similarly low, variable degradation of diclofenac under different culture conditions (Onesios and Bouwer. 2012, Onesios-Barry *et al.* 2014). On the other hand, ozonated soil-batch reactors inoculated with secondary effluent from wastewater treatment degraded approximately 85% of the diclofenac, indicating that removal depends on microbial populations that are enhanced by specific culturing conditions. Thus, data reported herein suggests that microbial community composition is a critical variable that likely affected removal rates in previous studies, although the majority of studies to date have not conducted detailed microbial community characterization, and have instead focused on varying culture conditions that could lead to different microbial community structures.

Although results indicate that microbial community composition matters, two cultures with diverging community structures both showed efficient PPCP removal. The systems with AS- and Sd-derived inoculum were similarly efficient at removing PPCPs. However, the abundant phylotypes differed; AS-derived inoculum was dominated by unknown *Cytophagaceae,* unknown *Myxococcales,* and *Beijerinckia,* while Sd-derived inoculum was dominated by *Nevskia, Sphingomonas, Hyphomicrobium,* and unknown *Bradyrhizobiaceae.* Thus, data suggested two hypotheses: (1) PPCPs are degraded by multiple phylotypes with redundant function, or (2) the PPCPs are degraded by rare phylotypes which were present in both the AS- and Sd-derived inoculum.

Microbial community composition did not have an impact on degradation of one model PPCP, 5-fluorouracil, which was readily degradable by all inocula. All inoculum sources studied here were very efficient at removing 5-fluorouracil (>90% removal after 2 days). This is consistent with another study, which found 5-fluorouracil was easily degraded under multiple conditions (>90% removal in SAT columns under both low acetate conditions (50 μg/L) and

high acetate conditions (1,000 μg/L) (Onesios and Bouwer, 2012). This fits with the acetate concentration in our cultures (100 μg/L), and indicates that this compound was readily degraded even under relatively carbon-starved conditions. Removal of 5-fluorouracil in this study was found to be more effective than other study. For example, Yu *et al.* (2006) obtained less than 60% of removal of the compound with 50 μg/L of initial concentration.

Previous studies have shown that when the concentration of a PPCP is sufficiently high, it has a toxic effect on the microbial community and degradation is inhibited. However, the exact concentration at which a toxic effect is seen differs between compounds, and for some compounds toxicity is not apparent even at milligram per liter concentrations. Onesios-Barry *et al.* (2014) reported that the removal efficiency of 5-fluorouracil was optimal at low concentration (10 μg/L), with decreasing removal at 100 μg/L or 1,000 μg/L (Onesios-Barry *et al.* 2014). This result is consistent with our finding, indicating that this compound is easily degraded at 10-50 μg/L but is potentially toxic to biodegradative microbes at 1,000 μg/L. Interestingly, in our preliminary tests with the unacclimated AS inoculum, 5-fluorouracil was not degraded over a 35-day period (Fig. 11); although these tests were conducted with a lower biomass, results and literature collectively suggest that microbial community composition is a key variable for 5-fluorouracil degradation. However, required microbes may be common and easily selected for during acclimation. Further research is needed to identify required microbial community members and those that directly mediate biotransformation of all PPCPs including 5-fluorouracil. Overall findings suggested that engineering approaches targeted at changing reactor microbiomes, via inoculation strategies or using selective pressures, will result in improved biotransformation performance. One potentially viable way to select for desired microbes is by providing a carbon source that favors PPCP-degrading phylotypes.

3.4.2. Impact of carbon source on PPCP biotransformation

PPCP biotransformation was also observed to depend on the primary carbon source provided. The CA-utilizing culture removed four out of six PPCPs within six days. The OA-utilizing culture removed three out of six PPCPs within six days and one PPCP within eight days. The PN-utilizing culture removed three out of six PPCPs within six days and another one PPCP within twelve days. The ML-utilizing culture removed two out of six PPCPs within six days and another one PPCP by 16 days. Of the carbon sources tested, phenol, casamino acids, and the organic acid mixture supported relatively rapid PPCP biotransformation for many of the model compounds, while molasses and both mixtures of humic acids and peptone showed relatively poor removal. Not surprisingly, however, results varied by compound. Phenol was found to be the most effective carbon source of those tested. The PN-utilizing culture degraded three out of six PPCPs (5-fluorouracil, diclofenac, and triclosan) to >90% within six days and an additional PPCP (gemfibrozil) by 30 days. This indicates that phenol may be capable of supporting biotransformation of multiple PPCPs. However, the other two PPCPs (gabapentin and ibuprofen) were only partially degraded even after 30 days (14.2% and 36.5% remaining, respectively). Similarly, the presence of phenol instead of glucose in the media resulted in increased degradation (75.14±1.71% with glucose, 86.27±2.09% with phenol) of the PPCP naproxen by the bacterium *Planococcus* sp. Strain S5 (Domaradzka *et al.* 2015). Domaradzka *et al.* (2015) hypothesized that phenol may induce enzymes such as phenol monooxygenase that are responsible for aromatic ring cleavage, which may be acting on the naproxen. A similar phenomenon may be occurring here, which may explain why gabapentin, the only PPCP in this study without an aromatic ring, was generally so poorly degraded; interestingly, gabapentin degradation appeared to be the best in the PN-utilizing culture, despite analytical issues. The

OA-, CA-, and ML-utilizing cultures degraded two out of six PPCPs (5-fluorouracil and triclosan) within a week and an additional PPCP (ibuprofen) by 30 days. Gemfibrozil and diclofenac showed different removal result corresponding to the carbon sources. Gabapentin showed poor removal results in all conditions. Casamino acids were a better primary carbon source than molasses or acetate for supporting the growth of bacterial isolates that were capable of degrading triclosan, ibuprofen, or gemfibrozil (Zhou *et al.* 2013). In our study, molasses was a less effective primary carbon source than others, especially for degradation of 5-fluororuacil and triclosan. As expected, microbial community compositions varied significantly as a function of carbon source provided. Thus, differences in PPCP biotransformation could be due to differences in microbial growth and microbial community composition as results of different carbon sources. Additionally, different carbon sources induce expression of different genes, so the favorable carbon sources may have induced expression of critical genes for PPCP biotransformation.

Both mixtures of humic acids and peptone as the primary carbon source were the least efficient for removing PPCPs. Like the other cultures, HP46- and HP64-utilizing cultures degraded 5-fluorouracil readily, with none remaining within 0.25 days and 6 days, respectively. However, all other PPCPs remained at near-initial levels for at least 6 days. Ultimately, both cultures degraded an additional two PPCPs (ibuprofen and triclosan) by 30 days. Interestingly, the removal patterns were generally similar regardless of the ratio of humic acids to peptone. This finding is contrast to that of Alidina *et al.* (2014b), who, in a series of studies, found that a higher portion of humic acid relative to peptone resulted in better removal of PPCPs, including diclofenac and gemfibrozil (Alidina *et al.* 2014b). One possible cause of this difference could be that the initial concentrations of PPCPs in the system differed (0.3 – 0.5 μg/L in Alidina *et al.* (2014b), Drewes *et al.* (2014) and Li *et al.* (2014); 50 μg/L in this study), suggesting that the

impact of humic acid and peptone as primary carbon sources may only appear when the PPCP concentration is relatively low. Additionally, the HP mixtures are less favorable carbon sources for growth, and the HP cultures showed lower biomass over time than the other cultures. Thus, lack of growth over the 30-day time period may have contributed to the minimal PPCP removal observed suggesting that degradation rates were limited by biomass.

3.4.3 Identification of microbial phylotypes linked with PPCP biotransformation

Certain phylotypes were positively related to PPCP degradation. By comparing different inoculum sources, we found that *Sphinogomonas,* unknown *Myxococcales, Beijerinckia, Methylophilus,* and unknown *Cytophagaceae* were linked with efficient PPCP biotransformation (Fig. 9). *Nevskia* was negatively correlated; however, despite its decreasing concentration during degradation, *Nevskia* may play a role in PPCP transformation because it was highly abundant during degradation. Considering that better biotransformation was shown with the AS- and Sd-derived inocula, unknown *Myxococcales, Beijerinckia, Methylophilus, Sphingomonas* and unknown *Cytophagaceae* may impact the PPCP biotransformation. PCA result showed that different primary carbon sources linked a suite of phylotypes to PPCP biotransformation. Unclassified *Myxococcales,* unknown *Methlyophilus,* unknown *Beijerinckia, Methlyovorus glucosotrophus*, unknown *Sphingomonas,* unknown *Bacteroidetes*, unknown *Planctomycetales,* unknown *Beijerinckiaceae,* and *Beijerinckia mobilis* were found to be related to PPCP biotransformation (Fig. 10). Considering that the highest removal was in the PN-, OA-, and CA-utilizing cultures, unknown *Beijerinckia,* unknown *Bacteroidetes*, unknown *Planctomycetales,* and unknown *Beijerinckiaceae* may be key microorganisms related to PPCP biotransformation. Some phylotypes that were unclassified or unknown at the genus level were also detected.

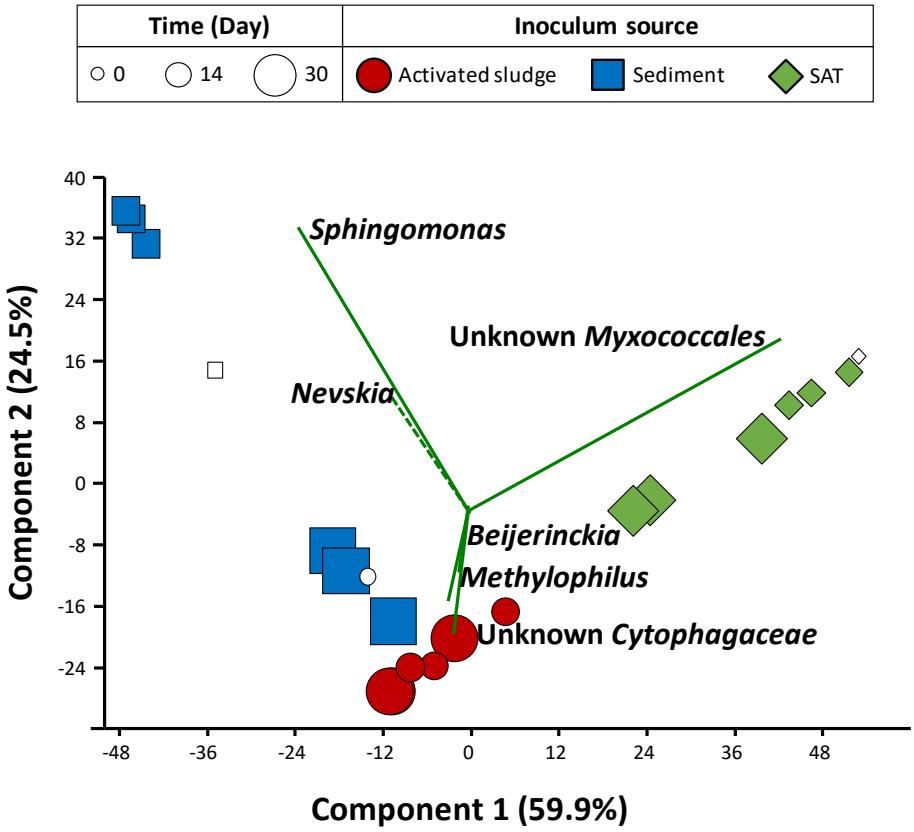


Figure 9 PCA result with microbial phylotypes change over time. Circles are the AS-derived inocula, squares are Sd-derived inocula, and diamonds are SAT-derived inocula. Small-sized labels are at day 0, medium-sized labels are at day 14, and large-sized labels are at day 30. Dotted line for *Nevskia* was from drastic decrease in Sd-derived inocula between day 0 and day 14.

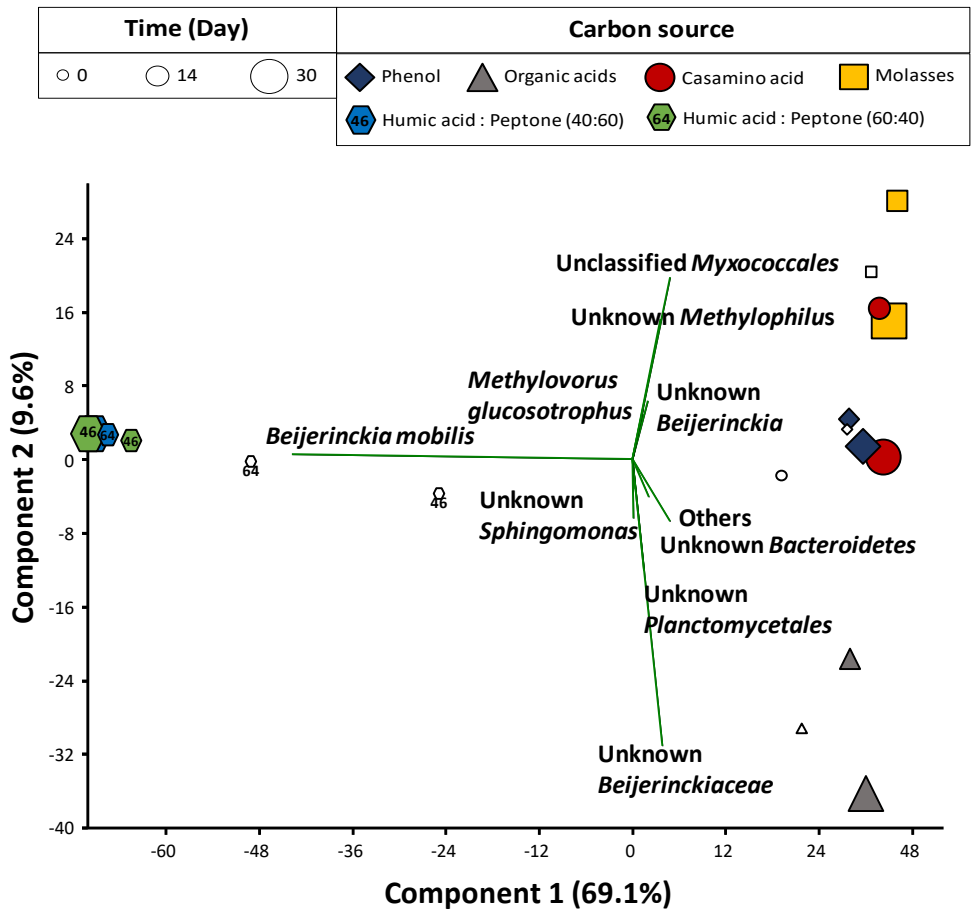


Figure 10 PCA result with microbial phylotypes change over time. Circles are CA-utilizing culture, squares are ML-utilizing culture, triangles are OA-utilizing culture, diamonds are PN-utilizing culture, and hexagons are HP46- and HP64-utilizing culture. The ratio of the mixture used are 40:60 and 60:40, respectively labeled 46 and 64 within the figures. Small-sized labels are at day 0, medium-sized labels are at day 14, and large-sized labels are at day 30.

