Microbial Transformations of Naphthalene and Pharmaceuticals under Anaerobic Conditions

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A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Environmental Sciences

Written under the direction of

Professor Lily Y. Young

And approved by

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New Brunswick, New Jersey

May 2017

ABSTRACT OF THE DISSERTATION

Microbial Transformations of Naphthalene and Pharmaceuticals under Anaerobic Conditions

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Anaerobic microbes are integral to the fate of organic contaminants in the environment. Polyaromatic pollutants are regularly found in anoxic environments, including wastewater treatment and subsurface sediments. In this dissertation, I combine microbial and chemical techniques to understand how microorganisms metabolize aromatic contaminants in anoxic marine sediment and anaerobic wastewater treatment. The first substrate described is naphthalene, the model polycyclic aromatic hydrocarbon (PAH) that established the field of anaerobic PAH degradation. Stable isotope probing of a new sulfidogenic naphthalene degrading culture revealed two organisms responsible for initial naphthalene degradation and a third probable initial naphthalene degrader. Two were closely related to the known isolated anaerobic naphthalene degraders, while the third was distantly related to its closest described organism. This third organism likely represents a new taxon of naphthalene degraders. Using classic anaerobic enrichment techniques, I also enriched for communities able to transform the pharmaceuticals naproxen and diphenhydramine. Unlike naphthalene, however, microbes did not

mineralize the pharmaceutical substrates to CO_2. Rather, microbes removed the methyl substituent, leaving demethylated metabolites. These accumulated in culture with no further metabolism of the carbon skeleton. Naproxen was readily O-demethylated by microbes in both methanogenic wastewater and marine sediment. Community analysis of each culture illustrated the heterotrophic communities enriched during demethylation. In the methanogenic naproxen culture, bacterial and archaeal sequencing revealed a three-tiered trophic transfer of the methyl carbon from acetogens to syntrophic acetate oxidizing bacteria/methanogens. In the marine naproxen transforming culture, a diverse fermentative community was enriched. The microbial community in the wastewater diphenhydramine culture was also enriched for fermenting organisms. In all cultures, the removal of the methyl substituent supported heterotrophic microbial communities. As seen in naproxen, the phenylmethyl ether structure is also abundant in many other pharmaceuticals and personal care products (PPCPs). Because of this, I also examined the ability of the methanogenic and marine naproxen cultures to O-demethylate diverse PPCPs. Both marine and methanogenic naproxen enrichments O-demethylated oxybenzone, guaifenesin, and methylparaben. We thus propose that in anoxic environments, phenylmethyl ether contaminants will be demethylated, and the desmethyl metabolites will comprise a critical portion of total contaminants. Systematic phenylmethyl ether O-demethylation can be extrapolated to a variety of PPCPs, with unknown ecosystem impacts.

Dedication

This dissertation is dedicated to my parents. I'm here because of the *National Geographic*, high expectations, freedom, and support.

Mike, my family, friends, and the best lab any researcher can work with, this was a team effort. Thank you.

Acknowledgements

I gratefully acknowledge my funding sources, NOAA NERRS Graduate Research Fellowship and the NSF IGERT Sustainable Fuels Fellowship NSF DGE 0903675.

I also thank the Graduate School-New Brunswick, whose travel grants allowed me to see the world and talk about science.

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Chapter 1

Introduction

1.1 Introduction

Microbes are the only organisms able to utilize hazardous organic chemicals for food and degrade the contaminants to innocuous products such as cells, CO_2 and H_2O. Their ability to naturally remediate contaminants may serve as an important mechanism in maintaining the integrity of the ecosystem. In contrast, microorganisms may also incompletely transform contaminants. By altering the chemical structure, microbes may change their physical, chemical, and bioactive properties. These microbial transformation products of anthropogenic compounds may exert adverse effects in the environment and to nontarget organisms.

1.2 Anaerobic Microbial Degradation of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons are abundant in petroleum and are a part of Earth's natural chemical composition. The use of fossil fuels, however, has resulted in widespread release of PAHs into the environment.[1] In the environment, PAHs preferentially partition to organic solids, causing a portion to sorb to organic matter and fall to the sediment, where they become buried.[2] Saturated sediment quickly becomes anoxic, leading to PAHs in anaerobic environments. PAH degradation occurs more slowly in the absence of molecular oxygen.[3,4] The smallest PAH, naphthalene, is a bicyclic molecule utilized as a model compound for laboratory study of PAH

biodegradation. Anaerobic naphthalene mineralization was first and is most completely described under sulfate reducing conditions. Zhang & Young (1997) demonstrated that sulfate reducing bacteria from marine environments activate naphthalene by the addition of a carboxyl group in the 2 position. The rings are sequentially reduced before complete mineralization to CO_2.[5,6]

Our studies in this work focuses on the bi-cyclic aromatic compound, naphthalene. To date only two axenic cultures capable of anaerobic naphthalene degradation have been established. One isolate, NaphS2, is a sulfate reducing organism isolated from the North Sea in Germany.[7] The other sulfate reducing naphthalene degrader, NaphS3, was isolated from a contaminated lagoon of the Mediterranean Sea in France.[8]

Only limited information is available about the capability of microorganisms indigenous to uncontaminated sediments to degrade naphthalene. Few cultures have been extensively characterized, however, and the transfer of naphthalene carbon through a sulfate reducing community has not been investigated. In Chapter 2 I describe a new sulfate reducing, naphthalene degrading culture enriched from unimpacted estuarine sediment. This highly enriched culture readily degrades naphthalene and 2-methylnaphthalene, though is incapable of degrading other hydrocarbon substrates. Work in the Young lab early on pioneered the use of isotope labeled substrates to characterize the anaerobic biodegradation pathways.[3,5,6] In this manner, unambiguous identification of isotope labeled metabolites along the naphthalene degradation pathway were confirmed, and the first reports of anaerobic naphthalene degradation was described.[3]

Carboxylation to naphthoic acid is a crucial step in the initiation and activation of anaerobic naphthalene metabolism. Two fundamental observations provided important

results to the understanding of the initial attack. First, carboxylation occurred only in the 2 position, no other carboxylated intermediate was observed.[3,5,6] Second, the source of the carbon was from free carbonate/bicarbonate in solution. This could be proven by ^{13}C labeling of carbonate in solution with subsequent detection of ^{13}C carboxyl group on naphthoic acid.[5,6] After carboxylation, the aromatic rings are sequentially reduced. The addition of deuterated water resulted in heavier mass shifted hydrogenated metabolites, demonstrating that water is the source of hydrogen for ring reduction.[5] While naphthalene degradation is initiated by the addition of a carboxyl in the 2 position, 2-methylnaphthalene is activated by a fumarate addition.[9] Following activation, naphthalene and 2-methylnaphthalene degradation proceeds via a common pathway of ring reduction.[6] The ability of sulfidic naphthalene degraders to metabolize both substrates is therefore anticipated, and was observed in the enrichment described in Chapter 2. The addition of fumarate to 2-methylnaphthalene is similar to that of the anaerobic toluene degradation pathway. It was therefore unexpected that the naphthalene degrading enrichment, capable of readily degrading 2-methylnaphthalene, did not also mineralize toluene.

To identify community members responsible for naphthalene degradation in this enrichment culture, DNA stable isotope probing (SIP) of $^{13}C_{10}$-naphthalene was utilized. Similar to the identification of intermediate metabolites by 13C substrate, DNA SIP of labeled contaminants was developed to identify organisms incorporating aromatic hydrocarbons into their cellular material.[10,11] Originally demonstrated in our lab, the degradation of labeled 2-methylnaphthalene confirmed the complete mineralization of 2-methylnaphthalene to CO_2.[6] In combining labeled hydrocarbon degradation with ^{13}C

DNA concomitant with substrate loss, it became possible to couple degradation with unambiguous substrate incorporation by bacteria.[10] Phylogenetic analysis of this labeled ^{13}C, incorporated into cells by benzene degraders, demonstrated benzene carbon transfer in an anoxic community. As detailed in Chapter 2, the identification of organisms responsible for naphthalene degradation builds upon this foundational PAH degradation research. By amending the culture with ^{13}C naphthalene substrate, the identification of microorganisms responsible for anaerobic naphthalene degradation was demonstrated.

1.3 Microbial Transformation of Pharmaceutical Compounds

Even less is known about the microbial degradation and transformation of pharmaceutical compounds. Antibiotics, non-steroidal anti-inflammatory drugs, and beta blockers have been found in the plasma of fish inhabiting streams near wastewater treatment plant.[12] These new contaminants are particularly concerning because they are designed to elicit a biological response in humans, and may also affect nontarget organisms. Diphenhydramine, for example, was demonstrated to suppress swimming and feeding behavior in crucian carp.[13] In contrast to point source organic contaminants like petroleum and dry cleaning fluids, pharmaceutical compounds are diffusely excreted by humans on a continual basis. Before entering the environment, pharmaceutical compounds undergo biological wastewater treatment. Because wastewater treatment plants were designed to remove BOD and COD, not complex organics, pharmaceuticals often escape wastewater treatment.[14,15] Pharmaceuticals are thus introduced to the environment through treated wastewater products, such as treated water and biosolids.

The change in chemical structure can affect organisms in unforseen ways. For example, this phenomenon is well documented in nonylphenol polyethoxylate transformation in

wastewater treatment (Geiger 1984).[16] During anaerobic digestion, nonylphenol polyethoxylated is fully de ethoxylated. Anaerobic wastewater bacteria sequentially remove O-ethyl groups, forming 4-nonylphenol. This recalcitrant end product is released from wastewater treatment into the environment, where it is detected in surface and groundwater.[17,18] This new compound is toxic to aquatic life, posing a threat to ecosystems downstream of wastewater treatment plants.

Pharmaceutical compounds may behave in a similar manner to 4-nonylphenol during wastewater treatment. Few of these emerging contaminants, however, have been carefully studied. Complete mineralization of ibuprofen has been demonstrated by microorganisms in activated sludge, the aerobic microbial process of wastewater treatment.[19] Several studies have investigated loss of select pharmaceutical compounds before and after wastewater treatment.[20,21] Incomplete removal of pharmaceutical compounds, however, during wastewater treatment means the compounds escape intact.[14] For example, wastewater microorganisms may transform a portion of the parent compound into modified metabolites, as is seen with nonylphenol polyethoxylate. The fate of pharmaceuticals in the environment will therefore depend on transformation during wastewater treatment and the chemical and physical properties of both the parent compound and any resulting metabolites. Because wastewater treatment is a highly engineered biological system, we require a nuanced understanding of the microbial ecology and biochemical processes of contaminant transformation.

1.3.1 Microbial Naproxen Transformation

Naproxen, sold under the trade name Aleve®, is an over the counter non-steroidal anti-inflammatory drug. It is among the most commonly detected compounds during surveys

of pharmaceutical and personal care products in the environment.[22–24] Transformation of naproxen by wastewater treatment microorganisms has been described under aerobic and anaerobic conditions. Quintana et al.[25] first described microbial transformation of naproxen during cometabolism with powdered milk in an aerobic membrane bioreactor. In this study, microorganisms cleaved the ether bond of naproxen, resulting in the demethylated metabolite 6-O-desmethylnaproxen. Lahti & Oikari et al.[26] found the same naproxen demethylation occurred during incubation with microbes from an anaerobic digester sludge under methanogenic conditions. In both of these studies, naproxen was supplied in μM concentrations. In chapter 3 I describe the microbial strategy of naproxen transformation by a methanogenic culture enriched from anaerobic digester sludge. Demethylation of naproxen by this culture resulted in 6-O-desmethylnaproxen accumulation as well as methane generation, indicating that the removed methyl group was utilized as carbon substrate through the anaerobic community. Naproxen demethylation proceeded by acetogenesis, which subsequently enriched for a community of syntrophic acetate oxidizing bacteria and methanogens. The complex syntrophy involved in naproxen demethylation to reveals that pharmaceuticals can drive niche microbial food webs during wastewater treatment.

1.3.2 Microbial Diphenhydramine Transformation

Diphenhydramine is a pharmaceutical used as an antihistamine, anti-nausea medication, and non-habit forming sleep aid. As such, it is present in a multitude of popular over the counter drugs, including Benadryl®, Tylenol-PM®, Advil-PM®, and ZzzQuil®. Because of its extensive use, diphenhydramine is prevalent in wastewater.[27] During wastewater treatment diphenhydramine is only partially removed.[20] This incomplete removal leads to

diphenhydramine release into environmental surface waters in treated wastewater effluent, with unknown effects to the ecosystem.