Genus *Sphingomonas* was found in both biotransformation tests and was positively involved in PPCP biotransformation. *Sphingomonas mali* and unknown *Sphingomonas* species were abundant in the cultures that were highly efficient at degrading a range of PPCPs. *Sphingomonas mali* may be particularly relevant to the removal of diclofenac and gemfibrozil (Table 7). Unknown *Sphingomonas* species were the most abundant in the Sd-derived inoculum, which was highly efficient at degrading 5-fluorouracil, triclosan, and diclofenac (Fig. 5). Various

Sphingomonas species have been reported in the context of PPCP biotransformation. *Sphingomonas* sp. Rd1 can degrade triclosan (Hay *et al.* 2001), and *Sphingomonas* sp. strain Ibu-2 can degrade ibuprofen (Murdoch *et al.* 2005). Zhou *et al.* (2013) reported that *Sphingomonas* isolates (TrD23, TrD34) were able to degrade triclosan to ng/L concentrations and utilize a wide range of carbon sources including several types of protein-rich substrates, sugars, or others such as acetate. *Sphingomonas* sp. strain P2 is able to degrade phenanthrene as a sole carbon source via a dioxygenase that may have broad substrate specificity (Pinyakong *et al.* 2000). Additionally, the genus *Sphingomonas* (45D) was found in bacterial communities that can degrade chlorinated flame retardants, such as tris(1,3-dichloro-2-propyl) phosphate (TCEP) and tris(2-chloroethyl) phosphate (TDCPP) (Takahasi *et al.* 2013). Therefore, this genus may be capable to metabolize a wide range of compounds, including several PPCPs.

Additionally, *Beijerinckia* species appear to be useful for removing PPCPs. *Beijerinckia mobilis* and unknown *Beijerinckia* showed varying patterns with respect to PPCP biotransformation in this study. Although low in abundance in the culture originally inoculated from activated sludge, the genus increased in abundance over time, correlating with efficient degradation of PPCPs. This phylotype may be relevant to higher removal efficiency for PPCP biotransformation. It is noteworthy that OA-utilizing culture also featured unknown *Beijerinckiaceae* in high abundance. HP46- and HP64-utilizing cultures were highly dominated by *Beijerinckia mobilis*, but these cultures were poor degraders of several PPCPs. However, growth rates were slower for HP-utilizing cultures (Fig. 17), and thus poor removal in these reactors was likely due to limited biomass. Therefore, *Beijerinckia mobilis* also may actually be capable of PPCP biotransformation. The genus *Beijerinckia* is known as an aerobic, chemo-heterotrophic bacteria, which is able to fix atmospheric dinitrogen, and *Beijeringkia mobilis* is

capable of methylotrophic metabolism (Dedysh *et al.* 2005). One strain of this species, *Beijerinckia mobilis* 1f Phn(+), is capable of degrading phenanthrene, and could cometabolize naphthalene and dibenzohiophene (Surovtseva *et al.* 1999).

Methylophilus, unknown *Cytophagaceae,* and unknown *Planctomycetales* were also linked with PPCP biotransformation. Based on the PCA conducted in this study, *Methylophilus* and unknown *Cytophagaceae* are likely to positively impact biotransformation for all compounds studied. *Methylophilus* is a methylotrophic bacterial strain found in soil and sediments contaminated by pollutants such as aromatics or TCE (trichloroethylene) (De Marco *et al.* 2004). Additionally, a *Methylophilus* sp. isolated from a humic lake degraded phenol and humic matter (Hutalle-Schmelzer *et al.* 2010). Genuse *Methylophilus* has been implicated in the biodegradation of anthracene in municipal solid waste composting soil (Zhang *et al.* 2011) and polychlorinated biphenyls (PCB) in bulk soil (Uhlik *et al.* 2009), so members of this genus may be capable of degrading other aromatic hydrocarbons and xenobiotic compounds. We could not determine the species of the *Cytophagaceae* present in this study, but this family is large and diverse. Although the *Cytophagaceae* family is known for cellulose degradation, the genus *Dyadobacter* within this family degrades heterocyclic aromatic hydrocarbons but not polysaccharides (Willumsen *et al.* 2005), indicating that this group may be capable of a wider range of metabolism than previously appreciated. In contrast, a previous study found that *Cytophagaceae* did not contribute to the degradation of 30 trace organic compounds tested in a membrane bioreactor (Phan *et al.* 2016). It is possible that the specific genera within the *Cytophagaceae* differed between their study and ours, In this study, unknown *Planctomycetales* significantly correlated to the removal of diclofenac and triclosan (Table 7). A previous study found that *Planctomycetes* may be involved in biotransformation of trace organic compounds,

including diclofenac and triclosan (Phan *et al.* 2016). Furthermore, a genomic comparison of the members of the *Planctomycetaceae* family within the *Planctomycetales* determined that every family member possessed metabolic pathways for degrading a range of toxic compounds including polycyclic aromatic hydrocarbons (Guo *et al.* 2014).

A phylotype related to poor removals was unknown *Myxococcales.* Unknown *Myxococcales* was related to poor PPCP biotransformation in this study, being particularly abundant in the SAT-derived inoculum and ML-utilizing culture. This is surprising given that *Myxococcales* was identified as a potential remover of trace organic contaminants (including gemfibrozil, ibuprofen, and triclosan) in a membrane bioreactor (Phan *et al.* 2016). However, diclofenac was poorly transformed in their system, fitting with our finding that cultures with high levels of *Myxococcales* (SAT-derived and molasses-utilizing) were poor degraders of diclofenac (Phan *et al.* 2016).

Table 7 Spearman's correlation between PPCPs and microbial species in biotransformation tests. Not significant ($p>0.1$, r value not reported), weakly significant $0.05<p<0.1$, significant $p<0.05$.

	Diclofenac	5-Fluorouracil	Gabapentin	Gemfibrozil	Ibuprofen	Triclosan
Unclassified (*Armatimonadetes*)	-	-	-	-	-	-0.4796
Unknown (*Ohtaekwangia*)	0.4073	-	-	-	-	
Unknown (*Flavobacterium*)	-	-	-	-	-	
Unknown (*Chitinophagaceae*)	-	-	-	-	-	-0.4152
Unclassified (*Bacteroidetes*)	-0.4532	-	-	-	-	-
Unknown (*Bacteroidetes*)	-	-	-	-	-	-
Unclassified (*Chlorobi*)	-	-	-	-	-	-
Hassallia byssoidea	-	-	-	-	-	-
Unclassified (*Planctomycetales*)	-	-	-	-	-	-
Unknown (*Planctomycetales*)	-0.4784	-0.4083	-	-0.4507	-	-0.5013
Asticcacaulis sp	-	-	-	-	-	-
Phenylobacterium sp	-	-	-	-0.3985	-	-0.5045
Beijerinckia mobilis	-	-	-	-	-	-
Unknown (*Beijerinckia*)	-	-	-	-	-	-
Unknown (*Beijerinckiaceae*)	-	-	-	-	-	-
Unclassified (*Rhodospirillaceae*)	-	-	-	-	-	-
Unknown (*Rhodospirillaceae*)	-	-	-	-	-	-
Sphingomonas mali	-0.4859	-	-	-0.6926	-	-
Unknown (*Sphingomonas*)	-	-	-	-	0.4985	-
Ideonella sp	-	-	-	-		-
Unknown (*Methylophilus*)	-	-	-	-		-
Methylotenera sp	-	0.5242	-	0.5245	0.5245	0.5777
Unknown (*Methylovorus*)	-	-	-	-	-	-
Methylovorus glucosotrophus	-	-	-	-	-	-
Unknown (*Methylophilaceae*)	-	-	-	0.4144	-	-
Unknown (*Myxococcales*)	-	-	-	-	-	-
Methylomonas sp	-	0.5972	-	0.3972	0.6044	0.5520
Unknown (*Nevskia*)	-	-	-	-	0.5286	0.4356
Unknown (*Bacteria*)	-	-	-	-	-	-
Others	-	-	-	-	-	-

3.4.4 Conclusion and future work

The hypotheses of this study were that microbial community and primary carbon sources influence PPCP biotransformation. Thus, engineering approaches that target specific microbial communities could result in improved biotransformation performance. The overall conclusion from this study is that microbial community composition, controlled by the inoculum source and the primary carbon source, impacted PPCP biotransformation. Of inoculum sources tested, microbial communities derived from AS and Sd resulted in acclimated cultures that degraded PPCPs with higher removal efficiencies (>90% for all compounds except gabapentin) than cultures from SAT, under consistent biomass concentration. Of primary carbon sources tested, casamino acids, the organic acids mixture, and phenol supported degradation of PPCPs with higher removal efficiencies. Unknown *Sphingomonas* and unknown *Beijerinckia* may have a positive impact on PPCP biotransformation. Further study is needed to identify specific phylotype(s) that are directly involved in PPCP biotransformation, i.e.,with serial dilution to develop less diverse cultures and culturing to find expression of critical genes playing a key role. Elucidating biotransformation pathways and intermediates using metabolomics and metatranscriptomics would also be necessary to advance fundamental understanding of PPCP biotransformation.

4. PPCP BIOTRANSFORMATION: THE IMPACT OF ACCLIMATION

4.1 Introduction

Biotransformation is affected by microorganisms present in the system, as shown via the study described in Chapter 3. Microbial community structure can be easily shifted by several environmental factors (e.g. temperature, pH, redox potential) (Hoppe-Jones *et al.* 2012). Previous studies suggested that adaptation of the microbial community could be one of the important factors to improve microbial biodegradation (Alidina *et al*. 2014b, Drewes *et al.* 2006, Hoppe-Jones *et al.* 2012, Rauch-Williams *et al*. 2010, Wilson *et al*. 2013). It has been shown that acclimation may help to stabilize and even increase microbial activity in biodegradation process. Rauch-Williams *et al.* (2010) reported that higher removal of the tested compounds including gemfibrozil and diclofenac was due to the microbial community adapted to metabolizing those compounds. Acclimation of the microbial community to single or multiple chemicals may produce microbial communities adapted to tolerate and degrade PPCPs (Wang *et al.* 2016).

The objective of the study presented in ths chapter was to investigate the hypothesis that PPCP biotransformation ability is dependent on the acclimation of the microbial community. This study was performed as a preliminary study prior to the study described in Chapter 3. Seven PPCP compounds (biosol, diclofenac, 5-fluorouracil, gabapentin, gemfibrozil, ibuprofen, and triclosan) were investigated. We evaluated degradation of these PPCPs and analyzed the structure changes of the microbial communities over time during biotransformation. Activated sludge was used for inoculum source. Acetate was used as a primary carbon source. PPCP detection method (GC-MS) and microbial analysis (16S rRNA gene amplicon sequencing via Illumina next-generation sequencing) described in Chapter 3 were also used for this experiment.

4.2 Material and methods

4.2.1 Inoculum source

Activated sludge (AS) was taken from Drake Water Reclamation Facility (DWRF) (Fort Collins, Colorado, United States) and was used for inoculum source. Acclimated inoculum data obtained in Chapter 3 is reproduced here for comparison with unacclimated inoculum data.

4.2.2 Reactor setup and operation

As described in Chapter 3.2.2, reactor setup and operation were the same including chemical composition and concentration, amount of sand, and sterilization.

Biotransformation systems were setup in 250 mL-flask batch reactor which consisted of 1 mL of AS, 50 µg/L of PPCPs, 100 µg/L of carbon source (acetate), trace mineral solution, and water. Acclimation for biotransformation systems with acclimated AS was conducted for 8 weeks. Incubation period for biotransformation systems with unacclimated AS and with acclimated AS were 36 days and 30 days, respectively. Killed control reactors were autoclaved before adding PPCPs. The tests were maintained at room temperature (25°C). All reactors were incubated in triplicate and were kept on a shaker with mixing speed at 120 rpm to maintain aerobicity.

4.2.3 Chemical analysis

Same analytic method with GC-MS described in Chapter 3.2.4 (except for using the isotope labeled standards) was used for PPCP detection. The targeted PPCPs are biosol, diclofenac, 5-fluorouracil, gabapentin, gemfibrozil, ibuprofen, and triclosan.

4.2.4 Microbial community analysis

Microbial communities in the unacclimated AS and in the acclimated AS were analyzed with the same method as described in Chapter 3.2.5. Samples for the biotransformation systems with unacclimated AS were taken at day 0 and day 36. Samples for the biotransformation systems with acclimated AS were taken at day 0, day 14, and day 30.

4.3 Results and discussion

4.3.1 PPCP biotransformation

With the unacclimated AS culture, 4 out of 7 PPCPs (biosol, gabapentin, ibuprofen, and triclosan) were removed >90% by the end of the incubation period (Fig. 11). Biosol was removed well (~90% removal), gabapentin was removed well (~85% removal), ibuprofen was removed >90% very fast within 3 days, and triclosan was removed >90% within 9 days. 5-Fluorouracil was removed moderately (~49% removal) as was gemfibrozil (~43% removal). Diclofenac was not removed: at day 30 remaining fraction was 1.19, with increase likely due to some media evaporation over the course of the study. Removal trends in this unacclimated study are less accurate than those in the acclimated study since the isotope labeled internal standards were not used at the time of the unacclimated study.

With the acclimated AS culture, 6 out of 7 PPCPs were removed >90% during the incubation period. No significant degradation was observed in the killed control reactors indicating that observed losses were due to biotransformation and not sorption or volatilization. For >90% removal, 2 days was taken for 5-fluorouracil and triclosan, 4 days was taken for diclofenac, 6 days was taken for gemfibrozil, 12 days was taken form ibuprofen, and 18 days was taken for biosol. Gabapentin appeared to be approximately 72% removed by day 16, but

then concentrations rebounded and only 20% removal was observed at the end of the incubation period.

For most PPCPs tested (except for gabapentin), biotransformation rates depended on acclimation. Comparison of biotransformation results for the unacclimated AS culture and the acclimated AS culture shows that acclimation enhanced biotransformation of PPCPs including biosol, diclofenac, 5-fluorouracil, gemfibrozil, and triclosan. For example, Ibuprofen was removed well both in the unacclimated AS culture and in the acclimated AS culture, but the time taken for >90% removal was different (3 days with the unacclimated AS culture, 12 days with the acclimated culture). In this study, acclimation did not affect biotransformation of gabapentin; however, analytical issues may have obscured trends as noted in Chapter 3.

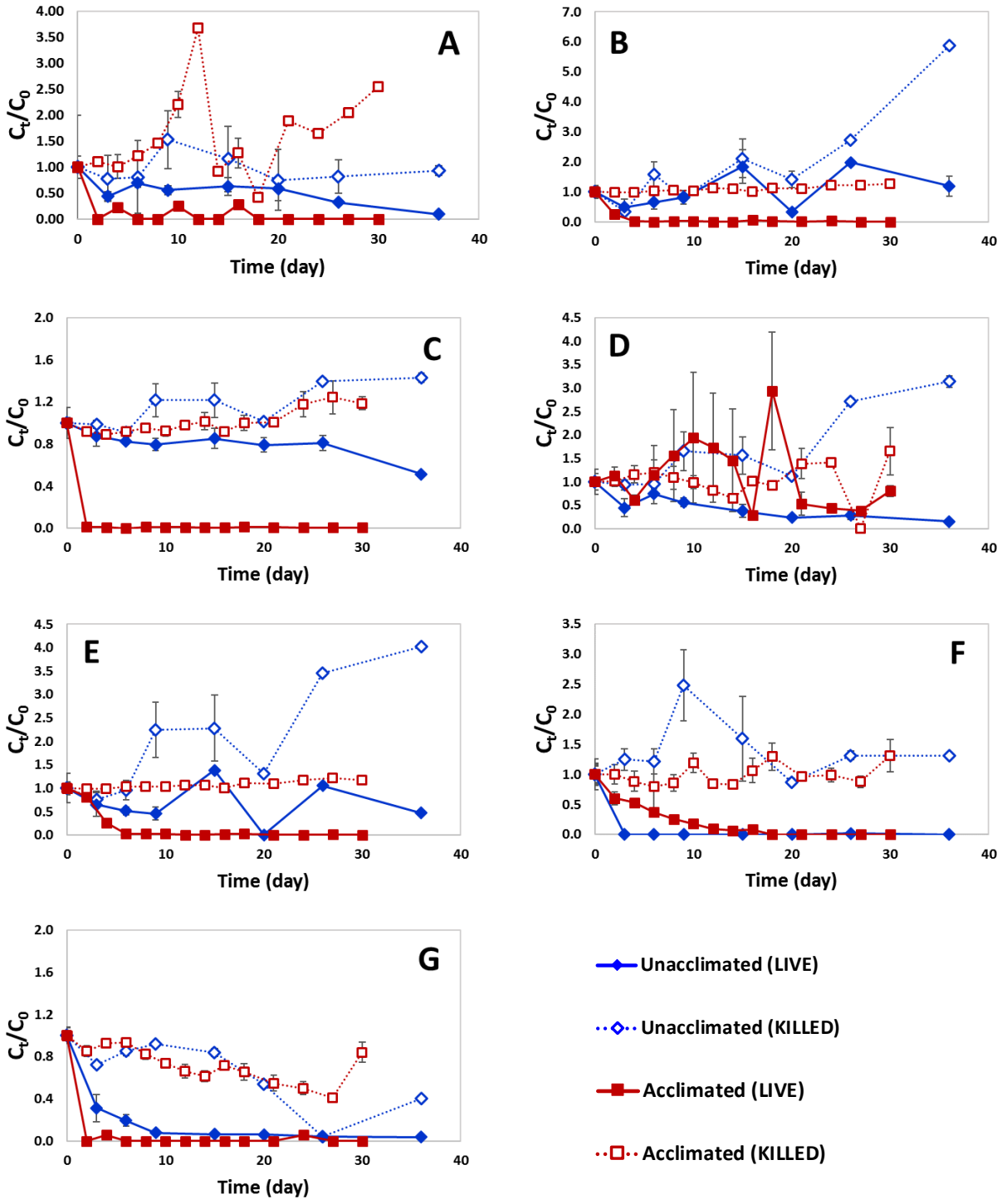


Figure 11 PPCP concentrations with unacclimated inoculum and with acclimated inoculum. Inoculum source used was activated sludge. Compounds monitored are (A) biosol, (B) diclofenac, (C) 5-fluorouracil, (D) gabapentin, (E) gemfibrozil, (F) ibuprofen, and (G) triclosan. Acclimated inoculum data presented is from Fig. 3 in Chapter 3 and is reproduced here for comparison. Error bars represent standard deviations for biological triplicates.

4.3.2 Microbial community composition

Acclimated inoculum data obtained in Chapter 3 is reproduced here for comparison with unacclimated inoculum data. The major genera in the unacclimated AS culture were *Flavobacterium* (9.55%), *Candidatus Microthrix* (8.05%), unknown *Comamonadaceae* (7.71%), unclassified *Saprospiraceae* (7.56%), and unknown *Xanthomonadales* (6.11%) at day 0 (Fig. 12). Although these phylotypes were abundant at day 0, they all diminished by the end of incubation (day 36). However, *Asticcaculis* (13.33% at day 36) and *Methylophilus* (69.09% at day 36) were observed only at day 36, and they were dominant genera in the community at that time.

The major phylotypes in the acclimated AS culture were unknown *Cytophagaceae*, *Beijerinckia*, and unknown *Myxococcales* (Fig. 12). This result is also described in the previous section, in Chapter 3. These phylotypes were present dominantly in the system throughout the incubation period of the biotrasnformation test. *Beijerinckia* (5.93% at day 0, 9.84% at day 14, and 14.21% at day 30) kept increasing during the incubation. Unknown *Cytophagaceae* (8.94% at day 0, 28.89% at day 14, and 21.19% at day 30) and unknown *Myxococcales* (5.01% at day 0, 13.58% at day 14, and 5.17% at day 30) were increased to day 14, but then decreased. *Flavobacterium* (6.30% at day 0 and 8.23% at day 30) and unclassified *Planctomycetales* (12.14% at day 0 and 6.60% at day 30) were shown at day 0 and day 30 but at day 14. *Gloeobacter* (7.17% at day 0, 5.86% at day 14, and 1.93% at day 30) and *Sphingomonas* (8.72% at day 0, 1.29% at day 14, and 0.09% at day 30) kept decreasing throughout the incubation period. Phylotypes capable of efficient PPCP degradation were likely those that were more abundant in the acclimated inoculum.

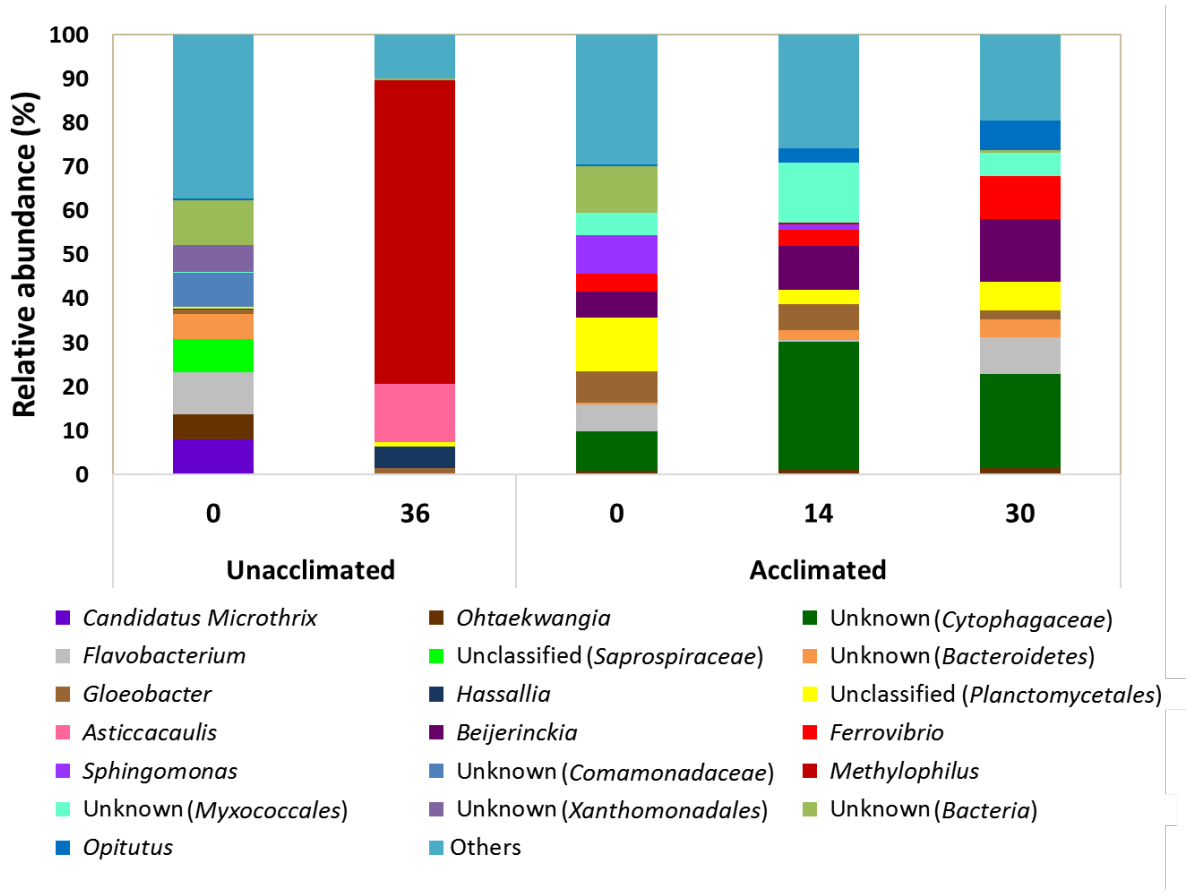


Figure 12 Relative abundance of microbial phylotypes analyzed at the genus level. Inoculum source used was activated sludge (AS). Microbial community samples from unacclimated inoculum were taken at day 0 and 36. Microbial community samples from acclimated inoculum were taken at day 0, 14, and 30; data presented is from Fig. 5 in Chapter 3 and is reproduced here for comparison. Abundance less than 5% was cutoff and classified as others.

4.4 Conclusion

The hypothesis of this study was that acclimation of the microbial community influences PPCP biotransformation. To conclude, acclimated microbial communities showed improved biotransformation for several PPCPs. The acclimated AS culture resulted in >90% removal of 6 compounds (biosol, diclofenac, 5-fluorouracil, gemfibrozil, ibuprofen, and triclosan) within 6 days. Microbial community composition was different between the unacclimated AS culture and

the acclimated AS culture. The microbial community composition of the unacclimated AS culture was diverse and its change from the start (day 0) to the end (day 36) was substantial; indicating that the community began acclimating over the course of this biotransformation study. The microbial community composition of the acclimated AS was also diverse, but its change from the start (day 0) to the end (day 30) was relatively less. Given the results of this preliminary study, we employed pre-acclimation to all inoculum sources for biotransformation tests described in Chapter 3.

5. SUMMARY AND RECOMMENTATIONS FOR FUTURE WORK

PPCPs are contaminants of growing concern because of their increasing detection in wastewater treatment plant effluent and surface water, including drinking water sources. PPCP contamination can cause adverse effects in ecosystems and in human health even at low concentrations (μg/L or ng/L). Various biological treatment technologies (e.g. activated sludge process, biofiltration, soil aquifer treatment, and managed aquifer recharge system) have been investigated for PPCP removal; however, reported removal rates are variable and many compounds are poorly removed**.** Further, to date, the rational design of efficacious and robust biological treatment technologies has been hindered by limited knowledge of the types of microorganisms capable of PPCP biotransformation and the conditions that promote their growth and activity. Many environmental factors and reactor operating conditions such as pH, temperature, retention times (e.g. HRT, SRT), biomass concentration, microbial community composition, substrate concentration and composition, and PPCP concentrations likely influence microbial community composition and PPCP removal rates. Among those factors, we investigated the impact of inoculum source and primary carbon source on PPCP biotransformation rates and on the microbial community. Results of the experiments conducted herein indicate that PPCP biotransformation is substantially impacted by inoculum source and primary carbon source. Moreover, specific microbial phylotypes were linked with transformation of specific PPCPs.

Of the three inoculum sources tested, cultures derived from activated sludge and sediment degraded PPCPs with dramatically higher removal efficiencies than cultures from SAT. Within 6 days, AS-derived inoculum, Sd-derived inoculum, and SAT-derived inoculum removed

>90% of 4, 3, and 2 out of 6 PPCPs, respectively. Gabapentin was not well degraded by any inoculum and approximately 80% remained at day 30, but analytical issues make it difficult to draw conclusions. Of the primary carbon sources tested, CA-, OA-, and PN-utilizing cultures degraded PPCPs with relatively good removal. Within 6 days, CA-utilizing culture removed >90% of 4 out of 6 PPCPs. In the same period, OA-utilizing culture and PN-utilizing culture removed >90% of 3 out of 6 PPCPs; ML-utilizing culture removed >90% of 2 out of 6 PPCPs; and both HP46- and HP64-utilzing culture removed >90% of 1 out of 6 PPCPs. The results based on next-generation 16S rRNA gene amplicon sequencing analysis indicated that microbial community composition impacts PPCP degradation. Abundant phylotypes were linked with biotransformation including unknown *Sphingomonas,* and unknown *Beijerinckia,* unknown *Myxococcales, Methylophilus,* and unknown *Cytophagaceae*. However, two cultures (AD-derived and Sd-derived) with diverging community structures both showed efficient PPCP removal. This implies two possibilities: that PPCPs were biotransformed (1) by multiple phylotypes with redundant function, and/or (2) by rare phylotypes present in both inocula that performed well.