To the author's knowledge, the microbial transformation of diphenhydramine has not been reported. Chapter 4 details the microbial transformation of diphenhydramine by anaerobic digester sludge microbes. Diphenhydramine was demethylated to N-desmethyl diphenhydramine, a metabolite formerly only known to be generated by mammals and fungi.[28] An anaerobic toxicity assay demonstrated that while the parent compound, diphenhydramine, suppressed both fermentative and methanogenic activity, the microbial suppressed only methanogenic activity. Differing activities of diphenhydramine compared with N-desmethyl diphenhydramine demonstrate the unforseen effects microbial metabolites can exert on nontarget organisms.

1.3.3 Anaerobic Demethylation of Phenylmethyl Ether Pharmaceuticals

Like naproxen, many pharmaceutically active compounds contain a methyl group ether linked to an aromatic ring. The enrichment culture described in Chapter 2 was also shown to perform syringic acid and vanillic acid demethylation. In Chapter 5, a second anaerobic naproxen transforming enrichment is described. This culture was enriched under sulfate reducing conditions from anoxic estuarine sediment. As was seen in the methanogenic enrichment, this new sulfidic culture readily demethylates naproxen to 6-O-desmethyl naproxen. The ability of these two naproxen transforming cultures to also demethylate other common phenylmethyl ether pharmaceuticals was investigated. Included were the expectorant guaifenesin, the active ingredient in Mucinex®, methylparaben, a preservative, and oxybenzone, a UV filter used in sunscreen and cosmetics. All compounds were demethylated by the methanogenic and sulfidic cultures.

As a result of the transformation, demethylated metabolites of the parent pharmaceuticals were detected in all cultures. In the case of oxybenzone, the demethylated metabolite was 2,4-dihydrobenzophenone, which is a plasticizer with known estrogenic activity.[29] Little information is available concerning the demethylated metabolite of guaifenesin or its impact on organisms. Methylparaben was demethylated to 4-hydroxybenzoate. As demonstrated by the substrate diversity utilized by the cultures, phenylmethylether pharmaceutical compounds can be expected to be demethylated in anoxic environments. Their demethylated metabolites may continue to exert pharmaceutical activity. It is therefore insufficient to merely survey for intact pharmaceutical compounds, their demethylated metabolites are of interest as well.

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Chapter 2

Naphthalene Degradation in a Sulfate Reducing Marine Enrichment Culture

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are common organic contaminants in anaerobic environments. The impact of naphthalene into sediment to enrich, select for, and sustain microbial communities specialized in the utilization of PAHs was investigated. Sediment was obtained from the Jacques Cousteau National Estuarine Research Reserve in Tuckerton, NJ, where PAHs are introduced through nonpoint deposition to an ecosystem otherwise relatively unimpacted by hydrocarbons. Primary anaerobic enrichment cultures completely degraded naphthalene within 139 days. Subsequent transfer cultures mineralized naphthalene within 21 days with stoichiometric sulfate loss. To determine the organisms responsible for naphthalene degradation, stable isotope probing was utilized on cultures amended with fully labeled ^{13}C-naphthalene as substrate. Three organisms were found to unambiguously incorporate ^{13}C-carbon from ^{13}C-naphthalene within 7 days. Phylogenetic analysis revealed that two of these organisms are closely related to the known naphthalene degrading isolates NaphS2 and NaphS3. A third organism, likely degrading naphthalene or early naphthalene metabolites is only distantly related to its closest related organism and may be a novel naphthalene degrading microbe.

2.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) have become ubiquitously distributed to the environment through the widespread use of petroleum for combustion, pesticides, and

numerous other applications.[1] These toxic chemicals are of concern because of commonplace, continual anthropogenic introduction. Burning fossil fuels leads to global atmospheric deposition, even in pristine environments historically unimpacted by point source pollution.

Of the major PAH transformation pathways in sediment, including volatilization, adsorption, and chemical degradation, microbial mineralization is the most important degradation process.[2] Aerobic PAH biodegradation is well characterized. PAHs, however, quickly enter anoxic environments via sorption to organic particulates in the water column and subsequent burial in the sediment.[3] Once PAHs enter anoxic regions of soils and sediments, aerobic degradation is no longer available and anaerobic processes become important. Naphthalene, the smallest PAH, is degraded under anaerobic conditions, of which sulfate reducing is the best characterized.[3] Two sulfate reducing naphthalene degraders, NaphS2 and NaphS3, have been isolated in pure culture from heavily polluted marine sediment in the Etang de Berre, a Mediterranean lagoon, and the North Sea[4,5]. Sulfate reducers from freshwater aquifers contaminated with hydrocarbon have also been described, including strain N47.[6,7] All of these organisms belong to the family *Desulfobacteraceae*. In addition to naphthalene, characterized sulfate reducing naphthalene degraders also metabolize 2-methylnaphthalene.[8] Besides these two substrates and common central metabolites, the metabolic diversity of anaerobic naphthalene degraders has not yet been explored. In the environment naphthalene will be found along with other petroleum constituents, so understanding the ability of naphthalene degraders to also mineralize other hydrocarbons is of interest.

Other investigations of PAH degrading microbes have focused on sites with known hydrocarbon contamination from oil spills or industry (Table 1). This project differs in that we assessed anaerobic naphthalene degradation from sediment not impacted by large scale hydrocarbon contamination. The study site has no natural oil seeps and no history of substantial hydrocarbon contamination.[9] Instead, like many environments on Earth, over the last century nonpoint sources continuously introduce low concentrations of PAHs to an ecosystem otherwise relatively unimpacted by hydrocarbons.[1] By tracking carbon transfer of naphthalene through a microbial community indigenous to an unimpacted environment, this study elucidates the contaminant food chain in pristine sediments. Exposing the naphthalene degrading culture to crude oil also demonstrated the limited substrate diversity of this culture, and related naphthalene degraders.

2.2 Methods

2.2.1 Chemicals

Chemicals were purchased from Sigma Aldrich (St. Louis, MO) and Thermo Scientific (Waltham, MA). Fully labeled $^{13}C_{10}$-naphthalene with chemical and isotopic purity of 99% was purchased from Sigma Aldrich.

2.2.2 Site Description

Enrichment cultures were established using sediment from the Jacques Cousteau National Estuarine Research Reserve, a protected marine salt marsh in Tuckerton, NJ. The concentration of total PAHs in this inoculum is lower than total PAHs in inoculum of previous naphthalene degrading studies (Table 1). Anoxic subsurface sediment was used as inoculum. This estuarine system is highly productive and as a result the sediment is

highly reducing. Sediment was maintained under a N_2 atmosphere at 4°C for 24 hours before enrichment cultures were established.

2.2.3 Enrichment Cultivation

A 20% (vol/vol) sediment slurry was added to anaerobic sulfate reducing marine medium as described previously.[10] ^{12}C-naphthalene was the sole added carbon source to primary enrichment cultures. Naphthalene was evaporated onto the bottom of 160mL glass serum bottles in a hexane carrier to a final concentration of 500μM. 100mL of the inoculated media slurry was dispensed into the naphthalene coated serum bottles and overlain with a 70% N_2/30% CO_2 atmosphere. Bottles were sealed with butyl rubber stoppers and aluminum crimps. Enrichments were established in triplicate with duplicate sterile controls, which were autoclaved on three consecutive days. All bottles were incubated statically at room temperature in the dark.

Primary enrichment cultures were transferred after one year. Transfers were established with 3% primary culture in 97% sulfate reducing marine media. The first transfer received naphthalene evaporated onto each serum bottle to a final concentration of 500μM. To facilitate naphthalene reamendment, subsequent naphthalene amendments were provided via silica carrier. Naphthalene dissolved in hexane was mixed with silica particles and then hexane was evaporated, leaving a naphthalene-sorbed silica stock of a theoretical concentration of 50μM per gram silica. A portion of the naphthalene volatilized during solvent evaporation. Following the initial 3% transfer, subsequent transfer cultures received 50% culture and 50% fresh media. These consortia were

amended with naphthalene three times before transfer. Complete naphthalene disappearance was confirmed before each reamendment. Molybdate (MoO_4) was supplied to duplicate subcultures at 20mM to determine the effects of sulfate reduction inhibition.[3]

2.2.4 Oil Biodegradation

Subcultures of sediment free enrichments were amended with two crude oils to determine the range of petroleum hydrocarbon substrate diversity.[11] Artificially weathered Alaska North Slope crude oil (API = 29º), depleted in monoaromatic compounds and low molecular weight alkanes, was amended at 5uL/30mL culture. Alba crude oil (API = 19.4) was also amended to a separate set of subcultures at 5uL/30mL culture. This crude oil was not weathered and contains monoaromatic constituents. Inactive controls of each treatment received molybdate to inhibit sulfate reduction. Entire bottles of triplicate active and duplicate inactive cultures were frozen and stored at each time point for hydrocarbon analysis.

2.2.5 Stable Isotope Probing Microcosms

Sediment-free ^{12}C-naphthalene degrading consortia were transferred to replicate fresh bottles and received uniformly labeled $^{13}C_{10}$-naphthalene as the sole carbon source. SIP cultures were amended with $^{13}C_{10}$-naphthalene sorbed to the bottom of the serum bottle at a final concentration of 390μM. Separate ^{12}Cnapthhalene control cultures were maintained. SIP culture bottles were sampled for DNA (1mL) every 2-3 days of incubation.

2.2.6 SIP Molecular Analysis

1mL of sample from one representative replicate was filtered onto a 13mm diameter 0.22um filter and immediate frozen until DNA extraction. Genomic DNA was extracted using a modified phenol-chloroform procedure with the addition of ^{12}C-labeled and ^{13}C-labeled archaeal carrier DNA from *Halobacterium salinarum* and ^{12}CDNA and ^{13}CDNA was separated by CsCl density gradient centrifugation[12]. Microbial diversity in the ^{12}C and ^{13}CDNA bands was analyzed by 16SrRNA amplification with Bacteria-specific primers 27F(5'-AGAGTTTGATCMTGGCTCAG-3'), labeled with a 6-carboxyfluoroscein at the 5' end, and 1100R(5'-GGGTTGCGCTCGTTG-3'). PCR product (20ng DNA) was digested using *Mnl1* endonuclease (New England Biolab, Beverly, USA) for 6 hours at 37ºC. DNA was precipitated and T-RFLP was performed using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, USA). Terminal restriction fragments (TRFs) were identified using a clone library of ^{13}C amplicons generated from DNA sampled at day 7. The relative abundance of each identified peak was calculated by normalizing its peak area to the total area of the entire sample as described previously.[13] 16S PCR products were cloned using a pGEM cloning kit and chemically transformed into *E. coli* JM109 competent cells (Promega, Madison, WI, USA) according to the manufacturer's instructions. Clone inserts were sequenced by Genewiz (Genewiz Inc., South Plainfield, NJ, USA) using T7F and M13R primers. Taxonomic identity was assigned by comparing cloned sequences with the NCBI database using BlastN.[14]

2.2.7 Chemical Analysis

Naphthalene concentration was monitored using a Hewlett-Packard 5890 Series II gas chromatograph with a flame ionization detector (Hewlett-Packard, Palo Alto, CA, USA) and a 30m x 0.32mm DBWax capillary column (J&W Scientific, Folsom, CA, USA). 0.75mL culture was extracted with an equal volume of hexane containing 500µM fluorene as an internal standard. Sulfate was measured using a Dionex model 100 ion chromatograph (Sunnyvale, CA) equipped with an IonPac AS9 column and conductivity detector. Eluent was composed of 2mM Na_2CO_3 and 0.75mM $NaHCO_3$ at 2ml min^{-1}.

Total hydrocarbon loss in crude oil subcultures was monitored using GC/MS. Thawed bottles were extracted with methylene chloride and processed as described previously.[11]

2.3 Results and Discussion

2.3.1 Naphthalene Degrading Enrichments

Enrichments capable of degrading naphthalene were established from the anoxic Tuckerton sediment. Primary enrichment cultures completely degraded naphthalene within one year (data not shown). Figure 1A illustrates that Primary transfer cultures completely degraded naphthalene in 139 days. Though 500µM naphthalene was delivered to bottles, little was recovered by hexane extraction due to low solubility of sorbed naphthalene in the aqueous media. Limited naphthalene loss was seen in sterile controls. After naphthalene was degraded by primary enrichments, cultures were transferred to fresh media and received naphthalene sorbed to silica for substrate delivery. The naphthalene-sorbed silica provides increased surface area and allows for a homogenous

dispersal throughout the liquid culture. Subsequent naphthalene amendments and transfers resulted in a sediment free enriched consortium capable of degrading naphthalene in 17 days (Figure 1B).

To determine whether naphthalene degradation was coupled to sulfate reduction, the electron acceptor concentration was monitored in primary transfer cultures. Due to the long primary enrichment incubation and low (3%) transfer volume, little background carbon is likely to have been present. Thus, all sulfate loss was expected to be a result of naphthalene metabolism. Three mM sulfate loss was observed in primary transfers, which received 500μM naphthalene (Table 2). This loss is consistent with the predicted electron acceptor loss based on stoichiometric values, which can also be seen in Table 2.

To further support that sulfate loss was due to naphthalene degradation and not background respiration, naphthalene concentration was monitored in the presence of molybdate, a specific inhibitor of sulfate reduction.[3] The addition of molybdate resulted in little naphthalene loss in active enrichment cultures (Figure 2). When sulfate reduction was inhibited, naphthalene concentrations remained stable. In the absence of molybdate, however, naphthalene was metabolized within 13 days.

2.3.2 Oil Biodegradation

As shown in Figure 3A, naphthalene degrading cultures amended with weathered Alaska North Slope crude oil readily degraded naphthalene and 2-methyl naphthalene. The two compounds were completely degraded within 2 weeks in active cultures, while no loss

was observed in cultures inhibited with MoO_4^{2-} (Figure 3A middle and bottom rows). Anaerobic naphthalene degrading bacteria are known to metabolize 2-methylnaphthalene.[3,8,15] Sullivan et al.[8] demonstrated that 2-methylnaphthalene is degraded via the same degradation pathway as naphthalene. Both compounds share the carboxylated intermediate metabolite 2-naphthoic acid before ring reduction and cleavage.[3,8] It thus follows that anaerobic naphthalene cultures, including the one enriched in this study, are able to degrade both naphthalene and 2-methylnaphthalene (figs 3A, B). This culture is unable to metabolize 1-methylnaphthalene, which is consistent with observations in other naphthalene degrading studies.[11] After an additional 14 weeks of incubation, no loss of any components besides naphthalene and 2-methylnaphthalene was observed (data not shown). This study presents the first comprehensive investigation of a highly enriched sulfate reducing, naphthalene degrading consortium.

Cultures were also incubated with Alba crude oil, which contains alkanes and monoaromatics in addition to PAHs. As shown in Figure 3B, during incubation with Alba crude oil, monoaromatic compounds were not degraded, even while naphthalene and 2-methylnaphthalene loss occurred. In these cultures, benzene, toluene, and xylenes persisted. Additionally, 1-methylnaphthalene, polyaromatic hydrocarbons, and alkanes all remained (data not shown). Recovery of these volatile constituents confirmed that volatilization did not occur, and that naphthalene and 2-methylnaphthalene were the only compounds used as substrate by this enriched consortium.

What is compelling, however, is that the mechanism of attack on 2 methylnaphthalene mimics that of toluene because of its similar physical structure. 2-methylnaphthalene and toluene are both activated via fumarate addition to the methyl group.[16] It is therefore noteworthy that despite degrading 2-methylnaphthalene, this culture did not degrade toluene (Figure 3). Similar mechanisms of activation would suggest that toluene would also be activated via the same pathway. In cultures amended with Alba crude oil, however, no toluene loss was observed over the 16 week experiment. It is possible that further time is needed to adapt to the substrate change, and eventually toluene may be degraded.

2.3.3 Stable Isotope Probing

$^{13}C_{10}$-naphthalene, not unexpectedly, was completely mineralized in SIP subcultures (data not shown). The labeled naphthalene was incorporated into DNA, validating that naphthalene was used as a carbon source by enriched bacteria.[17] Total genomic DNA included both ^{12}C DNA, present from previous enrichment on unlabeled naphthalene, and ^{13}C DNA, generated by bacteria consuming $^{13}C_{10}$-naphthalene. Figure 4 displays the PCR amplification of ^{12}C controls and ^{13}C SIP incubations. In the ^{12}C naphthalene fed control, no ^{13}C DNA was amplified (Figure 4 band 3), confirming that there was no contamination between the ^{12}C band and ^{13}C band. A minimum incubation time of 7 days was required to observe ^{13}Cnaphthalene incorporation with distinct differences between the ^{12}C and ^{13}C DNA TRFLP fingerprint. By day 7, a faint but distinct band of 16S amplicon was seen in the ^{13}C band (Figure 4 band 5). TRFs were compared at days 7, 14, and 17 of incubation to visualize changes in organisms incorporating ^{13}Cnaphthalene. As

displayed in Figure 1B, theses time points are related to the typical rate of naphthalene degradation. Screening 130 clones yielded four TRFs prominent in the ^{13}C fraction, including TRFs 120, 215, 233, and 275.

The naphthalene degrading community composition, before SIP incubation, is seen in Figure 5A. The four TRFs comprising 80% of the community were identified by clone library screening. The remaining portion, designated "other," contains minor TRFs not recovered in the clone library. TRFs 120, 233, 275, and 215 represent four distinct microorganisms, all found to incorporate ^{13}C carbon into their cellular material when amended with ^{13}C-naphthalene.

Figure 5B illustrates the relative abundance of RFs in the ^{13}C DNA at days 7, 14, and 17. The community composition incorporating ^{13}C naphthalene is relatively stable over time. Even after incorporation of ^{13}C naphthalene, the community composition remains largely the same as the starting community, which is expected for a highly enriched consortium receiving naphthalene as the sole carbon source. This suggests that the majority of the community is directly involved in naphthalene cycling.

The ^{13}C community profile at day 7 closely reflects that before SIP incubation, though exclusively includes TRFs with unambiguous ^{13}C incorporation from ^{13}C naphthalene. TRFs 120 and 233 incorporate ^{13}C into their DNA by day 7. TRF 233 comprised 5% of the relative abundance throughout the incubation, while TRF 120 was detected at 15% days 7 and 14, then only slightly rose to 18% by day 17. Both TRF 120 and 233 shared

100% and 99% identity, respectively, to NaphS2 (AJ132804) and NaphS3 (EU908726). NaphS2 and NaphS3, which belong to Deltaproteobacteria and are related to *Desulfobacteriaceae,* comprise 2 of the 3 naphthalene utilizing sulfate reducers that have been isolated to date.[5] The low percent presence of TRFs 120 and 233 is consistent with those of primary aromatic ring degraders observed in previous SIP studies. Gallagher et al.[17] demonstrated that the organisms responsible for ring cleavage were not the most abundant in the culture.

The selective increase of TRF 275 in the ^{13}C fraction also demonstrates early incorporation of ^{13}C from ^{13}C-naphthalene. Present as 22% of the relative abundance at day 7, TRF 275 increased to 28% by day 14 then fell to 17% by day 17. Notably, the relative abundance of TRF 275 is highest at day 14, concurrent with naphthalene consumption.

The relative abundance of TRF 275 corresponds to naphthalene degradation rates (Figure 1B), indicating that this organism is also likely responsible for naphthalene degradation. This is the first reporting of naphthalene incorporation in an anaerobic organism with sequence differing from the known anaerobic naphthalene degraders. TRF 275 was found to be part of a poorly characterized taxon. It shares 86% identity with its most closely related curated organism, *Desulfomicrobium thermophilum* (NR_042924.1).[18] While its ability to degrade hydrocarbons has not been reported, *D. thermophilum* has been identified as an important SRB in petroleum reservoirs and oil production facilities.[20–23] When uncultured organisms were included in the BLAST search, TRF 275 shared 89%

and 88% identity to two clones identified in a petroleum contaminated aquifer (JQ086867 and JQ086797).[19] Common detection in petroleum contaminated environments suggests a link between *Desulfomicrobium* and hydrocarbons. Their relation to TRF 275 provides further evidence of this association.

In addition to TRFs 120, 233, and 275, TRF 215 was also prevalent in both ^{12}C and ^{13}C fractions. It was present at 34% at days 7 and 14. While it did incorporate ^{13}C into its DNA by day 7, it increased from 34% to 46% between days 14 and 17. This increase in relative abundance occurred after naphthalene is almost completely exhausted in cultures (Figure 1B). Thus, between days 14 and 17, little intact naphthalene is left to incorporate into DNA. Gallagher et al.[17] also demonstrated that organisms consuming downstream metabolites of cleaved aromatic ring products were present in higher relative abundance and increased later in the incubation. In the naphthalene cultures, this trend similarly was observed in TRF 215. Downstream ^{13}C metabolites of ^{13}Cnaphthalene degradation, produced by primary naphthalene degraders, would continue to circulate in the culture. Though it successfully incorporated ^{13}C into its DNA, it is unlikely that TRF 215 degrades naphthalene, as it did not increase until most naphthalene is consumed.

TRF 215 was identified as a member of the genus *Prosthecochloris*, sharing 99% sequence identity with several strains of *P. vibrioformis* (Pfenning-B1, KX417801.1; isolate GS8B, AM690794.2) and other members of the genus.
Prosthecochloris is a member of *Chlorobi*, obligately phototrophic green sulfur bacteria that are surprisingly common in dark habitats. Its ability to utilize extremely low levels of

radiation (0.015 µmol quanta m^{-2} s^{-1}) allows *Prosthecochloris* to thrive in deep sediment, hydrothermal vents, and dark bioreactors.[20–22] *Prosthecochloris* fixes CO_2 and can photoheterotrophically assimilate acetate, propionate, peptone, and yeast extract. Gallagher et al.[17] demonstrated that organisms enriched after substrate disappearance in SIP cultures obtain ^{13}C from excreted metabolites of the original ^{12}C-substrate. Given its known metabolism and slow enrichment, *Prosthecochloris* most likely assimilated ^{13}C into its DNA, in the form of downstream ^{13}C intermediate metabolites excreted by primary naphthalene degraders.

Phylogenetic relatedness of the sequenced clones and their closest BLAST organisms are displayed in Figures 6 and 7. The *Proteobacteria* tree (Figure 6) shows that TRFs 120 and 233 cluster with each other, and together are most closely related to NaphS2 and NaphS3. The close phylogenetic relationship with NaphS2/S3, the isolated naphthalene degraders, provides further evidence that TRFs 120 and 233 are involved in initial naphthalene degradation. TRF 275, which showed a ^{13}C enrichment pattern consistent with expected naphthalene loss, is also a member of *Deltaproteobacteria*. It is not, however, closely related to the known naphthalene degraders. Rather, it is a member of phylogenetic group previously unknown to mediate naphthalene degradation. By demonstrating that this uncharacterized phylogenetic group likely is degrading naphthalene, these SIP results have identified a new taxon and added to the diversity of likely naphthalene degraders. Figure 7 displays the phylogeny of TRF 215 within *Chlorobi*. It is most closely related to *Prostecochloris*, a taxon not previously known to be involved in contaminant cycling.

To the authors' knowledge, all previously studied anaerobic marine naphthalene degraders, including isolates NaphS2 and NaphS3, were enriched from highly contaminated sediments (Table 1). The naphthalene degrading consortium in this study, in contrast, was enriched from sediment unimpacted by high levels of hydrocarbon pollution. Despite geographical distance and pollution levels, highly similar organisms were enriched from sediments in distant marine ecosystems. This suggests that sulfate reducing naphthalene degraders are widely dispersed in sediments. Since petroleum is a natural product that seeps into the marine environment, it makes sense that similar organisms have evolved to degrade naphthalene.

SIP allowed for identification of organisms involved in the natural attenuation of naphthalene in an unimpacted estuary. TRFs 120, 233, and 275 demonstrated early and relatively stable enrichment on ^{13}C from naphthalene. The close phylogenetic relationship with known anaerobic naphthalene degraders indicates that TRFs 120 and 233 are responsible for initial naphthalene degradation. TRF 275 shares similar enrichment pattern, supporting that it may be either an additional initial naphthalene degrader or early consumer of naphthalene metabolites. The fact that its relative enrichment peaks at day 14, concurrent with naphthalene consumption, suggests that it is a new anaerobic naphthalene degrader. Further study is necessary to confirm TRF 275's role in naphthalene degradation. The *Prosthecochloris* member, TRF 215, is also a dominant member of this community. Its presence and ^{13}C incorporation in the highly enriched culture demonstrates the involvement of contaminant-tolerant non-degraders in carbon

cycling. In the environment, naphthalene and other hydrocarbons serve as both a contaminant stress and carbon source. By tracking naphthalene carbon in the form of ^{13}C through the enriched community, we can elucidate how contaminants can be expected to move through anaerobic microbial trophic systems.