Future work is required to test these hypotheses and prove that specific microbial phylotypes are responsible for PPCP biotransformation. Identification of specific phylotype(s) could be done with serial dilution and further enrichment of the cultures followed by additional biotransformation tests. Pure cultures selected by distinguishing important microorganisms (e.g. dilution-to-extinction approach) or mixed cultures adapted under controlled conditions could clarify unknown pathways in the biotransformation (Franklin *et al.* 2001; Hutalle-Schemelzer *et al.* 2010). Elucidating biotransformation pathways and intermediates using metabolomics and metatranscriptomics would also be necessary to advance fundamental understanding of PPCP

biotransformation; this knowledge could be used to develop biomarkers and process monitoring assays. Gene expression is possibly correlated with functional biodegradation activity (Lee *et al.* 2006). Furthermore, understanding of engineered and controlled microbial communities would provide for efficient design and stable operation of PPCP treatment (Zhao *et al.* 2015). Thus, improved data on the key phylotypes and gene expression would further support improvement of PPCP biotransformation, as well as lessening environmental risks related to the contaminants.

6. REFERENCES

Alidina M, Li D, Drewes JE (2014a) Investigating the role for adaptation of the microbial community to transform trace organic chemicals during managed aquifer recharge. *Water Research* 56: 172-180 DOI 10.1016/j.watres.2014.02.046

Alidina M, Li D, Ouf M, Drewes JE (2014b) Role of primary substrate composition and concentration on attenuation of trace organic chemicals in managed aquifer recharge systems. *Journal of Environmental Management* 144: 58-66 DOI 10.1016/j.jenvman.2014.04.032

Almeida B, Kjeldal H, Lolas I, Knudsen AD, Carvalho G, Nielsen KL, Crespo MTB, Stensballe A, Nielsen JL (2013) Quantitative proteomic analysis of ibuprofen-degrading *Patulibacter* sp strain I11. *Biodegradation* 24: 615-630 DOI 10.1007/s10532-012-9610-5

Bedner M, Maccrehan WA (2006) Transformation of acetaminophen by chlorination produces the toxicants 1,4-benzoquinone and N-acetyl-p-benzoquinone imine. *Environmental Science & Technology* 40: 516-522 DOI 10.1021/es0509073

Bisceglia KJ, Yu JT, Coelhan M, Bouwer EJ, Roberts AL (2010) Trace determination of pharmaceuticals and other wastewater-derived micropollutants by solid phase extraction and gas chromatography/mass spectrometry. *Journal of Chromatography A* 1217: 558-564 DOI 10.1016/j.chroma.2009.11.062

Blair B, Nikolaus A, Hedman C, Klaper R, Grundl T (2015) Evaluating the degradation, sorption, and negative mass balances of pharmaceuticals and personal care products during wastewater treatment. *Chemosphere* 134: 395-401 DOI 10.1016/j.chemosphere.2015.04.078

Boxall ABA, Rudd MA, Brooks BW, Caldwell DJ, Choi K, Hickmann S, Innes E, Ostapyk K, Staveley JP, Verslycke T, Ankley GT, Beazley KF, Belanger SE, Berninger JP, Carriquiriborde P, Coors A, DeLeo PC, Dyer SD, Ericson JF, Gagne F, Giesy JP, Gouin T, Hallstrom L, Karlsson MV, Larsson DGJ, Lazorchak JM, Mastrocco F, McLaughlin A, McMaster ME, Meyerhoff RD, Moore R, Parrott JL, Snape JR, Murray-Smith R, Servos MR, Sibley PK, Straub JO, Szabo ND, Topp E, Tetreault GR, Trudeau VL, Van Der Kraak G (2012) Pharmaceuticals

and Personal Care Products in the Environment: What Are the Big Questions? *Environmental Health Perspectives* 120: 1221-1229 DOI 10.1289/ehp.1104477

Caracciolo AB, Topp E, Grenni P (2015) Pharmaceuticals in the environment: Biodegradation and effects on natural microbial communities. A review. *Journal of Pharmaceutical and Biomedical Analysis* 106: 25-36 DOI 10.1016/j.jpba.2014.11.040

Dedysh SN, Smirnova KV, Khmelenina VN, Suzina NE, Liesack W, Trotsenko YA (2005) Methylotrophic autotrophy in *Beijerinckia mobilis. Journal of Bacteriology* 187: 3884-3888 DOI 10.1128/jb.187.11.3884-3888.2005

Dettmer-Wilde K, Engewald W (2014) Practical Gas Chromatography – A Comprehensive Reference. Springer.

Dhillon GS, Kaur S, Pulicharla R, Brar SK, Cledon M, Verma M, Surampalli RY (2015) Triclosan: Current Status, Occurrence, Environmental Risks and Bioaccumulation Potential. *International Journal of Environmental Research and Public Health* 12: 5657-5684 DOI 10.3390/ijerph120505657

Domaradzka D, Guzik U, Hupert-Kocurek K, Wojcieszynska D (2015) Cometabolic Degradation of Naproxen by *Planococcus* sp Strain S5. *Water Air and Soil Pollution* 226 DOI 10.1007/s11270-015-2564-6

Drewes JE, Hoppe C, Jennings T (2006) Fate and transport of N-nitrosamines under conditions simulating full-scale groundwater recharge operations. *Water Environment Research* 78: 2466-2473 DOI 10.2175/106143006x115408

Drewes JE, Li D, Regnery J, Alidina M, Wing A, Hoppe-Jones C (2014) Tuning the performance of a natural treatment process using metagenomics for improved trace organic chemical attenuation. *Water Science and Technology* 69: 628-633 DOI 10.2166/wst.2013.750

Eichhorn P, Ferguson PL, Perez S, Aga DS (2005) Application of ion trap-MS with QqTOF-MS in the identification H/D exchange and of microbial degradates of trimethoprim in nitrifying activated sludge. *Analytical Chemistry* 77: 4176-4184 DOI 10.1021/ac050141p

Espejo A, Aguinaco A, Amat AM, Beltran FJ (2014) Some ozone advanced oxidation processes to improve the biological removal of selected pharmaceutical contaminants from urban wastewater. *Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering* 49: 410-421 DOI 10.1080/10934529.2014.854652

Franklin RB, Garland JL, Bolster CH, Mills AL (2001) Impact of dilution on microbial community structure and functional potential: Comparison of numerical simulations and batch culture experiments. *Applied and Environmental Microbiology* 67: 702-712 DOI 10.1128/aem.67.2.702-712.2001

Fortunato MS, Abril NPF, Martinefski M, Tripodi V, Papalia M, Radice M, Gutkind G, Gallego A, Korol SE (2016) Aerobic degradation of ibuprofen in batch and continuous reactors by an indigenous bacterial community. *Environmental Technology* 37: 2617-2626 DOI 10.1080/09593330.2016.1156773

Gracia-Lor E, Martinez M, Sancho JV, Penuela G, Hernandez F (2012) Multi-class determination of personal care products and pharmaceuticals in environmental and wastewater samples by ultra-high performance liquid-chromatography-tandem mass spectrometry. *Talanta* 99: 1011-1023 DOI 10.1016/j.talanta.2012.07.091

Guo M, Zhou Q, Zhou YZ, Yang LF, Liu TX, Yang JL, Chen YL, Su LX, Xu J, Chen J, Liu F, Chen JP, Dai WK, Ni PX, Fang CX, Yang RF (2014) Genomic Evolution of 11 Type Strains within Family Planctomycetaceae. *Plos One* 9 DOI 10.1371/journal.pone.0086752

Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* 4(1):9.

Hay AG, Dees PM, Sayler GS (2001) Growth of a bacterial consortium on triclosan. *Fems Microbiology Ecology* 36: 105-112 DOI 10.1111/j.1574-6941.2001.tb00830.x

Herrmann M, Menz J, Olsson O, Kummerer K (2015) Identification of phototransformation products of the antiepileptic drug gabapentin: Biodegradability and initial assessment of toxicity. *Water Research* 85: 11-21 DOI 10.1016/j.watres.2015.08.004

Hoppe-Jones C, Dickenson ERV, Drewes JE (2012) The role of microbial adaptation and biodegradable dissolved organic carbon on the attenuation of trace organic chemicals during groundwater recharge. S*cience of the Total Environment* 437: 137-144 DOI 10.1016/j.scitotenv.2012.08.009

Hutalle-Schmelzer KML, Zwirnmann E, Kruger A, Grossart HP (2010) Enrichment and cultivation of pelagic bacteria from a humic lake using phenol and humic matter additions. *Fems Microbiology Ecology* 72: 58-73 DOI 10.1111/j.1574-6941.2009.00831.x

Kalsch W (1999) Biodegradation of the iodinated X-ray contrast media diatrizoate and iopromide. *Science of the Total Environment* 225: 143-153 DOI 10.1016/s0048-9697(98)00340-4

Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ (2008) Multiresidue methods for the analysis of pharmaceuticals, personal care products and illicit drugs in surface water and wastewater by solid-phase extraction and ultra performance liquid chromatography-electrospray tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* 391: 1293-1308 DOI 10.1007/s00216-008-1854-x

Kim SD, Cho J, Kim IS, Vanderford BJ, Snyder SA (2007) Occurrence and removal of pharmaceuticals and endocrine disruptors in South Korean surface, drinking, and waste waters. *Water Research* 41: 1013-1021 DOI 10.1016/j.watres.2006.06.034

Kjeldal H, Zhou NA, Wissenbach DK, von Bergen M, Gough HL, Nielsen JL (2016) Genomic, Proteomic, and Metabolite Characterization of Gemfibrozil-Degrading Organism *Bacillus* sp GeD10. *Environmental Science & Technology* 50: 744-755 DOI 10.1021/acs.est.5b05003

Koutsouba V, Heberer T, Fuhrmann B, Schmidt-Baumler K, Tsipi D, Hiskia A (2003) Determination of polar pharmaceuticals in sewage water of Greece by gas chromatography-mass spectrometry. *Chemosphere* 51: 69-75 DOI 10.1016/s0045-6535(02)00819-6

Kumirska J, Migowska N, Caban M, Lukaszewicz P, Stepnowski P (2015) Simultaneous determination of non-steroidal anti-inflammatory drugs and oestrogenic hormones in

environmental solid samples. *Science of the Total Environment* 508: 498-505 DOI 10.1016/j.scitotenv.2014.12.020

Kwon J-W, Rodriguez JM (2014) Occurrence and Removal of Selected Pharmaceuticals and Personal Care Products in Three Wastewater-Treatment Plants. *Archives of Environmental Contamination and Toxicology* 66: 538-548 DOI 10.1007/s00244-013-9979-0

Lahti M, Oikari A (2011) Microbial Transformation of Pharmaceuticals Naproxen, Bisoprolol, and Diclofenac in Aerobic and Anaerobic Environments. *Archives of Environmental Contamination and Toxicology* 61: 202-210 DOI 10.1007/s00244-010-9622-2

Langenhoff A, Inderfurth N, Veuskens T, Schraa G, Blokland M, Kujawa-Roeleveld K, Rijnaarts H (2013) Microbial Removal of the Pharmaceutical Compounds Ibuprofen and Diclofenac from Wastewater. *Biomed Research International* DOI 10.1155/2013/325806

Lee PKH, Johnson DR, Holmes VF, He JZ, Alvarez-Cohen L (2006) Reductive dehalogenase gene expression as a biomarker for physiological activity of *Dehalococcoides* spp. *Applied and Environmental Microbiology* 72: 6161-6168 DOI 10.1128/aem.01070-06

Li D, Alidina M, Drewes JE (2014) Role of primary substrate composition on microbial community structure and function and trace organic chemical attenuation in managed aquifer recharge systems. *Applied Microbiology and Biotechnology* 98: 5747-5756 DOI 10.1007/s00253-014-5677-8

Li D, Sharp JO, Saikaly PE, Ali S, Alidina M, Alarawi MS, Keller S, Hoppe-Jones C, Drewes JE (2012) Dissolved Organic Carbon Influences Microbial Community Composition and Diversity in Managed Aquifer Recharge Systems. *Applied and Environmental Microbiology* 78: 6819-6828 DOI 10.1128/aem.01223-12

Margot J, Kienle C, Magnet A, Weil M, Rossi L, de Alencastro LF, Abegglen C, Thonney D, Chevre N, Scharer M, Barry DA (2013) Treatment of micropollutants in municipal wastewater: Ozone or powdered activated carbon? *Science of the Total Environment* 461: 480-498 DOI 10.1016/j.scitotenv.2013.05.034

Moder M, Braun P, Lange F, Schrader S, Lorenz W (2007) Determination of endocrine disrupting compounds and acidic drugs in water by coupling of derivatization, gas chromatography and Negative Chemical ionization mass Spectrometry. *Clean-Soil Air Water* 35: 444-451 DOI 10.1002/clen.200720001

Murdoch RW, Hay AG (2005) Formation of catechols via removal of acid side chains from ibuprofen and related aromatic acids. *Applied and Environmental Microbiology* 71: 6121-6125 DOI 10.1128/aem.71.10.6121-6125.2005

Nieto A, Borrull F, Marce RM, Pocurull E (2009) Determination of personal care products in sewage sludge by pressurized liquid extraction and ultra high performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* 1216: 5619-5625 DOI 10.1016/j.chroma.2009.05.061

Onesios KM, Bouwer EJ (2012) Biological removal of pharmaceuticals and personal care products during laboratory soil aquifer treatment simulation with different primary substrate concentrations. *Water Research* 46: 2365-2375 DOI 10.1016/j.watres.2012.02.001

Onesios KM, Yu JT, Bouwer EJ (2009) Biodegradation and removal of pharmaceuticals and personal care products in treatment systems: a review. *Biodegradation* 20: 441-466 DOI 10.1007/s10532-008-9237-8

Onesios-Barry KM, Berry D, Proescher JB, Sivakumar IKA, Bouwer EJ (2014) Removal of Pharmaceuticals and Personal Care Products during Water Recycling: Microbial Community Structure and Effects of Substrate Concentration. *Applied and Environmental Microbiology* 80: 2440-2450 DOI 10.1128/aem.03693-13

Pavlovic DM, Babic S, Dolar D, Asperger D, Kosutic K, Horvat AJM, Kastelan-Macan M (2010) Development and optimization of the SPE procedure for determination of pharmaceuticals in water samples by HPLC-diode array detection. *Journal of Separation Science* 33: 258-267 DOI 10.1002/jssc.200900571