The selective metabolism of the enriched consortium for naphthalene and 2-methyl-naphthalene indicates that among those studied, sulfate reducing naphthalene degraders have a limited metabolism. In petroleum contaminated environments, however, naphthalene is present as one of many hydrocarbons.[11] Microorganisms will therefore be simultaneously exposed to a variety of hydrocarbons. As demonstrated by SIP, the naphthalene degraders in this study occupy a specific metabolic niche. Though not observed in this study, anaerobic degradation of alkanes, monoaromatics, and high molecular weight PAHs under sulfate reducing conditions have been documented.[3,18,23] This supports that in the environment a suite of organisms will be required for degradation of the hydrocarbons present, in addition to the known naphthalene degraders.

PAHs are naturally occurring compounds from very early in the earth's history. As demonstrated, even environments relatively unimpacted by anthropogenic contamination contain microbes capable of naphthalene degradation. Natural petroleum seeps, emitting hydrocarbons into marine environments for millions of years, selected for microbes capable of degrading naphthalene. Naphthalene degraders are thus likely to be present in widely distributed habitats, including pristine ecosystems. Adapted to degrading naturally occurring naphthalene, these organisms should also be able to metabolize anthropogenic

naphthalene upon introduction, even to unimpacted ecosystems. Phelps et al.[24] established that anaerobic naphthalene mineralization occurs in the subsurface environment by detecting known metabolites of the naphthalene degradation pathway in contaminated groundwater. A later study at the same contaminated site detected functional genes in addition to anaerobic naphthalene metabolites, further demonstrating the activity of anaerobic microbes in contaminated environments.[25] Laboratory studies of anaerobic naphthalene degradation are thus indicative of what occurs in the environment. In this study, two known degraders and one likely new naphthalene degrading taxon from unimpacted sediment demonstrated naphthalene biodegradation. The presence and activity of these organisms can be considered to be valuable to the health of the environment as they likely act to prevent naphthalene from accumulating, despite continual, ubiquitous deposition.

Inocula Water System	Range (µg/kg) Reported in Inocula Water System	Naphthalene Degradation	Oil Contamination?
Barnegat Bay, NJ[26]	Σ_{18}PAH = 37-1,696	Enriched in this study	-
Mullica River, NJ[27]	Σ_{25}PAH = 436-1,380	Least disturbed river in North East USA	-
Arthur Kill, NY/NJ[3,28]	Σ_{26}PAH = 3,192-11,484	1st anaerobic naphthalene degradation enrichment	+
North Sea, Germany/Denmark[5,29]	Σ_{22}PAH = 849-3,769	NaphS3	+
Etang de Berre, France[4,30]	Σ_{16}PAH = 1,595-3,359	NaphS2	+

Table 1. Total measured PAHs in Barnegat Bay sediment compared with Arthur Kill, North Sea, and Etang de Berre sediment, inoculum locations of previous anaerobic naphthalene degradation enrichments.

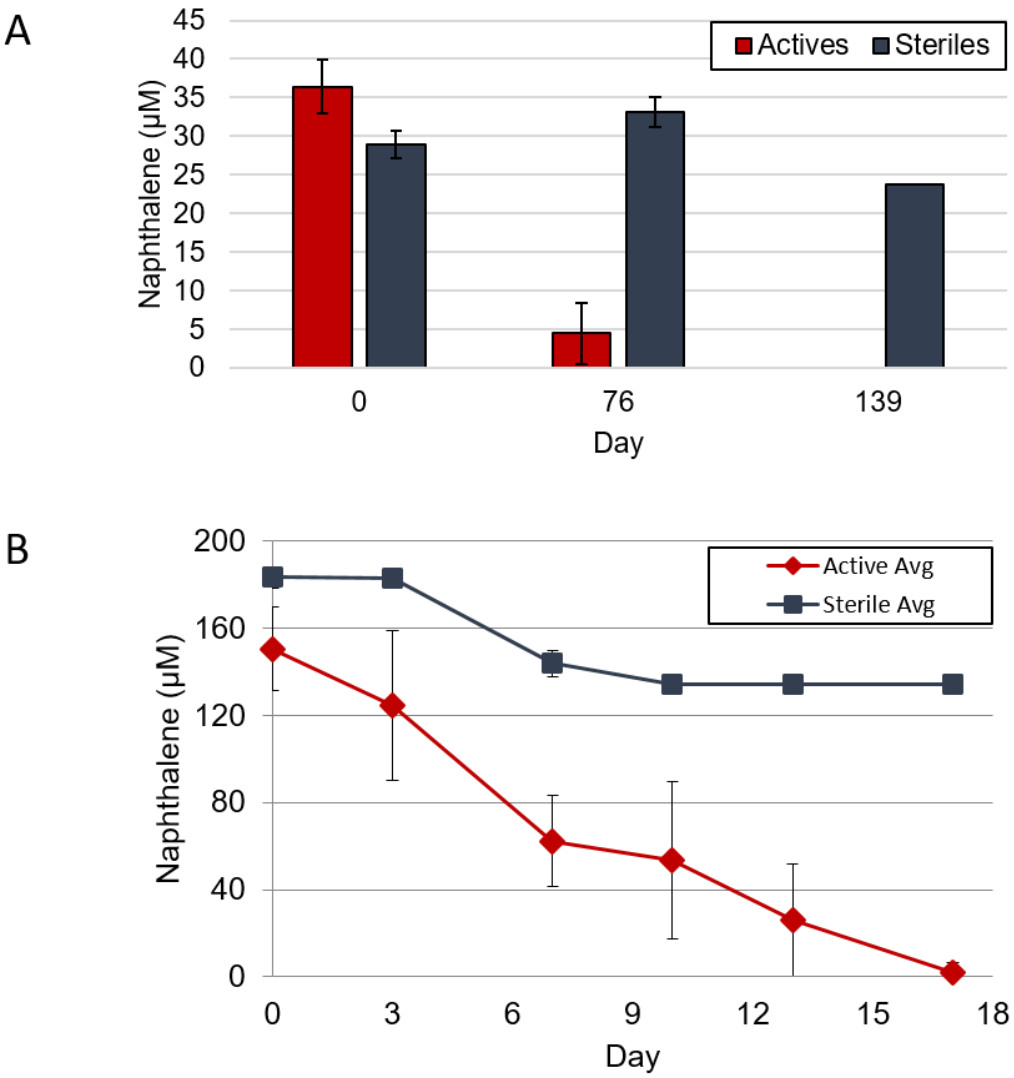


Figure 1. Average Naphthalene Loss in Anaerobic Cultures vs Sterile Controls. A. Primary Transfer Cultures, naphthalene sorbed to glass, triplicate actives and duplicate sterile controls. B. Enriched Cultures, naphthalene sorbed to silica, quadruple actives and duplicate sterile controls. (♦) Active Cultures (■) Sterile Controls. Error bars represent standard deviation between replicate cultures. Error bars are present for all data points, if they are not visible they are within the marker size.

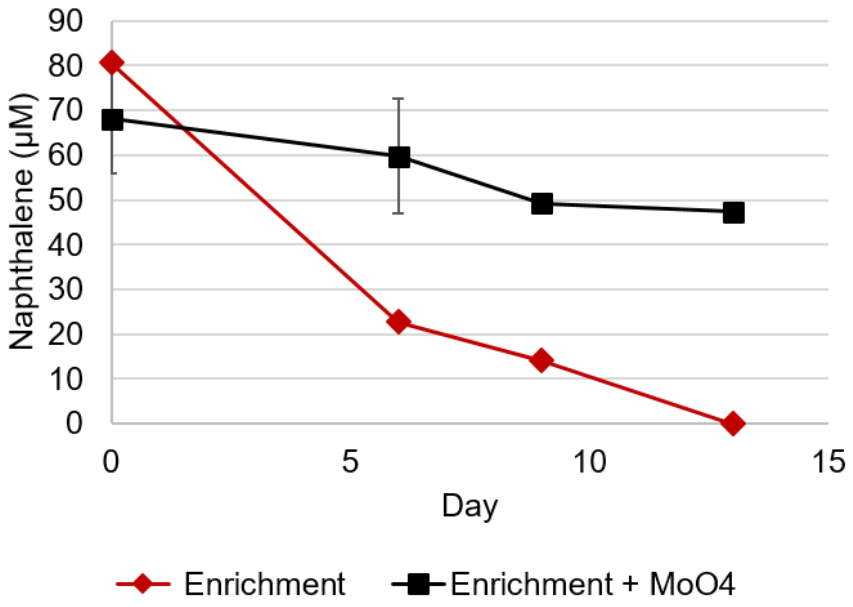
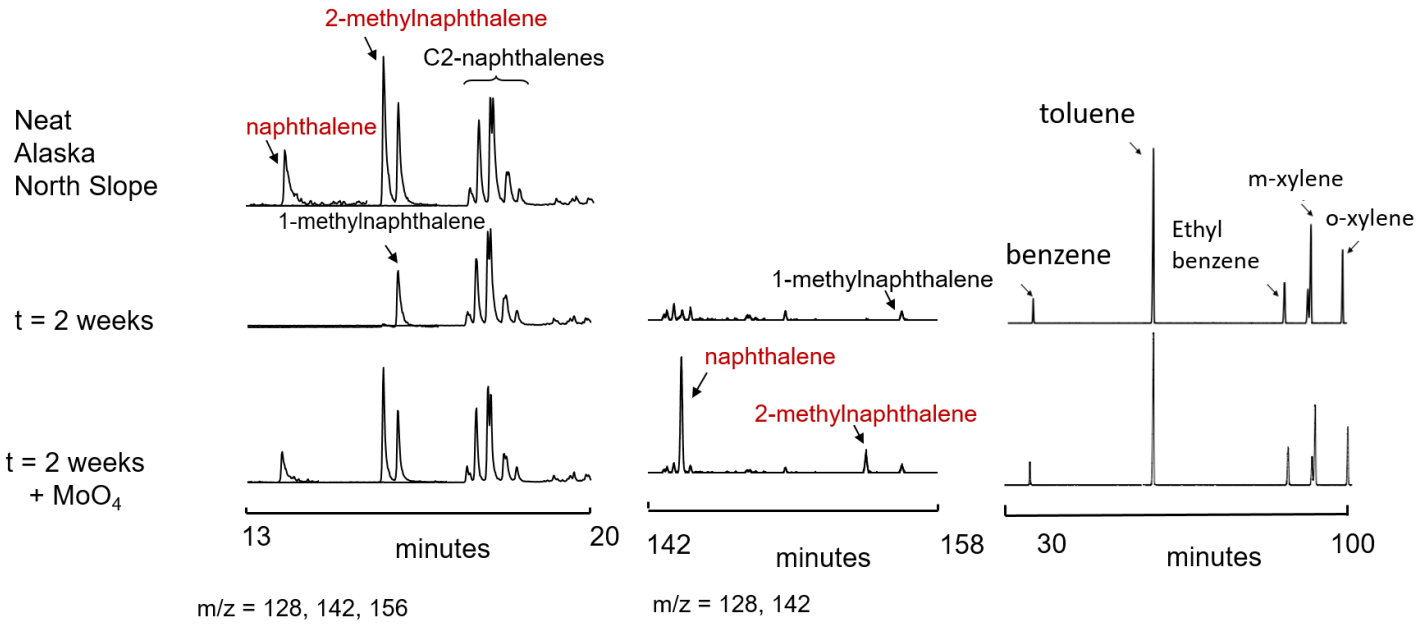


Figure 2. Naphthalene loss with and without molybdate amendment. (♦) Enrichment Cultures (■) Enrichment cultures with molybdate amendment. Error bars represent standard deviation between replicate cultures. Error bars are present for all data points, if they are not visible they are within the marker size.

	Predicted SO_4^{2-} Loss (mM)	Observed SO_4^{2-} Loss (mM)
Active Cultures	3.0	3.0 (±1.2)
Sterile Controls	0	0 (±0)
Stoichiometry	$C_{10}H_8 + 6H_2SO_4 \rightarrow 10CO_2 + 6H_2S + 4H_2O$	

Table 2. Sulfate loss during naphthalene degradation. Cultures were amended with 0.5mM naphthalene in triplicate active cultures and sterile controls. Stoichiometry of complete naphthalene degradation illustrated.



A. Alaska North Slope

B. Alba naphthalenes and monoaromatics

Figure 3. Selective naphthalene degradation in A. Alaska North Slope and B. Alba crude oils. Top row, neat substrate. Middle row, 2 week incubation. Bottom row, 2 week incubation of cultures amended with molybdate to inhibit respiration. Naphthalene and 2-methylnaphthalene are indicated in red. Unmetabolized aromatics are indicated in black. The m/z of 182, 142, and 156 are combined.

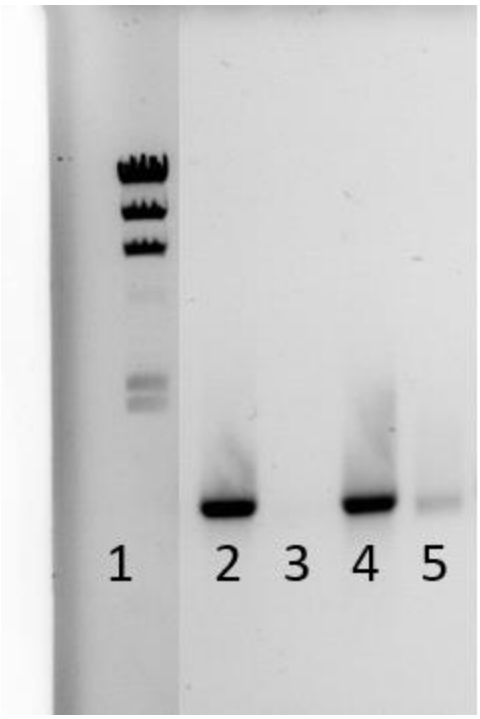
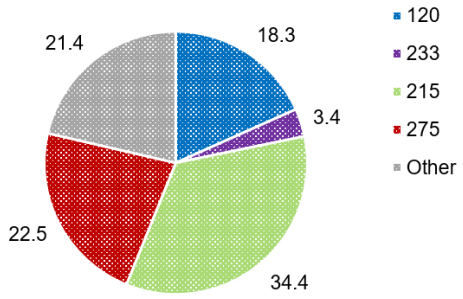


Figure 4. Agarose gel of bacterial 16S gene amplicons from either ^{12}Cnaphthalene control or ^{13}Cnaphthalene SIP incubation. 1) lambda DNA; 2) ^{12}C naphthalene control ^{12}C DNA 3) ^{12}C naphthalene control ^{13}C DNA; 4) SIP incubation day 7 ^{12}C DNA; 5) SIP incubation day 7 ^{13}C DNA

A



B

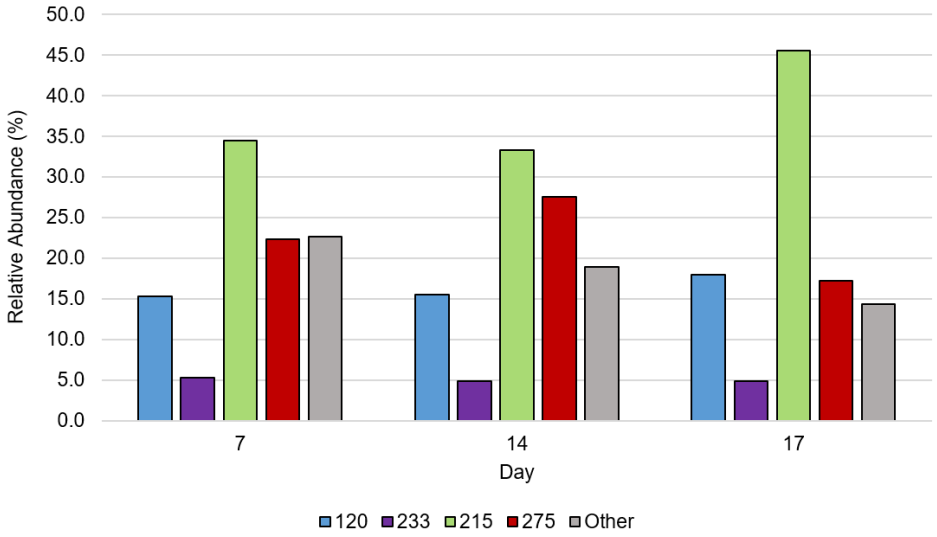


Figure 5. Community profile of naphthalene degrading enrichment culture. A. Relative abundance of identified TRF (120, 233, 215, and 275) and all other combined unidentified peaks before incubation with 13C naphthalene. B. Relative abundance of each identified TRF incorporating 13C during SIP incubation on days 7, 14, and 17.

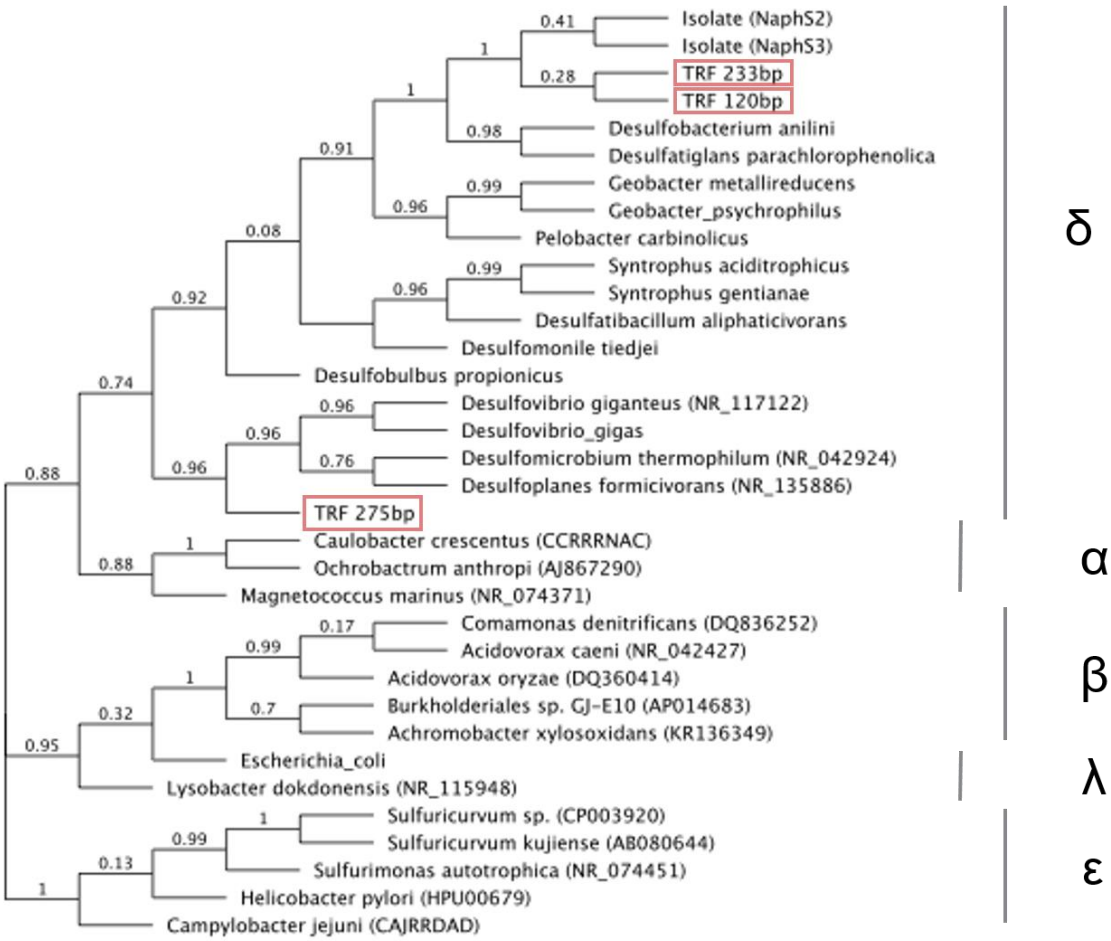


Figure 6. Figure Phylogeny of TRFs 120, 233, and 275. Partial sequence of the 16S rDNA gene of clones with sequences demonstrated to incorporate labeled naphthalene are reported. TRFs of these clones are indicated in red outline. Their closest matches in Genbank are included. Phylogenetic tree was constructed with MEGA 7 using neighbor joining. 600bp unambiguously aligned sequences were used for analysis. Bootstrap values are reported on brackets.

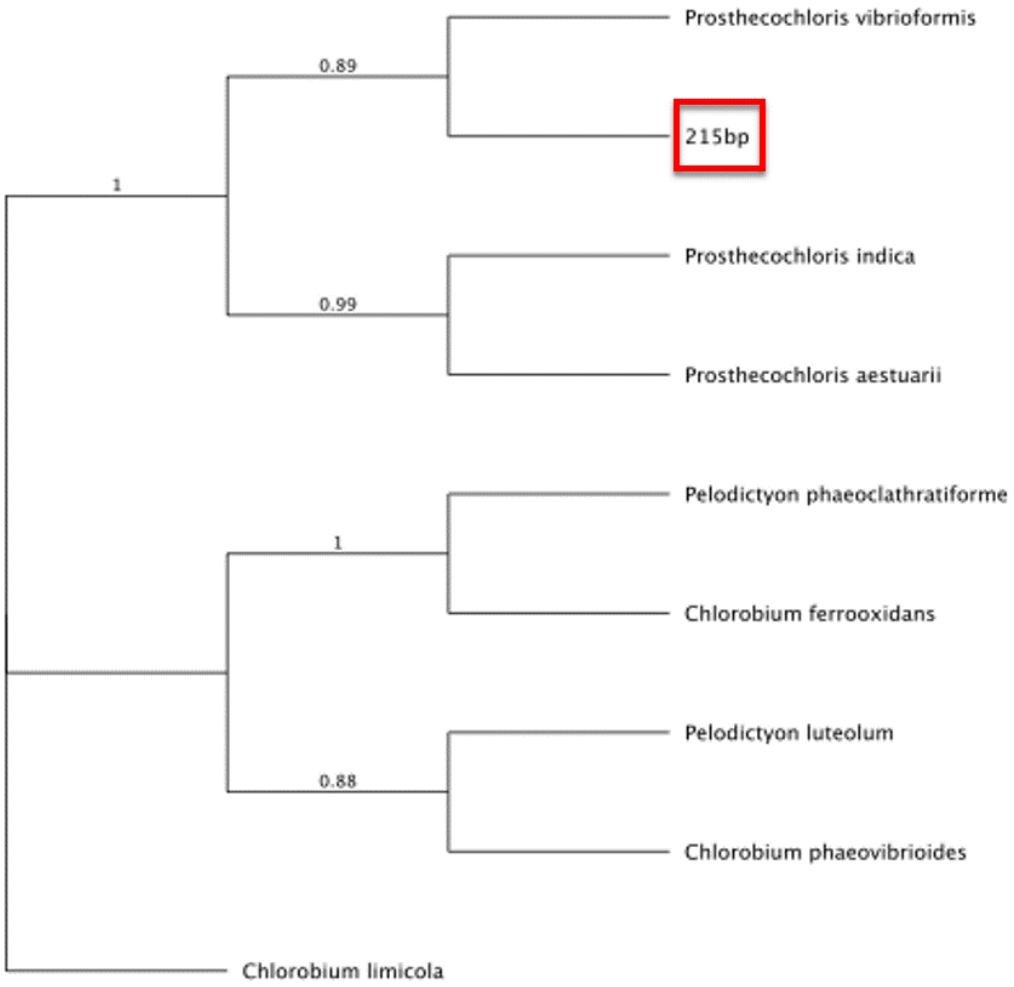


Figure 7. Figure Phylogeny of TRF 215. Partial sequence of the 16S rDNA gene of clones with sequences demonstrated to incorporate labeled naphthalene are reported. The closest matches in Genbank are included. Phylogenetic tree was constructed with MEGA 7 using neighbor joining. 770bp unambiguously aligned sequences were used for analysis. Bootstrap values are reported on brackets.

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Chapter 3

Naproxen is Transformed via Acetogenesis and Syntrophic Acetate Oxidation in a Methanogenic Wastewater Consortium

Abstract

Pharmaceutical compounds, now recognized as environmental contaminants, are potential carbon sources for microbes. Naproxen is a common nonsteroidal anti-inflammatory drug and has been detected in wastewater treatment influent, treated effluent, and environmental waters. In this work, the community ecology and biochemistry of naproxen demethylation was investigated in an anaerobic wastewater community. Enrichment cultures were established with anaerobic wastewater sludge inoculum and provided naproxen as the sole carbon source. Primary enrichment cultures transformed 1mM naproxen to 6-O-desmethylnaproxen (DMN) in 22 days. Continual enrichment, naproxen addition, and culture transfer resulted in consistent transformation of naproxen with no loss of DMN observed. Methane was also generated at 0.083mM per 1mM naproxen transformed. In addition to naproxen, the consortium readily demethylated syringic acid and vanillic acid. DNA analysis revealed a community of acetogenic bacteria and syntrophic acetate oxidizing archaea. Combined with the chemical data, this suggests the enriched consortium performs aromatic O-demethylation by a tight syntrophy between acetogenic bacteria and syntrophic acetate oxidizers. The

proposed model of carbon transfer through the anaerobic wastewater food web elucidates community level metabolism of aromatic O-methyl contaminants.