Petrovic M, Gonzalez S, Barcelo D (2003) Analysis and removal of emerging contaminants in wastewater and drinking water. *Trac-Trends in Analytical Chemistry* 22: 685-696 DOI 10.1016/s0165-9936(03)01105-1

Phan HV, Hai FI, Zhang R, Kang J, Price WE, Nghiem LD (2016) Bacterial community dynamics in an anoxic-aerobic membrane bioreactor - Impact on nutrient and trace organic contaminant removal. *International Biodeterioration & Biodegradation* 109: 61-72 DOI 10.1016/j.ibiod.2016.01.002

Pinyakong O, Habe H, Supaka N, Pinpanichkarn P, Juntongjin K, Yoshida T, Furihata K, Nojiri H, Yamane H, Omori T (2000) Identification of novel metabolites in the degradation of phenanthrene by *Sphingomonas* sp strain P2. *Fems Microbiology Letters* 191: 115-121 DOI 10.1016/s0378-1097(00)00380-3

Quintana JB, Weiss S, Reemtsma T (2005) Pathway's and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. *Water Research* 39: 2654-2664 DOI 10.1016/j.watres.2005.04.068

Radjenovic J, Petrovic M, Barcelo D (2007) Advanced mass, spectrometric methods applied to the study of fate and removal of pharmaceuticals in wastewater treatment. *Trac-Trends in Analytical Chemistry* 26: 1132-1144 DOI 10.1016/j.trac.2007.10.002

Radjenovic J, Petrovic M, Barcelo D (2009) Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. *Water Research* 43: 831-841 DOI 10.1016/j.watres.2008.11.043

Rauch-Williams T, Hoppe-Jones C, Drewes JE (2010) The role of organic matter in the removal of emerging trace organic chemicals during managed aquifer recharge. *Water Research* 44: 449-460 DOI 10.1016/j.watres.2009.08.027

Richardson D, Ortori CA, Chapman V, Kendall DA, Barrett DA (2007) Quantitative profiling of endocannabinoids and related compounds in rat brain using liquid chromatography-tandem

electrospray ionization mass spectrometry. *Analytical Biochemistry* 360: 216-226 DOI 10.1016/j.ab.2006.10.039

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology* 75: 7537-7541 DOI 10.1128/aem.01541-09

Selvi A, Salam JA, Das N (2014) Biodegradation of cefdinir by a novel yeast strain, *Ustilago* sp SMN03 isolated from pharmaceutical wastewater. *World Journal of Microbiology & Biotechnology* 30: 2839-2850 DOI 10.1007/s11274-014-1710-4

Simazaki D, Kubota R, Suzuki T, Akiba M, Nishimura T, Kunikane S (2015) Occurrence of selected pharmaceuticals at drinking water purification plants in Japan and implications for human health. *Water Research* 76: 187-200 DOI 10.1016/j.watres.2015.02.059

Spellman FR (2014) Personal Care Products and Pharmaceuticals in Wastewater and the Environment DEStech Publications, Inc. ISBN No. 978-1-60595-123-2.

Stasinakis AS, Petalas AV, Mamais D, Thomaidis NS, Gatidou G, Lekkas TD (2007) Investigation of triclosan fate and toxicity in continuous-flow activated sludge systems. *Chemosphere* 68: 375-381 DOI 10.1016/j.chemosphere.2007.01.047

Stokvis E, Rosing H, Beijnen JH (2005) Stable isotopically labeled internal standards in quantitative bioanalysis using liquid chromatography/mass spectrometry: necessity or not? *Rapid Communications in Mass Spectrometry* 19: 401-407 DOI 10.1002/rcm.1790

Surovtseva EG, Ivoilov VS, Belyaev SS (1999) Physiological and biochemical properties of *Beijerinckia mobilis* 1f Phn(+) capable of degrading polycyclic aromatic hydrocarbons. *Microbiology* 68: 746-750

Terzopoulou E, Voutsa D, Kaklamanos G (2015) A multi-residue method for determination of 70 organic micropollutants in surface waters by solid-phase extraction followed by gas

chromatography coupled to tandem mass spectrometry. *Environmental Science and Pollution Research* 22: 1095-1112 DOI 10.1007/s11356-014-3397-3

Tran NH, Urase T, Kusakabe O (2009) The characteristics of enriched nitrifier culture in the degradation of selected pharmaceutically active compounds. *Journal of Hazardous Materials* 171: 1051-1057 DOI 10.1016/j.jhazmat.2009.06.114

Trussell S, Tiwari S, Gerringer F, Trussell R (2015) Enhancing the Soil Aquifer Treatment Process for Potable Reuse. WateReuse Research Foundation. Project 12-12.

Uhlik O, Jecna K, Mackova M, Vlcek C, Hroudova M, Demnerova K, Paces V, Macek T (2009) Biphenyl-Metabolizing Bacteria in the Rhizosphere of Horseradish and Bulk Soil Contaminated by Polychlorinated Biphenyls as Revealed by Stable Isotope Probing. *Applied and Environmental Microbiology* 75: 6471-6477 DOI 10.1128/aem.00466-09

U.S. Environmental Protection Agency (2012) Pharmaceuticals and Personal Care Products. Washington, D.C. Retrieved 2015-07-23.

Vanderford BJ, Pearson RA, Rexing DJ, Snyder SA (2003) Analysis of endocrine disruptors, pharmaceuticals, and personal care products in water using liquid chromatography/tandem mass spectrometry. *Analytical Chemistry* 75: 6265-6274 DOI 10.1021/ac034210g

Wabaidur SM, AlOthman ZA, Siddiqui MR, Mohsin K, Bousiakou LG, Karikas GA (2015) UPLC-MS method for the simultaneous determination of naproxen, fluvastatin and ibuprofen in waste water samples. *Journal of Industrial and Engineering Chemistry* 24: 302-307 DOI 10.1016/j.jiec.2014.09.046

Wang XC, Shen JM, Chen ZL, Zhao X, Xu H (2016) Removal of pharmaceuticals from synthetic wastewater in an aerobic granular sludge membrane bioreactor and determination of the bioreactor microbial diversity. *Applied Microbiology and Biotechnology* 100: 8213-8223 DOI 10.1007/s00253-016-7577-6

Willumsen PA, Johansen JE, Karlson U, Hansen BM (2005) Isolation and taxonomic affiliation of N-heterocyclic aromatic hydrocarbon-transforming bacteria. *Applied Microbiology and Biotechnology* 67: 420-428 DOI 10.1007/s00253-004-1799-8

Wilson LP, Loetscher LH, Sharvelle SE, De Long SK (2013) Microbial community acclimation enhances waste hydrolysis rates under elevated ammonia and salinity conditions. *Bioresource Technology* 146: 15-22 DOI 10.1016/j.biortech.2013.06.081

Wu SJ, Zhang LL, Chen JM (2012) Paracetamol in the environment and its degradation by microorganisms. *Applied Microbiology and Biotechnology* 96: 875-884 DOI 10.1007/s00253-012-4414-4

Yoon MK, Drewes JE, Amy GL (2013) Fate of bulk and trace organics during a simulated aquifer recharge and recovery (ARR)-ozone hybrid process. *Chemosphere* 93: 2055-2062 DOI 10.1016/j.chemosphere.2013.07.038

Yu JT, Bisceglia KJ, Bouwer EJ, Roberts AL, Coelhan M (2012) Determination of pharmaceuticals and antiseptics in water by solid-phase extraction and gas chromatography/mass spectrometry: analysis via pentafluorobenzylation and stable isotope dilution. *Analytical and Bioanalytical Chemistry* 403: 583-591 DOI 10.1007/s00216-012-5846-5

Yu JT, Bouwer EJ, Coelhan M (2006) Occurrence and biodegradability studies of selected pharmaceuticals and personal care products in sewage effluent. *Agricultural Water Management* 86: 72-80 DOI 10.1016/j.agwat.2006.06.015

Yu Y, Wu LS (2012) Analysis of endocrine disrupting compounds, pharmaceuticals and personal care products in sewage sludge by gas chromatography-mass spectrometry. *Talanta* 89: 258-263 DOI 10.1016/j.talanta.2011.12.023

Yu YY, Huang QX, Cui JL, Zhang K, Tang CM, Peng XZ (2011) Determination of pharmaceuticals, steroid hormones, and endocrine-disrupting personal care products in sewage sludge by ultra-high-performance liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* 399: 891-902 DOI 10.1007/s00216-010-4295-2

Yu ZR, Peldszus S, Huck PM (2007) Optimizing gas chromatographic-mass spectrometric analysis of selected pharmaceuticals and endocrine-disrupting substances in water using factorial experimental design. *Journal of Chromatography A* 1148: 65-77 DOI 10.1016/j.chroma.2007.02.047

Zhang SY, Wang QF, Wan R, Xie SG (2011) Changes in bacterial community of anthracene bioremediation in municipal solid waste composting soil. *Journal of Zhejiang University-Science B* 12: 760-768 DOI 10.1631/jzus.B1000440

Zhao X, Chen ZL, Wang XC, Li JCZ, Shen JM, Xu H (2015) Remediation of pharmaceuticals and personal care products using an aerobic granular sludge sequencing bioreactor and microbial community profiling using Solexa sequencing technology analysis. *Bioresource Technology* 179: 104-112 DOI 10.1016/j.biortech.2014.12.002

Zhou NA, Lutovsky AC, Andaker GL, Gough HL, Ferguson JF (2013) Cultivation and characterization of bacterial isolates capable of degrading pharmaceutical and personal care products for improved removal in activated sludge wastewater treatment. *Biodegradation* 24: 813-827 DOI 10.1007/s10532-013-9630-9

Zwiener C, Seeger S, Glauner T, Frimmel FH (2002) Metabolites from the biodegradation of pharmaceutical residues of ibuprofen in biofilm reactors and batch experiments. *Analytical and Bioanalytical Chemistry* 372: 569-575 DOI 10.1007/s00216-001-1210-x

APPENDIX A: REACTOR OPERATION

A.1 Chemical information

Table 8 Chemical information for the medium

Compound		molar mass	Concentration			
Name	Formula	(g/mol)	mass/vol.		molar	
Monopotassium phosphate	KH_2PO_4	136.09	8.5	mg/L	62	µM
Potassium phosphate dibasic	K_2HPO_4	174.18	21.75	mg/L	125	µM
Sodium phosphate dibasic	Na_2HPO_4	141.96	33.4	mg/L	235	µM
Iron(III) chloride hexahydrate	$FeCl_3{\cdot}_6H2O$	270.3	0.33	mg/L	1	µM
Ammonium chloride	NH_4Cl	53.49	2.7	mg/L	50	µM
Magnesium sulfate	$MgSO_4$	120.37	22.5	mg/L	187	µM
Calcium chloride dihydrate	$CaCl_2{\cdot}2H_2O$	147.01	36.4	mg/L	248	µM

Table 9 Chemical information for the primary carbon sources

Primary carbon source		Molar mass	Compound conc.	
Name	Formula	(g/mol)	(µg/L)	(µM)
Phenol	C_6H_6O	94.1	113	1.2
Casamino acid			100	
Humic acid:Peptone (60:40)	-	-	-	-
- humic acid	-		180	
- peptone	$C_{13}H_{24}O_4$	244.33	120	0.49
Humic acid:Peptone (40:60)	-	-	-	-
- humic acid	-		119	
- peptone	$C_{13}H_{24}O_4$	244.33	178	0.73
Molasses	$C_6H_{12}NNaO_3S$	201.2	411	1.2
Organic acids	-	-	-	-
- citric acid	$C_6H_8O_7$	192.1	79	0.41
- lactic acid	$C_3H_6O_3$	90.1	37	0.41
- succinic acid	$C_4H_6O_4$	118.1	49	0.41
Acetate	CH_3COONa	82	100	1.2

Table 10 Chemical information of the PPCPs

PPCPs			Molar mass (g/mol)	Concentration (ug/L)	Solvent used for making stock solution
Name	CAS No.	Formula			
Biosol	3228-02-2	$C_{10}H_{14}0$	150.2	50	Methanol
Diclofenac	15307-86-5	$C_{14}H_{11}Cl_2NO_2$	296.2	50	Water
5-Fluorouracil	51-28-8	$C_4H_3FN_2O_2$	130.1	50	Water
Gabapentin	60142-96-3	$C_9H_{17}NO_2$	171.2	50	Water
Gemfibrozil	25812-30-0	$C_{15}H_{22}O_3$	250.3	50	Ethanol
Ibuprofen	15687-27-1	$C_{13}H_{18}O_2$	206.3	50	Methanol
Triclosan	3380-34-5	$C_{12}H_7Cl_3O_2$	289.5	50	Ethanol

A.2 Reactor operation and measurements

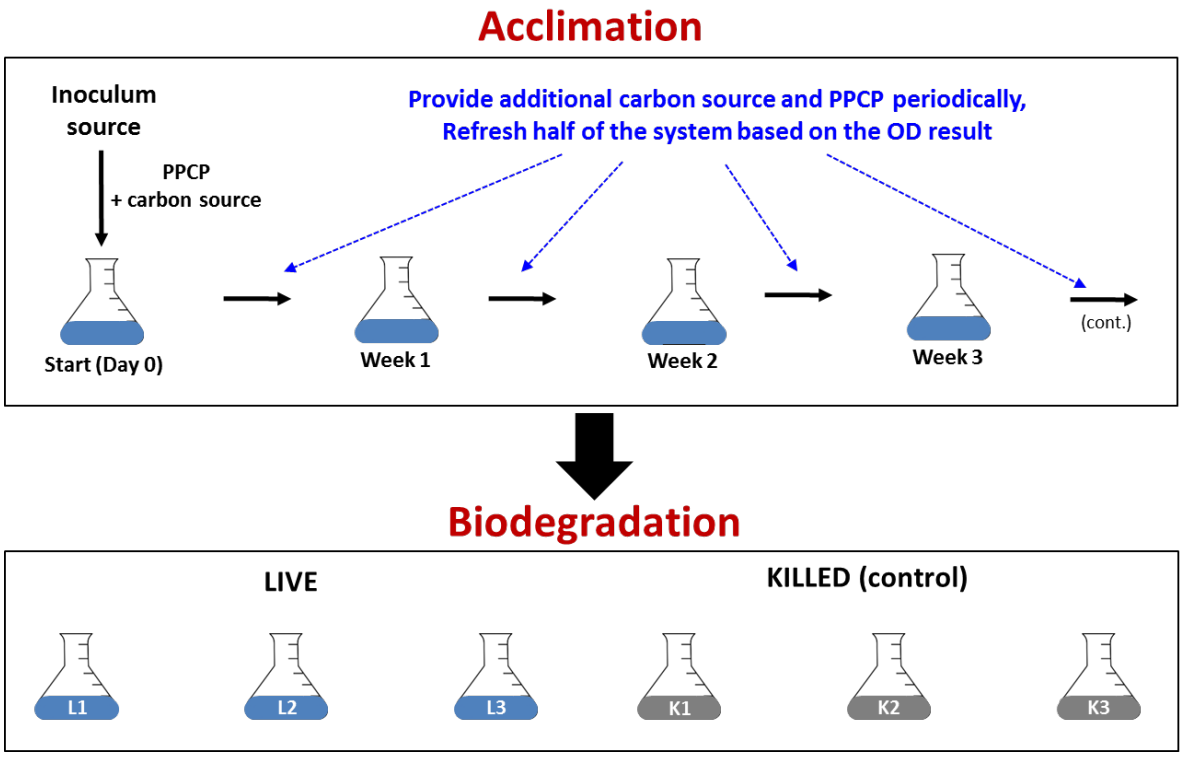


Figure 13 Schematic diagram of acclimation and biotransformation experiment. Acclimated inoculum was transferred to all biotransformation reactors. Killed control reactors were autoclaved before providing PPCPs to the system.