This work was coauthored with Dr. Lily Young, Dr. Abigail Porter, and Julia Campbell.

3.1 Introduction

Naproxen is a non-steroidal anti-inflammatory drug that is consistently among the most abundant pharmaceuticals detected in wastewater treatment effluent and environmental surface waters.[1-3] Incomplete removal of pharmaceuticals during wastewater treatment allows these emerging contaminants to enter the environment through a number of avenues, including treated effluent outflow, reclaimed water irrigation, and land application of sludge.[4] While it is water soluble at low concentrations, naproxen also sorbs to organic matter during wastewater treatment and in soils.[5,6] Such complex chemical interactions result in unanticipated ecosystem effects due to environmental transport. For example, naproxen can accumulate in plant tissue[4] and in the bile of fish inhabiting rivers downstream of wastewater treatment plants.[3]

Several studies have investigated microbial transformation of naproxen during biological wastewater treatment. In aerobic laboratory enrichments from wastewater sludge, bacteria only partially demethylated 10µM naproxen to 6-O-desmethylnaproxen (DMN) under co-metabolic conditions.[7] Anaerobic demethylation of 0.43µM naproxen to DMN was also seen in wastewater under methanogenic conditions after 161 days.[8] The metabolite was detected in streams receiving treated wastewater effluent.[2] The extent to which DMN is further degraded and its ecosystem impacts are as of this time unknown.

Naproxen contains an ether linked methyl group to the aromatic ring, structurally similar to naturally occurring plant methoxyaromatic compounds. Bacteria are capable of demethylating such compounds by acetogenesis, a strictly anaerobic process in which microbes conserve energy through the transfer of a methyl group to form acetate.[9] This study investigates the community ecology and biochemistry of a methanogenic naproxen-

transforming consortium enriched from anaerobic wastewater treatment. We report that demethylation of naproxen to methane is mediated by a commensal relationship between 3 groups of anaerobic microbes – acetogens that mediate the demethylation, along with acetate oxidizers that are in a tight syntrophic relationship with methanogens.

3.2 Methods

3.2.1 Anaerobic Toxicity Assay

An anaerobic toxicity assay (ATA)[10] was carried out in 60mL serum bottles using anaerobic sludge inocula from the Rockland County Sewage Treatment Plant (Rockland, NY) obtained 24 hours prior to culturing. 35mL cultures received 20% vol/vol inoculum in methanogenic media under a 25mL 30% CO_2 : 70% N_2 atmosphere. Five sets of cultures were established: 1) unamended controls, 2) active controls, 3&4) 2 sets of cultures amended with naproxen (1mM or 0.5mM), and 5) one set amended with 6-O-desmethylnaproxen (1mM). Each culture set consisted of triplicate active and duplicate autoclaved sterile controls. Unamended controls received seed inocula and defined methanogenic media to determine total gas and methane produced from background carbon present in the anaerobic sludge. Active controls received inocula, media, and the addition of 18.3mM acetate + 5.5mM propionate. Amended cultures received inocula, media, acetate+propionate, and either 0.5mM naproxen, 1mM naproxen, or 1mM DMN. DMN was synthesized according to Andersen and Hansen 1992[11] and confirmed using GC-MS (TQ8040, Shimadzu, Columbia, MD).

3.2.2 Enrichment Cultures

Anaerobic sludge used as inoculum for methanogenic cultures was obtained from the anaerobic digester of the municipal wastewater facility Joint Meeting of Union and Essex

Counties (Elizabeth, NJ). 100mL slurries of 20mL anaerobic sludge and 80mL defined methanogenic media were incubated in 160mL serum bottles under a 30%CO_2 : 70%N_2 atmosphere.[12] Bottles were sealed with butyl rubber stoppers and aluminum crimps. Enrichments received 1mM naproxen (Sigma-Aldrich, St. Louis, MO) as the sole carbon source. To maximize surface area for microbial access, a stock of naproxen sorbed to silica was prepared. Naproxen was dissolved in methanol and added to dry sterile silica at 1mmol naproxen per gram silica. The methanol carrier was then evaporated off under nitrogen gas. Enrichment cultures were established in triplicate, with duplicate sterile and background controls. Sterile controls were autoclaved three times on three successive days. Background cultures received defined media and inoculum but no added carbon substrate. All cultures were incubated in the dark at 35°C. Enrichments were reamended when naproxen concentrations were no longer detected, and transferred into 50% fresh media every 4th feeding. After five transfers, the concentration of vitamins and B12 was doubled and the enrichment culture is referred to as NPX consortium.

3.2.3 Inhibition of Methanogenesis

2-Bromoethane sulfate (BES, Sigma-Aldrich, St. Louis, MO) was added to 50% subcultures at 10mM to inhibit methanogenesis.[13] Naproxen was amended to a final concentration of 1.2mM.

3.2.4 Substrate Diversity

Enriched subcultures were supplied with methoxy-aromatic plant phenolic compounds to assess the diversity of aromatic o-demethylation. Syringic acid and vanillic acid (Sigma-Aldrich, St. Louis, MO) were amended to enrichment cultures at 0.5mM and monitored for substrate loss. To determine whether the enriched consortium could further degrade

the naproxen metabolite, 1mM 6-O-desmethyl naproxen was provided as the sole carbon source to 50% subcultures.

3.2.5 Analytical Procedures

Aqueous samples were taken periodically to monitor substrate loss and metabolite formation. Samples were extracted with an equal volume of methanol for 15 minutes and filtered through a 0.22µm Spin-X nylon filter (Corning Costar, Corning, NY) then analyzed by HPLC equipped with a UV-VIS detector (Shimadzu, Columbia, MD) and a C-18 reverse phase column (Phenomenex, Torrance, CA). Aromatic analytes were detected at 280nm. Naproxen and DMN were detected using a mobile phase of 70% methanol : 28% water : 2% glacial acetic acid at a flow rate of 1mL min-1. Syringic acid and vanillic acid were detected with a mobile phase consisting of 64% 5mM formic acid in water : 30% methanol : 6% acetonitrile at a flow rate of 1mL min-1. All solvents were HPLC-grade and purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Total gas volume was measured using a degassed glass syringe fitted with an 18 gauge needle as described previously.[10] Headspace gas composition was measured by injecting a sample of 0.5mL headspace gas using a Pressure-Lok gas-tight syringe into a Fischer Hamilton 1200 gas partitioner with a 3.35m x 4.76mm column packed with 80/100 mesh PoraPak Q (Supelco, Bellefonte, PA). CH_4 production at each time point was calculated by the percent CH_4 measured and headspace volume, with the residual headspace methane from the last time point subtracted.

Gibbs free energies at standard temperature and pressure were estimated according to ΔGf^o values from aromatic O-demethylation reactions.[15, 16] To confirm these values,

Gibbs free energies at T=298.1K and P=1atm were obtained using electronic structure calculations by Gaussian 09 computer programs as described by Laviska et al. 2014.[17]

3.2.6 MiSeq Amplicon Sequencing

Genomic DNA was extracted from the NPX consortium after several transfers that occurred over 1.5 years following the establishment of the primary cultures. Background cultures were sampled and DNA extracted on the same day. MoBio PowerSoil DNA Isolation kit was used (MoBio Laboratories, CA, USA). 16S rRNA was PCR amplified using bacterial 27F(5'-AGRGTTTGATCMTGGCTCAG-3')/530R (5'-CCGCNGCNGCTGGCAC-3') and archaeal 344F(5'-ACGGGGYGCAGCAGGCGCGA-3')/915R primers with barcode on the forward primer. MrDNA (Shallowater, TX, USA) performed MiSeq Illumina sequencing following manufacturer's guidelines. Sequences were processed using MrDNA pipeline, in which sequences were joined. Barcodes, sequences <150bp, and sequences with ambiguous base calls were removed.[14] Data was denoised and chimera removed. Operational Taxonomic Units (OTUs) were generated by clustering at 97% similarity and classified using BLASTn against the RDPII and NCBI databases.

3.3 Results

3.3.1 Anaerobic Toxicity Assay

The anaerobic toxicity assay is a method to evaluate the initial toxicity of a compound to methanogenesis and the potential of a methanogenic community to acclimate to the compound.[10, 18] Acetate and propionate supplied in known amounts serve as carbon substrates for fermentation and methanogenesis. Suppression of total gas (CO_2 + CH_4) and methane produced in cultures amended with the test compound relative to the active

control indicate toxicity of the test compound to the methanogenic community. After normalizing to unamended controls, the difference in gas produced with test compounds vs. active controls is displayed as a ratio (Table 1). Total Gas Ratios of <0.9 indicate inhibition to the overall community, and Methane Ratios of <1.0 indicate inhibition specifically to methanogens.[18] The gas ratios at day 2 are useful in determining acute impacts of target compounds, while the end ratio indicates the ability of the community to acclimate to the test compounds.

As summarized in Table 1, naproxen inhibited total gas during the active gas production phase at day 2, resulting in a maximum biogas ratio of 0.82 at 0.5mM and 0.77 at 1mM. Following day 2, total gas production was within one standard deviation of uncertainty of active controls and thus was not significantly inhibited. Naproxen did not suppress methane production. The methane ratio at day 2 and day 30 for both concentrations of naproxen was 1, reflecting the equal volumes of methane in naproxen amended ATA assays and active controls. In the ATA, carbon dioxide is produced by both fermenters and methanogens, while methane is exclusively produced by methanogens. The observed decrease in CO_2, concurrent with no decrease in methane, implies fermenters are suppressed, not the methanogens. Naproxen appears to be toxic to a portion of the acetate-propionate fermenting bacterial community while exerting no observed toxicity to methanogens.

Also shown in Table 1, ATA assays amended with DMN did not substantially inhibit total gas production, as seen in total gas ratio of 1.01 at day 2 and 0.92 at day 30. DMN did, however, suppress methanogenesis. The Maximum Methane Ratio for DMN of 0.59 and End Methane Ratio of 0.84 indicate acute initial suppression of methanogenesis

followed by slight acclimation by day 30. The rate of methane generation is seen in Figure S1, where methane production in DMN amended bottles is clearly inhibited at day 2, recovers by day 5, and plateaus by day 12. After plateauing, the accumulated volume of methane in DMN amended bottles remains lower than active controls.

Naproxen was transformed to DMN during the ATA assay by day 30 (data not shown). This did not suppress methane production, however, because by the time naproxen transformation to DMN was complete most of the total gas and methane had already been generated (Figure S1). Methane production rate in all cultures substantially slowed following day 12, indicating that acetate and propionate were almost exhausted. By that time only very low concentrations of DMN would be present. The presence of DMN at later timepoints therefore did not influence methane production from acetate and propionate.

3.3.2 Enrichments

Complete naproxen loss was observed within 22 days in primary enrichment cultures (Figure 1). Only active cultures demonstrated loss; no decrease in naproxen concentration was observed in sterilized controls. As naproxen decreased a metabolite appeared and increased with incubation time. The metabolite was identified as 6-O-desmethylnaproxen by GC/MS. This is the same intermediate metabolite observed under aerobic and anaerobic naproxen transformation reported in previous studies.[7,8]

Every three weeks cultures were amended with naproxen and 50% subsequently transferred into fresh media. The resulting sediment-free cultures are referred to as NPX consortium. NPX consortium consistently demethylated 4mM naproxen within 7 days with concomitant formation of DMN (data not shown). The transformation product

remained throughout the course of the experiment, that is, no further degradation of DMN was observed. In addition, the concentration of DMN increased with each naproxen amendment; only diluting with fresh media during culture transfer decreased DMN concentrations. Despite DMN accumulation, metabolism and loss of amended naproxen continued. This suggests that the transformation product is not toxic to the enriched NPX community.

To determine whether NPX consortium can further degrade DMN in the absence of naproxen, subcultures amended with DMN as the sole carbon source were monitored for DMN loss. The metabolite did not decrease in concentration over a one year incubation, thus the NPX consortium is unable to further metabolize DMN (data not shown).

In addition to the substrate and metabolite concentrations, methane generation was also monitored in NPX cultures. Transformation of 1mM naproxen resulted in the generation of 0.083mM methane (Figure 2). The net volume of methane from naproxen addition was determined by subtracting methane produced in background cultures from that produced in actively transforming NPX consortium. In this manner, we ensured only naproxen-derived CH_4 was included. After determining the net volume of methane, the gas law was used to convert volume to moles CH_4 at T=298K and P=1atm.

3.3.3 Inhibition of Methanogenesis

Because the NPX consortium consists of both fermentative heterotrophs and methanogenic archaea, the role of the methanogens was investigated. Complete inhibition of methane generation was achieved by adding BES, a specific inhibitor of methanogenesis.[13] As shown in Figure 2, naproxen demethylation proceeded at a slower rate in the presence of BES. Following the first 5 days of incubation, transformation

slowed in BES treated cultures relative to NPX. All 1.2mM naproxen was demethylated within 11 days in NPX consortium, while 0.4mM remained in BES amended cultures. By 17 days, 0.35mM naproxen, (30%) remained in BES amended cultures. Though transformation slowed, it did not completely stop. This suggests that methanogens are not directly responsible for naproxen demethylation, but perform a function that supports the transformation. In the NPX consortium, naproxen is demethylated by bacteria and the production of methane is a result of downstream carbon transfer of that methyl group through the community.

3.3.4 Methoxy-Aromatic Substrate Diversity

Subcultured NPX consortia were fed natural plant phenols to evaluate its ability to transform naturally occurring methoxyaromatic compounds. Syringic and vanillic acids, each containing a methyl group ether linked to the aromatic ring like naproxen, were readily demethylated (Figure 3). Both compounds were demethylated by NPX consortium within 14 days. Vanillic acid was demethylated to protocatechuic acid. Each of the two methyl groups of syringic acid were removed, forming gallic acid. These demethylated metabolites were not further degraded by the NPX consortium, just as DMN is not further degraded following naproxen demethylation. Anaerobic bacteria are known to demethylate vanillic and syringic acids by acetogenesis, a process in which microbes conserve energy by cleaving the ether bond and utilizing the methyl group to synthesize acetate. Acetogens, the polyphyletic group of bacteria responsible for acetogenesis, are unable to cleave the aromatic ring following demethylation.[25]

3.3.5 Microbial Community Analysis

Figure 4 summarizes the family-level microbial community composition of the NPX consortium after 78 weeks of enrichment and culture transfers, compared to that of the background control at the same time point. The bars display the percent of total sequences of families in NPX consortium (blue bars) and background (green bars). Bacterial families enriched in the naproxen consortium contain acetogens, including *Eubacteriaceae* (34%), *Thermoanaerobacteraceae* (30%), *Acidaminococcaceae* (8%), and *Spirochaetaceae* (5%) (Figure 4A).[19-22] Together, these acetogenic families comprise 77% of total NPX consortium sequences, while background controls contained lower abundances and more families. The only non-acetogen enriched in NPX consortium, *Bacteriodaceae* (5%), employ fermentative metabolism.[23] *Eubacteriaceae* and *Thermoanaerobacteriaceae* enrichment is noteworthy because they contain members known to acetogenically demethylate methoxyaromatic plant phenolics. This includes syringic acid and vanillic acid, which are also demethylated by the NPX consortium.[19] Together, these two taxa comprise 64% of NPX consortium. *Thermoanaerobacteraceae* is not detected in background controls and *Eubacteriaceae* accounts for only 3% of background sequences.

In Figure 4B, the methanogenic diversity of NPX consortium (blue bars) and background (green bars) is displayed. *Methanomicrobiaceae*, the only archaea enriched in NPX consortium, accounts for 53% of archaeal sequences. These organisms generate methane from H_2 and CO_2 but, notably, not directly from acetate.[24] In contrast, *Methanomicrobiaceae* is completely absent in background controls. The addition of naproxen therefore provides a source of H_2 that supports *Methanomicrobiaceae* in NPX consortium. *Methanobacteriaceae* and *Methanosaetaceae*, acetoclastic archaea capable

of transforming acetate to methane, were only half as abundant in NPX consortium (24% and 14%) compared to background controls (55% and 30%). These observations indicate that the dominant methanogens in the NPX consortium are unable to directly utilize acetate. Since the dominant bacteria are acetogens, as shown in Figure 4A, the expectation was that the methanogens in the NPX consortium would be acetoclastic. Given that the methanogens enriched in the NPX consortium are not acetate utilizers, there must be an additional step between acetogenesis and methanogenesis.

3.4 Discussion

The NPX consortium, enriched from an anaerobic digester community, rapidly demethylated naproxen. Complete naproxen transformation to DMN occurred within 22 days in primary enrichment cultures, even at 1mM concentration. This is substantially higher than the likely concentration encountered during wastewater treatment and yet transformation still occurred within the solids retention time of anaerobic digestion.[1,26] Demethylation also occurred at a faster rate than previously reported in naproxen transforming wastewater cultures.[7,8] Naproxen demethylation can therefore be expected to occur under fermentative and methanogenic conditions during wastewater treatment, resulting in the presence of both residual naproxen and DMN in treated wastewater and sludge biosolids.

In the ATA, naproxen decreased initial biogas production by the anaerobic microbial community without affecting methane generation. In contrast, DMN inhibited methanogens but not total gas production. Interestingly, DMN does not inhibit overall biogas production, indicating that DMN is not inhibitory to fermenters. Instead, the demethylated naproxen metabolite is inhibitory specifically to methanogens. Contrasting

patterns of microbial suppression illustrate the unforeseen effects of microbial pharmaceutical transformation.

Despite the dominance of acetogenic bacteria in the NPX consortium, the dominant methanogens in the NPX cultures are not known to generate methane directly from acetate. We propose that an intermediate metabolic step is thus necessary to link acetogenic naproxen demethylation to methanogenesis. To understand the interactions of this microbial community, we propose that two tightly linked metabolic strategies are at play, namely acetogenesis and syntrophic acetate oxidation. First, the acetogens demethylate naproxen to DMN and form acetate. By cleaving off the ether-linked methyl group of naproxen, acetogens supply the carbon that supports this methanogenic naproxen metabolizing community. This energetically favorable reaction involves the uptake of one mole of H_2 and forms one mole of acetate per mole of naproxen (Figure 5, Reaction 1). The low H_2 requirement allows the acetogens to thrive in an environment in which no H_2 was supplemented. Acetate generated by naproxen demethylation is then converted to methane via syntrophic acetate oxidation. Living in a tight syntrophy, acetate oxidizing bacteria couple with methanogens to produce energy.[27] These bacteria generate $4H_2$ per mole acetate oxidized (Figure 5, Reaction 2). Though this requires the input of energy, it provides the H_2 needed by methanogens to make methane and generate energy (Figure 5, Reaction 3). [27,28] In support of these interactions, *Methanomicrobiaceae*, the dominant archaeal family in NPX consortium seen in Figure 4B, is a member of the order *Methanomicrobiales,* which is found in syntrophic acetate oxidation.[29] Only a few bacterial syntrophs have been described, one being a member of *Thermoanaerobacteriaceae,* which is also dominant in the NPX consortium (Figure

4A).[30] To generate methane under low environmental H_2 conditions, *Methanomicrobiaceae* utilize the H_2 produced by their acetate oxidizing syntrophs. No H_2 was added to NPX consortium, so the hydrogenotrophic methanogens must obtain H_2 from acetate oxidizing syntrophs and by doing so maintain low H_2 partial pressure. Syntrophic acetate oxidation is thermodynamically favorable only under low H_2 partial pressure, precisely the conditions in the NPX consortium.[31] The acetogenic/syntrophic conversion of naproxen to methane is described and figuratively illustrated in Figure 5. Methane is produced at 0.083mM per 1mM naproxen transformed. If 100% of the -CH_3 removed from naproxen was metabolized to methane, 1mM CH_4 would be expected. Instead, the –CH_3 removed from naproxen is transferred through three trophic levels before release as CH_4. The initial step in this syntrophy, acetogenesis, proceeds at an observed 22-39% theoretical acetate yield during acetogenesis of C1 compounds in H_2-limited mixed cultures.[32-35] Only 0.22-0.39mM acetate is therefore predicted from acetogenic demethylation of 1mM naproxen. In the subsequent proposed steps, syntrophic acetate oxidation, both bacteria and archaea require carbon for biomass. Additionally, unaffiliated microbes "along for the ride" will sequester acetate, H_2, and CH_4 throughout the incubation. The -CH_3 removed from naproxen is the only source of carbon for heterotrophs in NPX consortium, and acetate oxidation is the only source of H_2. Any loss of substrate along the syntrophic process will decrease methane production, as that is the final step. The low CH_4 yield from naproxen carbon reflects these sequential transformations and demonstrates how –CH_3 from aromatic O-demethylation may be expected to move through a low-H_2 anaerobic food chain. The low methane yield is thus

consistent with the proposed transfer of the methyl group of naproxen from acetate to methane in this consortium.

Naproxen is not a naturally occurring compound. It is likely and reasonable, however, that acetogens are able to access the methyl group because of its structural similarity to naturally occurring compounds. Methyl tert-butyl ether (MTBE), a prevalent xenobiotic contaminant, is similarly demethylated under anaerobic conditions.[36] Notably, though MTBE is a branched methoxylated alkane, its demethylation is stimulated by methoxyaromatic plant phenolics, which are also demethylated in this study. The microbial imperative to find a carbon source drives this anaerobic community to demethylate methoxylated compounds, whether xenobiotic or natural. This also suggests that the transformation of other methoxyaromatic emerging contaminants is likely to take place. As demonstrated by this study, the transformation of naproxen to DMN is somewhat inhibitory to methanogenesis, a critical process mediated by the wastewater microbiome and anaerobic ecosystems. The opposing patterns of inhibition by naproxen and its demethylated metabolite illustrate the importance of understanding impacts of contaminants and their microbial metabolites. As a consequence, it is insufficient to only study the loss of parent compounds. To understand the effects of a xenobiotic compound on an engineered or a natural ecosystem, we also need to consider activities and effects of the products of microbial transformation. The model proposed in this study demonstrates the complex and coupled interactions of the anaerobic microbial communities that generate methane. This can have both positive and negative implications for understanding waste treatment and ecosystem sustainability, as well as carbon turnover in the biosphere and greenhouse gas production.

3.5 Acknowledgements

This project was supported by the USDA National Institute of Food and Agriculture Hatch Multistate project 1007899 through the New Jersey Agricultural Experiment Station, HatchMultistate NJ07212. Sarah Wolfson was supported by a US National Science Foundation Fuels IGERT (NSF DGE 0903675) from Rutgers University. The authors gratefully acknowledge Tom Villani and the labs of Dr. James Simon and Dr. Alan Goldman for analysis support.

	Total Gas Ratio Day 2	Total Gas Ratio Day 30	Methane Ratio Day 2	Methane Ratio Day 30
Naproxen 1mM	0.77§	0.87ǂ	1.00	1.07
Naproxen 0.5mM	0.82§	0.84ǂ	1.04	1.03
DMN 1mM	1.01	0.92	0.59§	0.84§
Total Gas Ratio	$\frac{\text{test compound ATA headspace} - \text{unamended control headspace}}{\text{active control headspace} - \text{unamended control headspace}}$			< 0.9, inhibited
Methane Ratio	$\frac{\text{test compound ATA methane} - \text{unamended control methane}}{\text{active control methane} - \text{unamended control methane}}$			<1.0, inhibited

Table 1. Total gas (CO_2 + CH_4) and methane ratios at day 2 (acute inhibition) and day 30 (acclimated inhibition). Equations display how the ratios were calculated.

§ Inhibition and significant difference between test compound and active control

ǂ Difference between test compound and active control is within one standard deviation of uncertainty; not significantly different

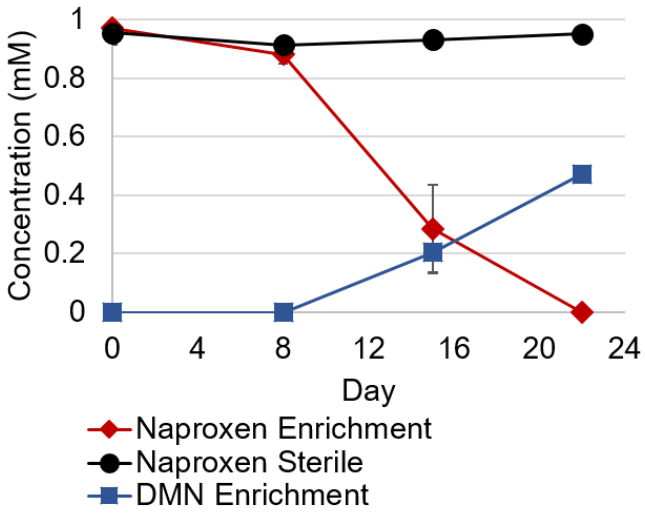
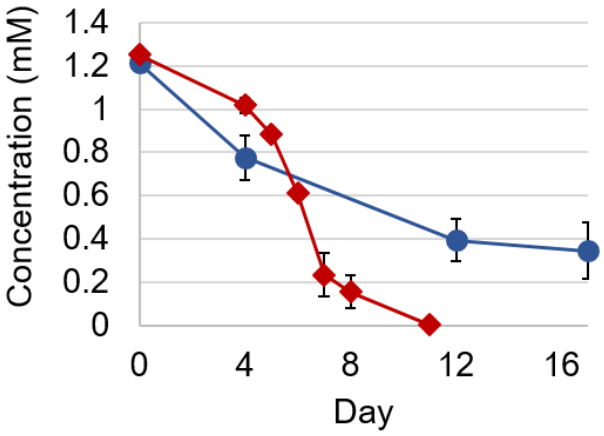


Figure 1. Naproxen loss and DMN appearance in primary enrichment cultures. (♦) Naproxen in primary enrichments; (●) Naproxen in steriles; (■) DMN in primary enrichments. Error bars are included for all data points, if they are not visible they are within the size of the symbol.