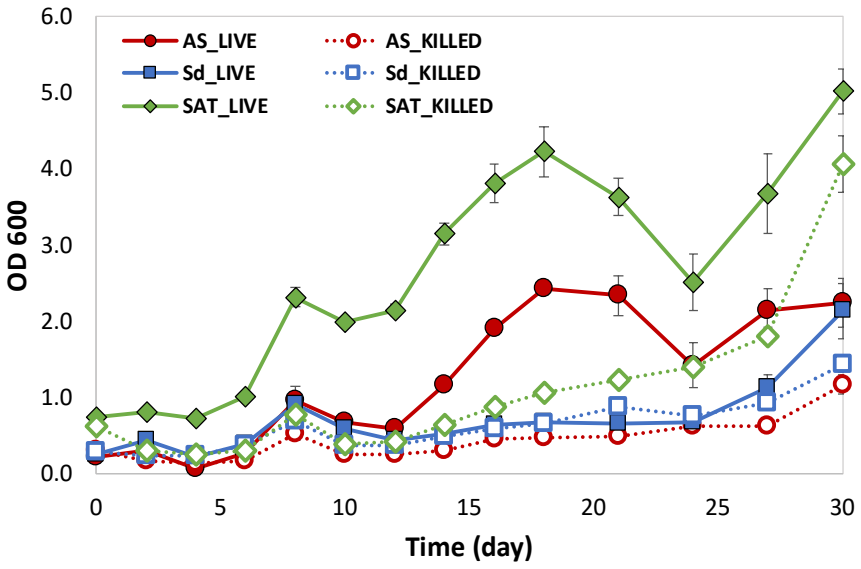


Figure 14 Optical density changes of biotransformation reactors for inoculum source experiment. Inoculum sources used were acclimated cultures originally seeded with activated sludge (AS), sediment (Sd), and soil from SAT reactor (SAT). Error bars represent standard deviations for biological triplicates.

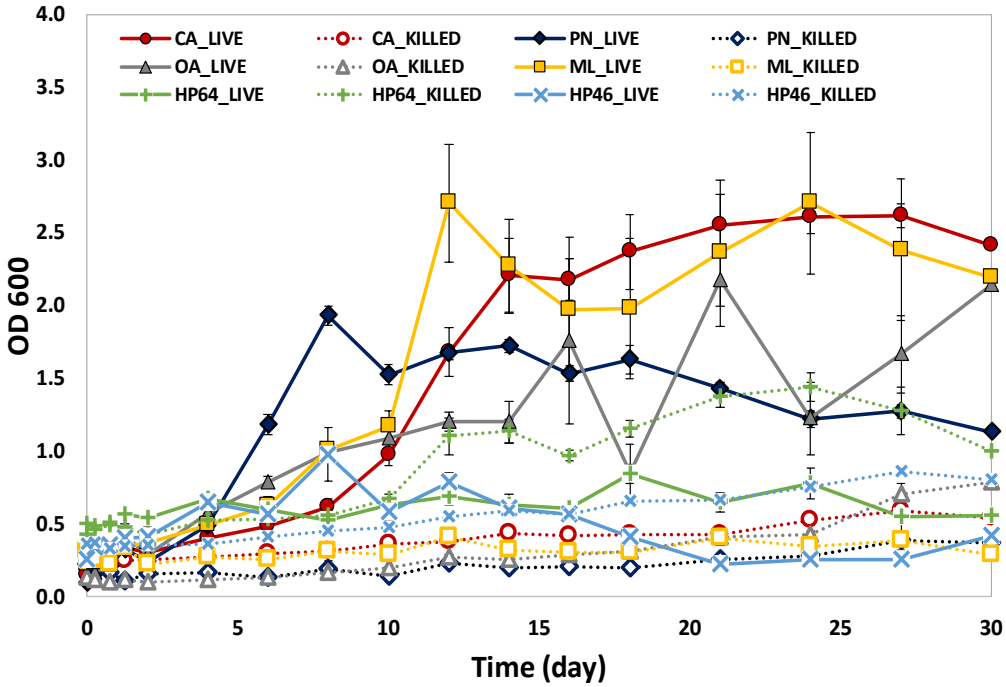


Figure 15 Optical density changes of biotransformation reactors for primary carbon source experiment. Carbon sources used were casamino acid (CA), phenol (PN) organic acids (OA), molasses (ML), and humic acid and peptone mixture (HP46, HP64). Error bars represent standard deviations for biological triplicates.

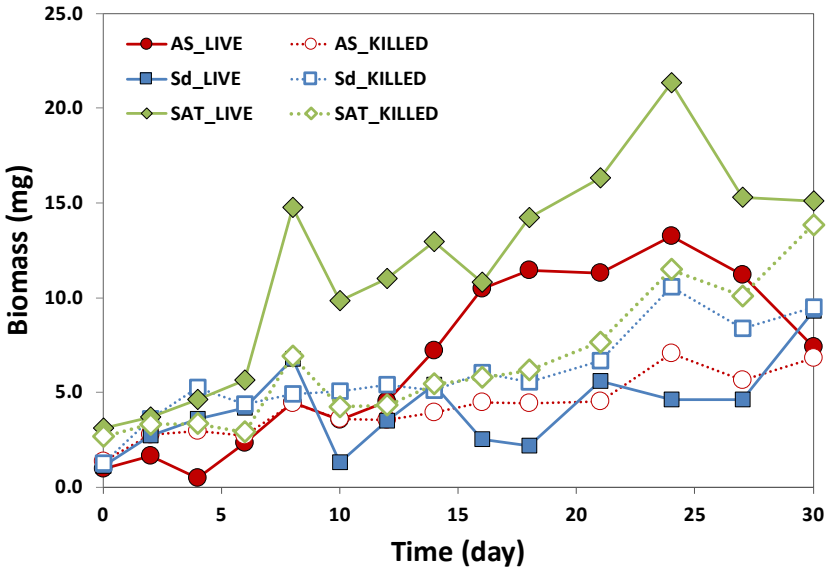


Figure 16 Biomass changes of biotransformation reactors for inoculum source experiment. Biomass was calculated from protein measurement results, and the protein fraction used for the calculation was assumed approximately 55%.

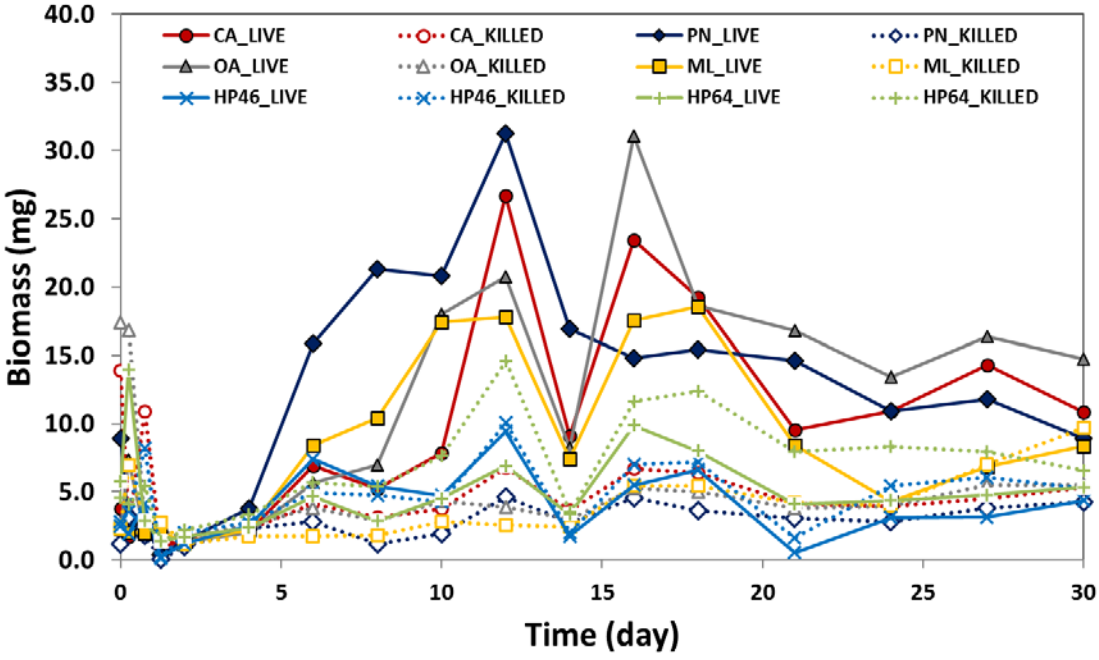


Figure 17 Biomass changes of biotransformation reactors for primary carbon source experiment. Biomass was calculated from protein measurement results, and the protein fraction used for the calculation was assumed approximately 55%.

APPENDIX B: MICROBIAL ANALYSIS AT ALL PHYLOGENETIC LEVELS

B.1 The impact of acclimation

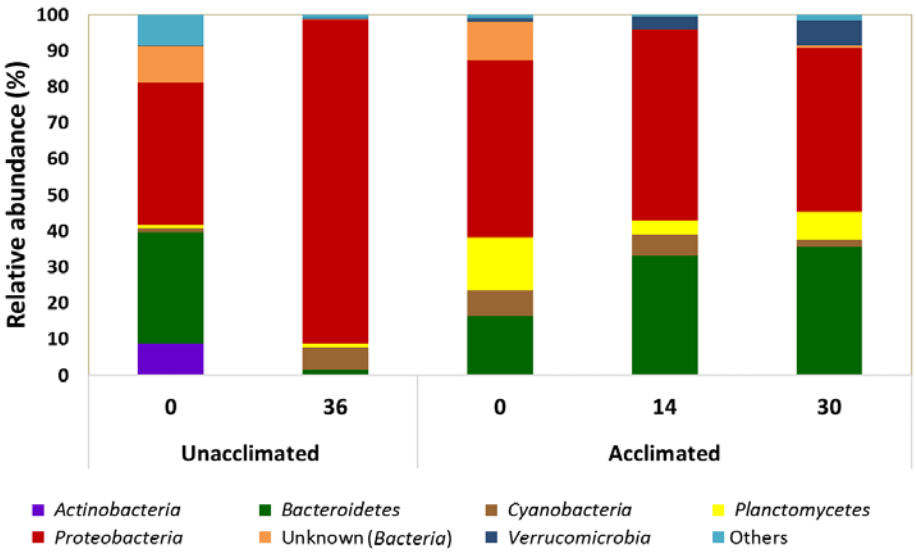


Figure 18 Microbial community structures analyzed at the phylum level. Inoculum source used was activated sludge (AS). Microbial community samples from unacclimated inoculum were taken at day 0 and 36. Microbial community samples from acclimated inoculum were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

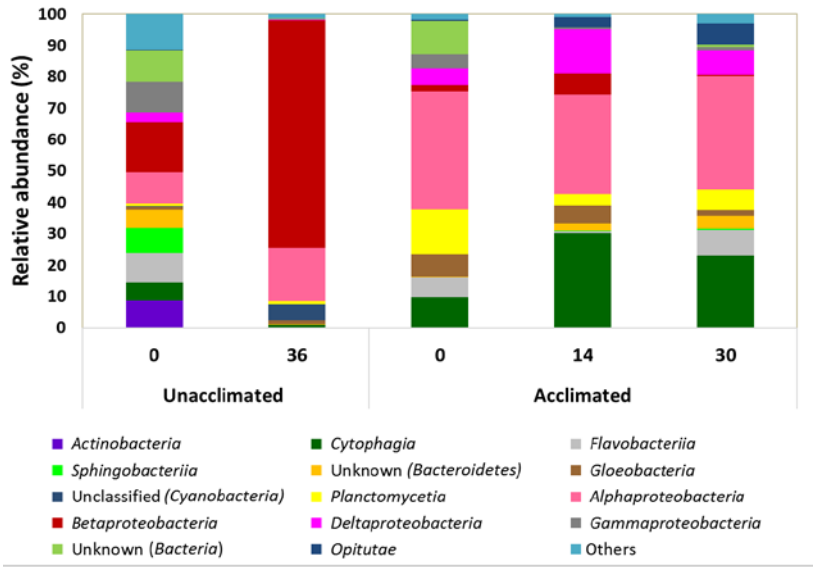


Figure 19 Microbial community structures analyzed at the class level. Inoculum source used was activated sludge (AS). Microbial community samples from unacclimated inoculum were taken at day 0 and 36. Microbial community samples from acclimated inoculum were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

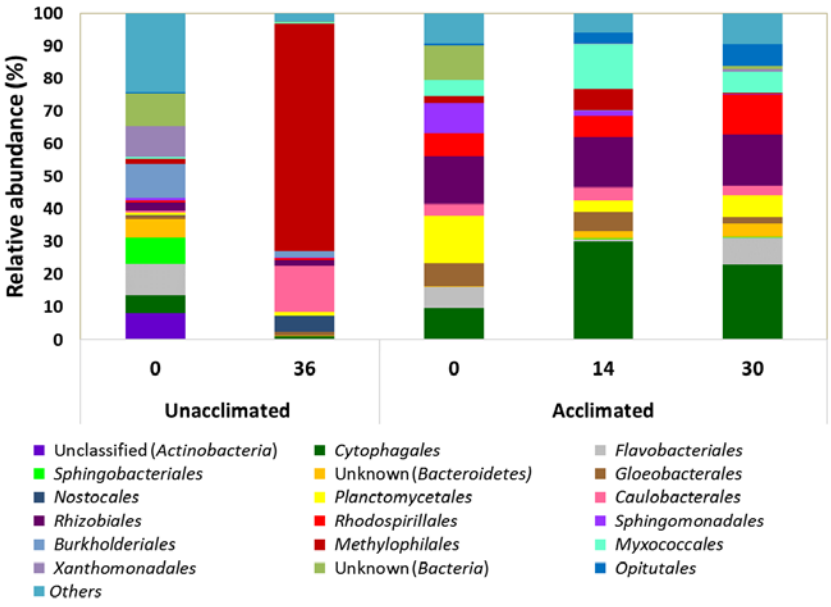


Figure 20 Microbial community structures analyzed at the order level. Inoculum source used was activated sludge (AS). Microbial community samples from unacclimated inoculum were taken at day 0 and 36. Microbial community samples from acclimated inoculum were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

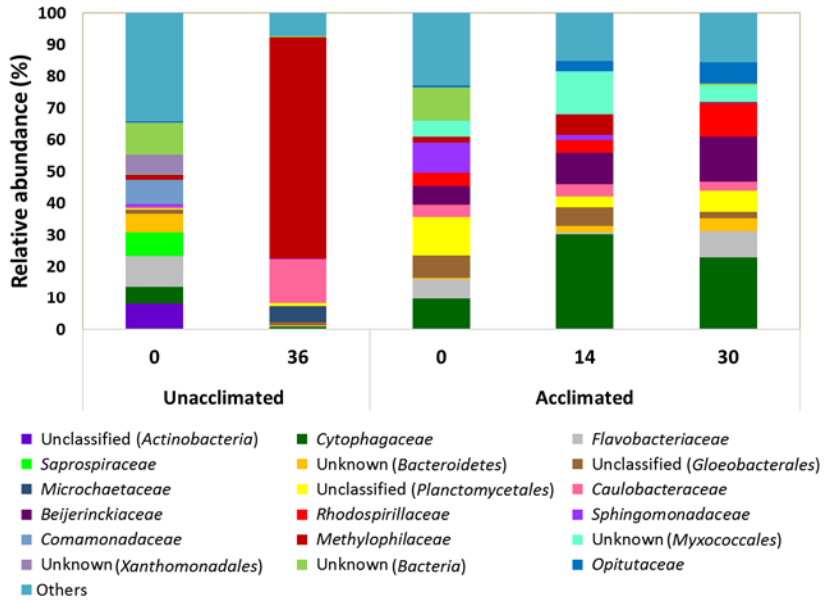


Figure 21 Microbial community structures analyzed at the family level. Inoculum source used was activated sludge (AS). Microbial community samples from unacclimated inoculum were taken at day 0 and 36. Microbial community samples from acclimated inoculum were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

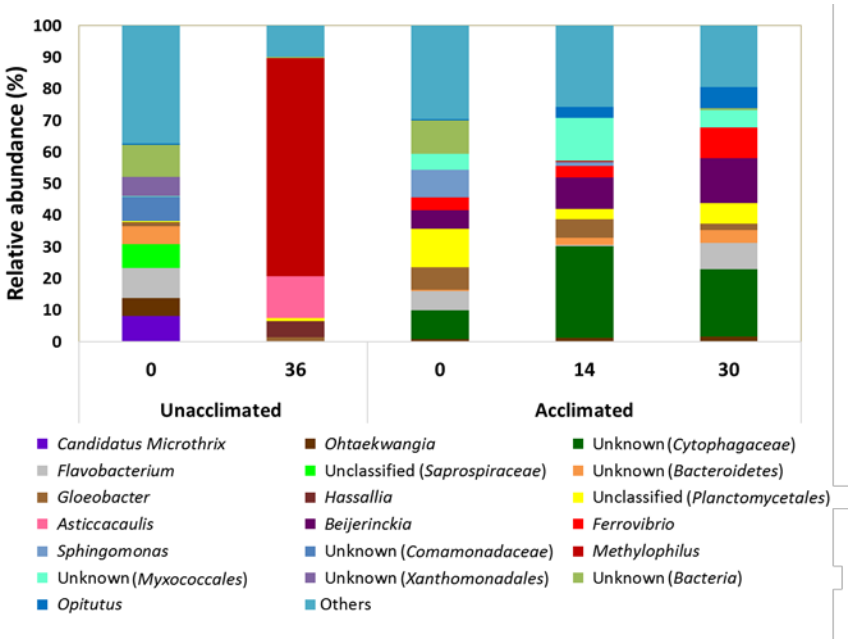


Figure 22 Microbial community structures analyzed at the species level. Inoculum source used was activated sludge (AS). Microbial community samples from unacclimated inoculum were taken at day 0 and 36. Microbial community samples from acclimated inoculum were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

B.2 The impact of inoculum source

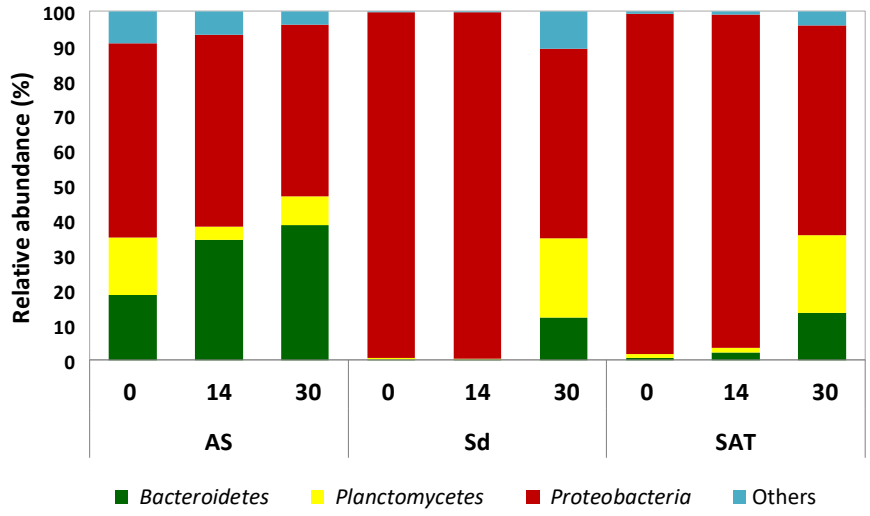


Figure 23 Microbial community structures analyzed at the phylum level. Microbial communities were pre-acclimated with different inoculum sources—activated sludge (AS), sediment (Sd), and soil from a SAT reactor (SAT). The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

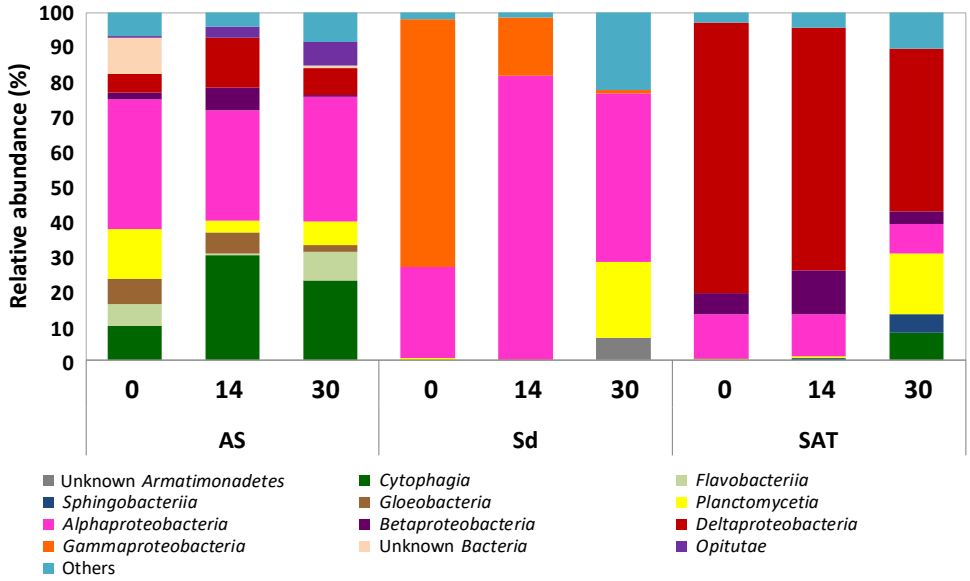


Figure 24 Microbial community structures analyzed at the class level. Microbial communities were pre-acclimated with different inoculum sources—activated sludge (AS), sediment (Sd), and soil from a SAT reactor (SAT). The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

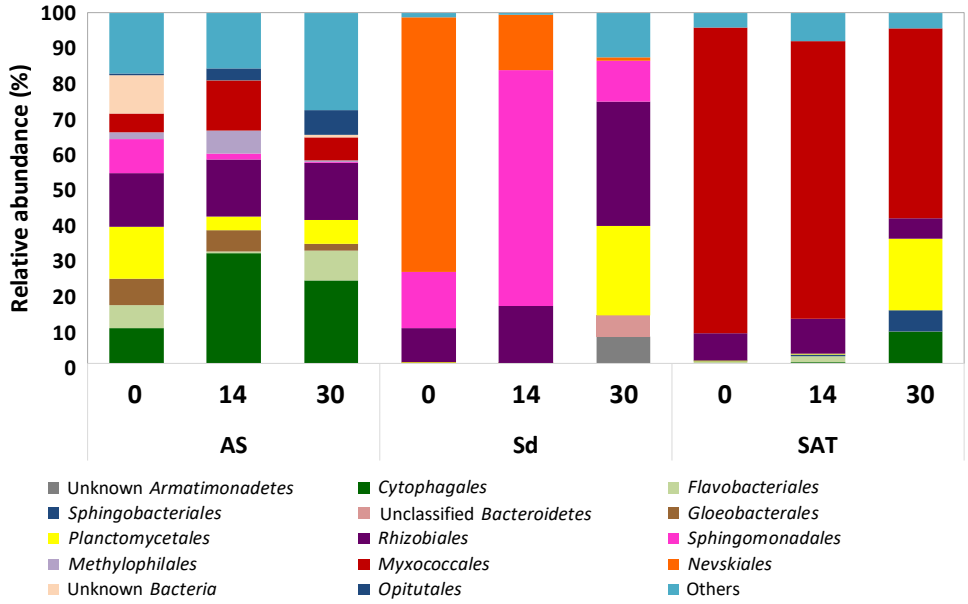


Figure 25 Microbial community structures analyzed at the order level. Microbial communities were pre-acclimated with different inoculum sources—activated sludge (AS), sediment (Sd), and soil from a SAT reactor (SAT). The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

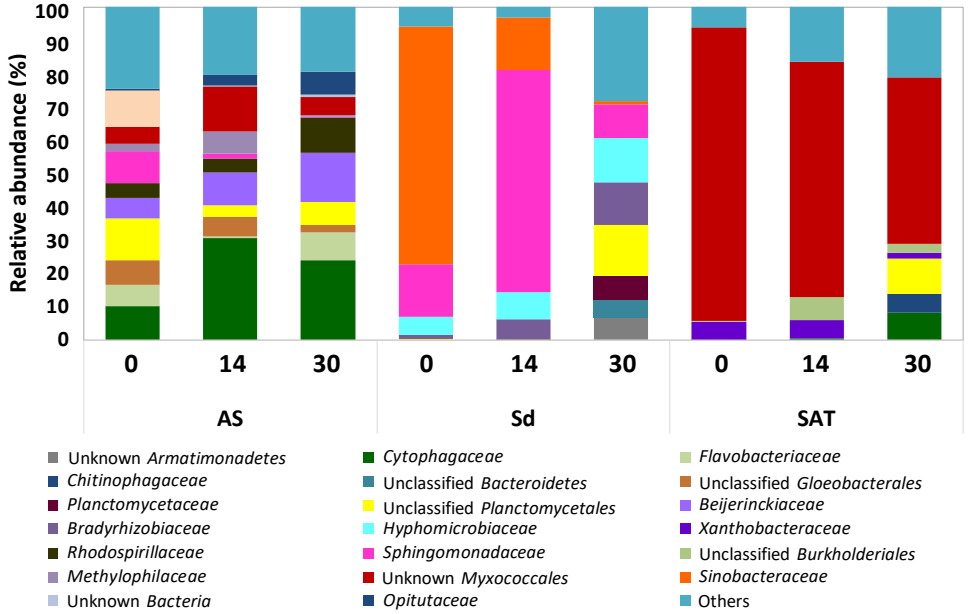


Figure 26 Microbial community structures analyzed at the family level. Microbial communities were pre-acclimated with different inoculum sources—activated sludge (AS), sediment (Sd), and soil from a SAT reactor (SAT). The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

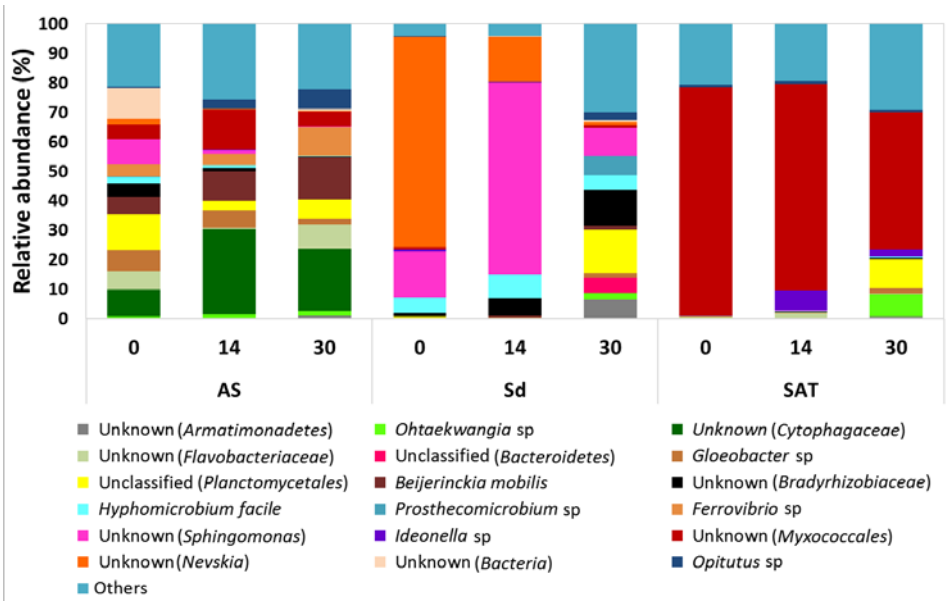


Figure 27 Microbial community structures analyzed at the species level. Microbial communities were pre-acclimated with different inoculum sources—activated sludge (AS), sediment (Sd), and soil from a SAT reactor (SAT). The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

B.3 The impact of primary carbon source

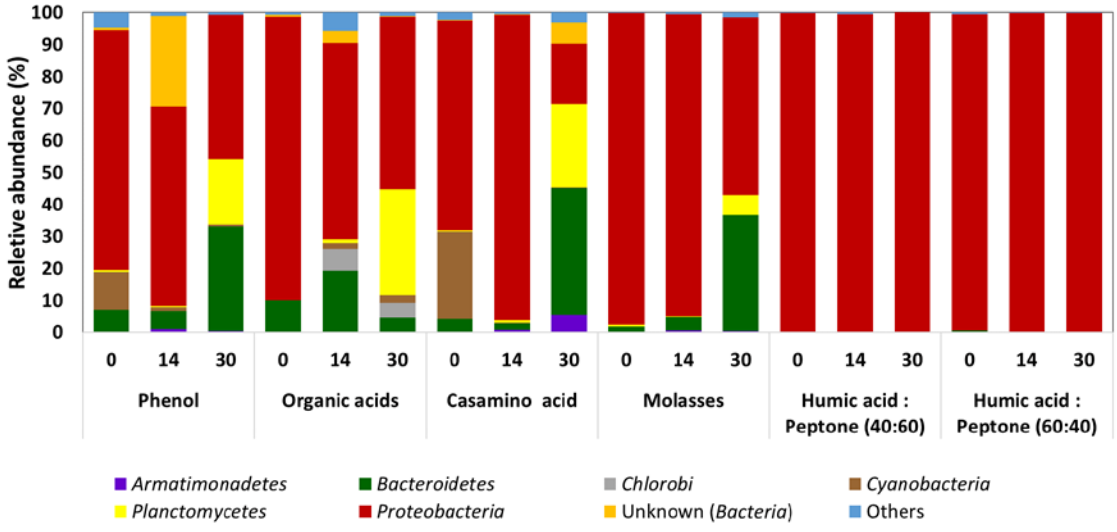


Figure 28 Microbial community structures analyzed at phylum level. Microbial communities were pre-acclimated with different carbon sources. The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

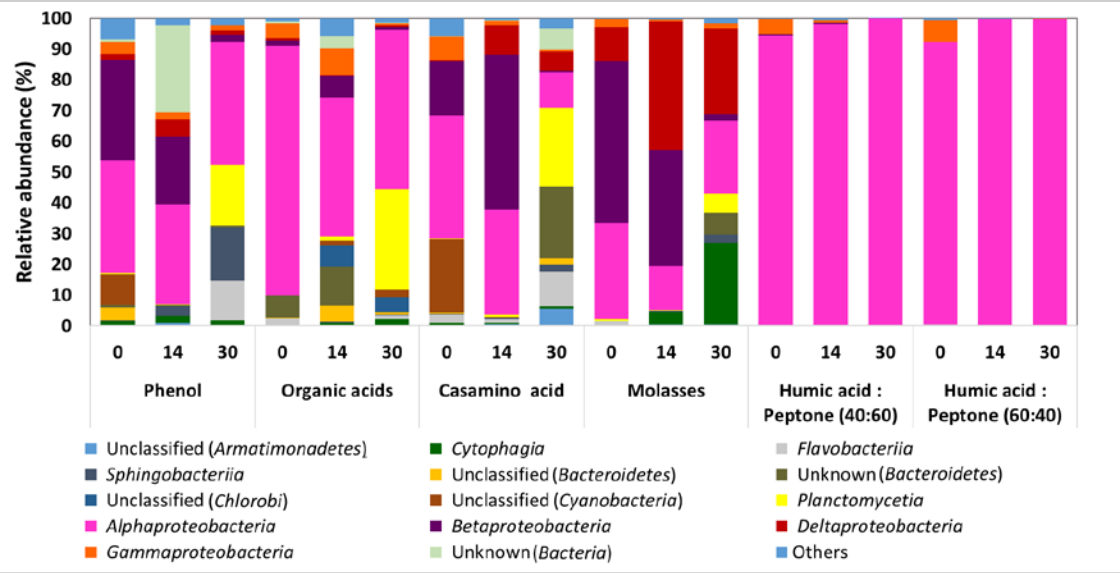


Figure 29 Microbial community structures analyzed at class level. Microbial communities were pre-acclimated with different carbon sources. The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

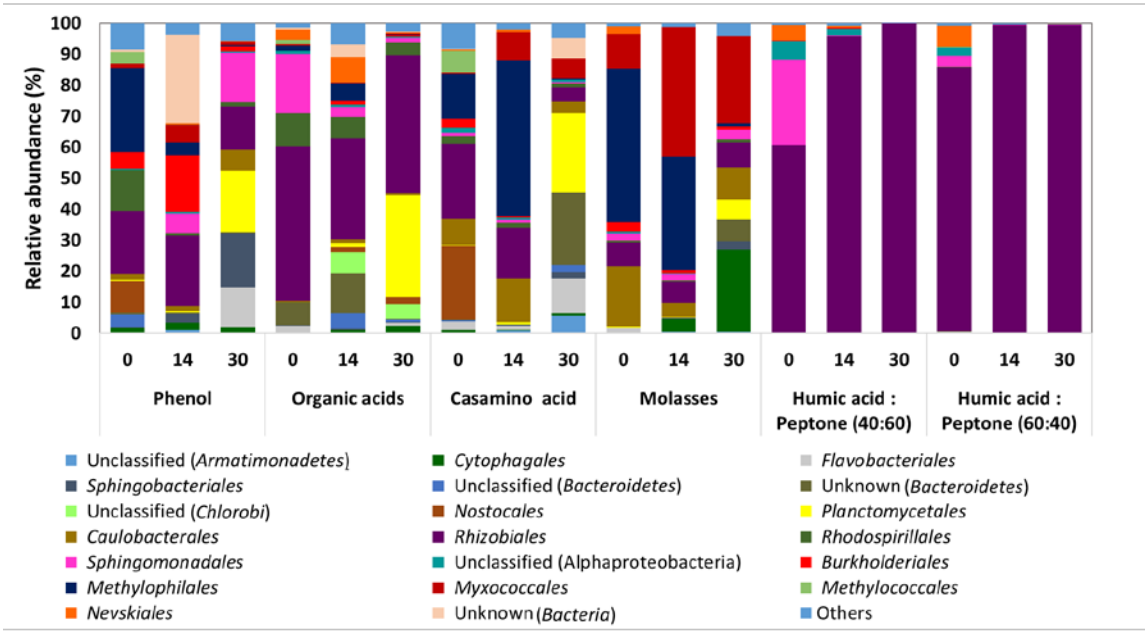


Figure 30 Microbial community structures analyzed at order level. Microbial communities were pre-acclimated with different carbon sources. The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

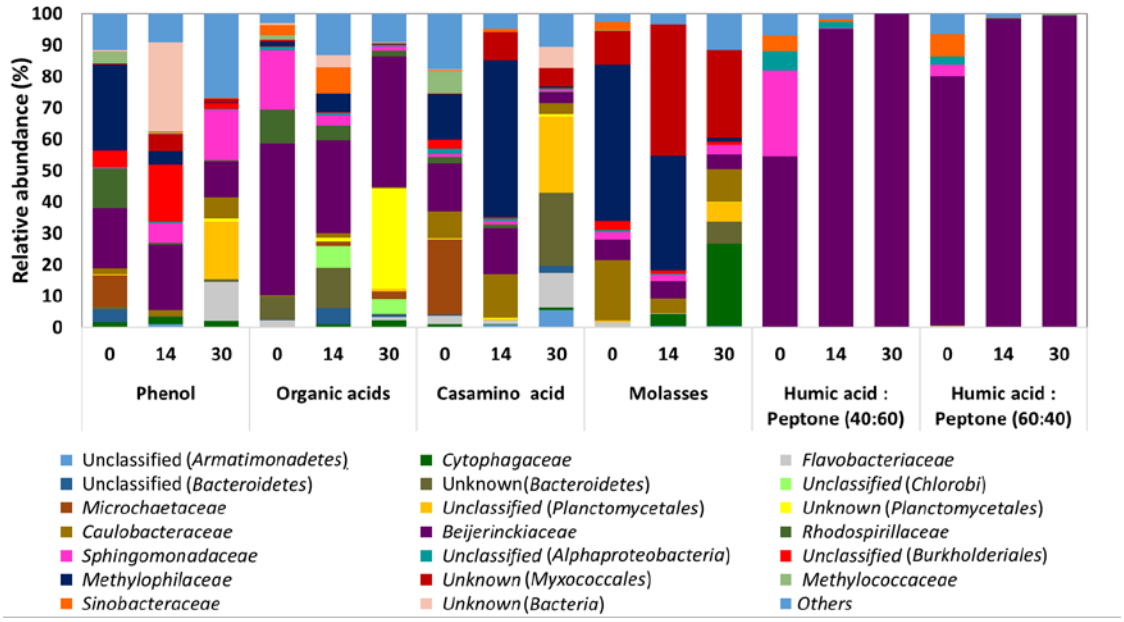


Figure 31 Microbial community structures analyzed at family level. Microbial communities were pre-acclimated with different carbon sources. The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

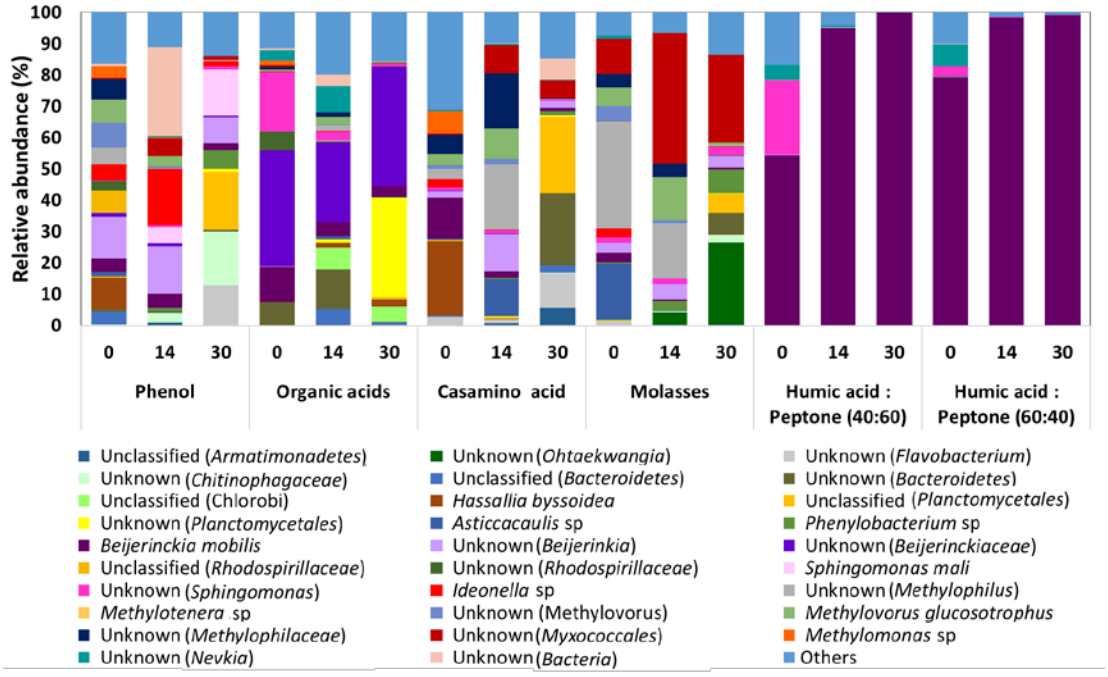


Figure 32 Microbial community structures analyzed at species level. Microbial communities were pre-acclimated with different carbon sources. The microbial samples were taken at day 0, 14, and 30. Abundance less than 5% was cutoff and classified as others.

LIST OF ABBREVIATIONS

AOP	Advanced Oxidation Processes
AS	Activated Sludge
CAS	Conventional Activated Sludge process
CI	Chemical Ionization
DGGE	Denaturing Gradient Gel Electrophoresis
DOC	Dissolved Organic Carbon
DRWF	Drake Water Reclamation Facility
EAAS	Extended Aeration Activated Sludge process
EI	Electron Impact Ionization
ESI	Electrospray Ionization
FISH	Fluorescence *in situ* Hybridization
GC	Gas Chromatography
HRT	Hydraulic Retention Time
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
MAR	Managed Aquifer Recharge
MBR	Membrane Biological Reactor
MLSS	Mixed Liquor Suspended Solids
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry

MTBSTTFA	N-Methyl-N-trimethylsilyltrifluoroacetamide
NSAID	Nonsteroidal anti-inflammatory drugs
PCA	Principal Component Analysis
PCB	Polychlorinated biphenyls
PCR	Polymerase Chain Reaction
PPCP	Pharmaceutical and personal care products
QC	Quality Control
RSD	Relative Standard Deviation
SAT	Soil Aquifer Treatment
SBR	Sequencing Batch Reactor
SIM	Selected Ion Monitoring
S/N	Signal to Noise ratio
SPE	Solid Phase Extraction
SRT	Sludge Retention Time
TBDMCS	Tertbutyldimetheylchlorosilane
TCEP	Tris(1,3-dichloro-2-propyl) phosphate
TDCPP	Tris(2-chloroethyl) phosphate
T-RFLP	Terminal Restriction Fragment Length Polymorphisms
WWTP	Wastewater Treatment Process