Condition	CH_4 (mM) per 1mM naproxen
NPX	0.083 (±0.025)
NPX + BES	0.0 (± 0)

Figure 2. Naproxen loss and methane generation in NPX consortium. (♦) NPX consortium; (●) NPX consortium with 10mM BES amendment. Error bars are present for all data points. Inset includes the methane generated (mM) by the NPX consortium with and without BES. Methane was measured after 1mM naproxen loss in NPX cultures and at day 17 in BES cultures.

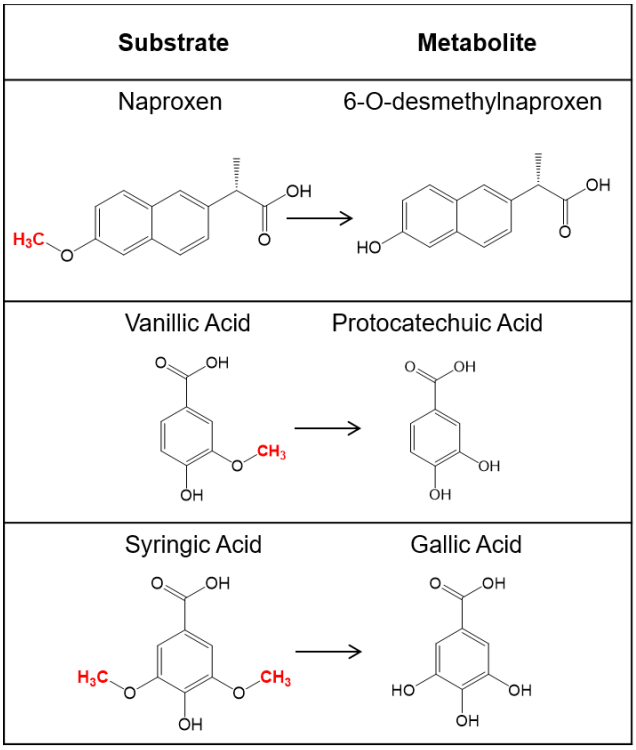


Figure 3. NPX Consortium Substrate Diversity. Left column is substrate (methyl group in red) and right column is the demethylated metabolite recovered following incubation. The removal of the ether linked methyl group results in a hydroxylated aromatic compound.

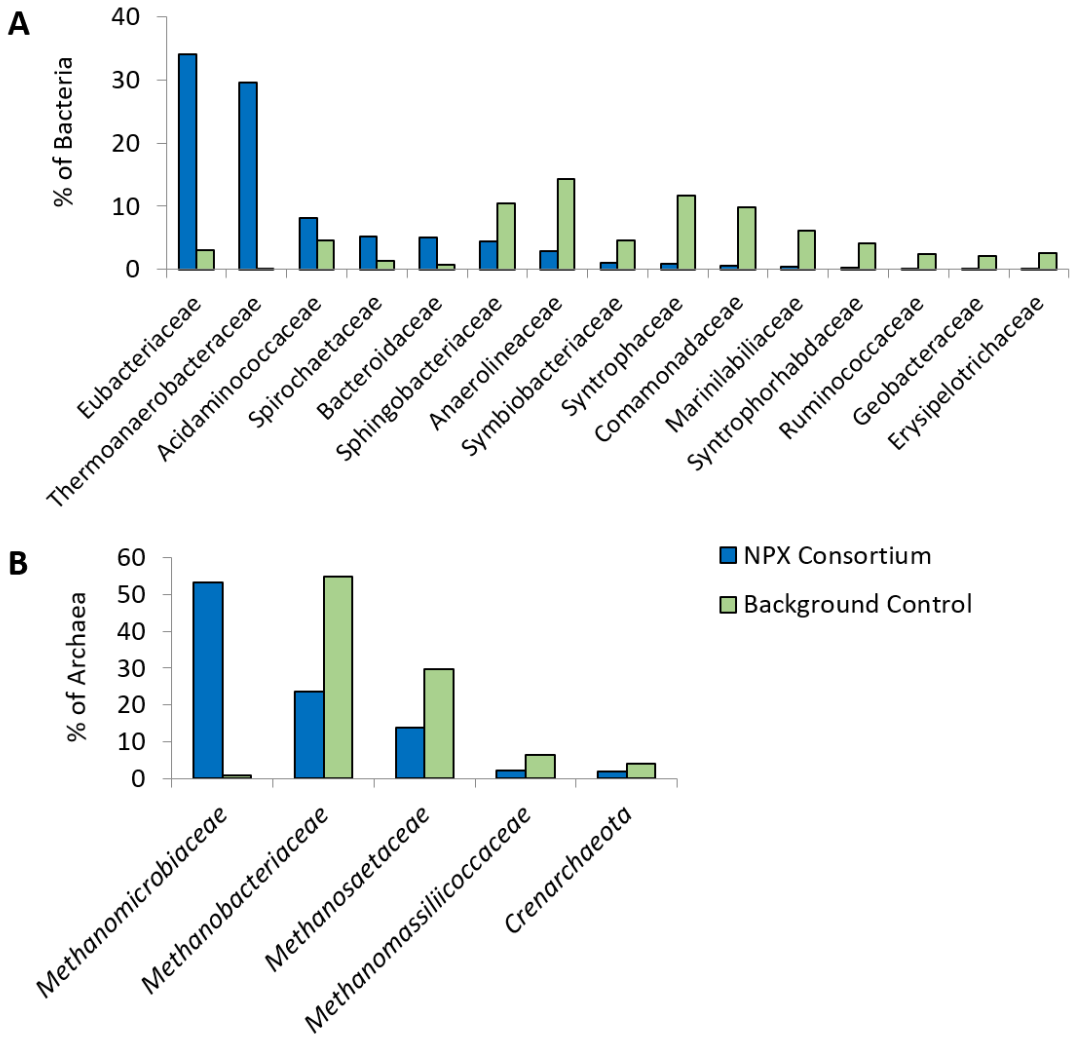


Figure 4. 16S rDNA family level community composition of NPX consortium and background control. Phylogenetic distribution is displayed as percent of total sequences in (A) Bacteria and (B) Archaea. Blue bars, NPX consortium; green bars, background control.

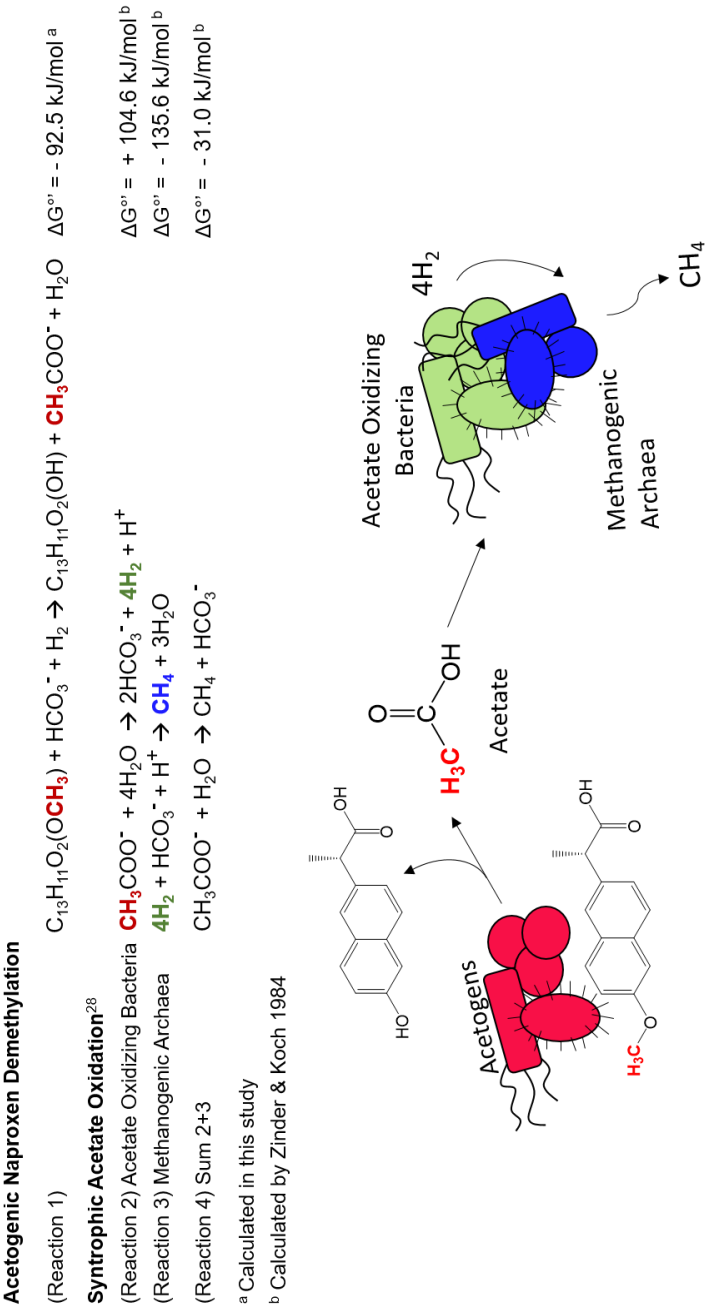


Figure 5. Naproxen metabolism by the NPX consortium. Naproxen demethylation by acetogens (Reaction 1), acetate oxidation by acetate oxidizing bacteria (Reaction 2), methanogenesis by syntrophic methanogenic archaea (Reaction 3). Sum of 2 and 3 (Reaction 4) is the net stoichiometry and kJ/mol of syntrophic acetate oxidation, calculated by Zinder & Koch 1984. Acetogens (red), acetate oxidizing bacteria (green), methanogens (blue).

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Chapter 4

Demethylation of the Antihistamine Diphenhydramine by Anaerobic Microbial Communities from Wastewater Treatment

Abstract

While emerging pharmaceutical contaminants are monitored in wastewater treatment and the environment, there is little information concerning their microbial metabolites. The transformation of diphenhydramine by microorganisms in anaerobic digester sludge was investigated using anaerobic cultures amended with 1mM diphenhydramine as the sole carbon source. Complete transformation of the parent compound to a persistent metabolite occurred within 191 days. Using GC/MS analysis, the metabolite was identified as N-desmethyl diphenhydramine. There was no observed decrease in metabolite concentration even after a further 120 days of incubation, suggesting that the metabolite resists further degradation during wastewater treatment. An anaerobic toxicity assay demonstrated that diphenhydramine has an inhibitory effect on both fermentative bacteria and methanogenic archaea in the wastewater community. The metabolite N-desmethyl diphenhydramine partially suppressed methanogens but did not impact the fermenting community. To our knowledge, this is the first report of diphenhydramine metabolism by a bacterial community. The limited transformation of diphenhydramine by wastewater microorganisms indicates that N-desmethyl diphenhydramine will enter the environment along with unmetabolized diphenhydramine.

This chapter was coauthored with Dr. Lily Young, Dr. Abigail Porter, Tom S. Villani, and Dr. James Simon and has been submitted to *Water Research*.

4.1. Introduction

Diphenhydramine is an extensively used over the counter antihistamine, non-habit forming sleep aid and anti-nausea medication. As such, large quantities are consumed, and excreted, by humans. The liver partially metabolizes diphenhydramine through N-demethylation, N-glucuronidation, and acetic acid conjugation, while 2-15% is excreted unchanged [1]. The fungi *Cunninghamella elegans* was shown to transform 74% of amended diphenhydramine to the same metabolites in humans under aerobic conditions [2]. N-desmethyl diphenhydramine is a pharmaceutically active metabolite, retaining some antihistaminic activity [3,4]. Despite these transformations, intact diphenhydramine is prevalent in wastewater influent and effluent, and commonly detected in surface waters [5]. Diphenhydramine is also present in the bodies of fish inhabiting streams that receive wastewater effluent [6,7]. Once in the environment, the pharmaceutical can pose a threat to aquatic ecosystems. Diphenhydramine has been demonstrated to suppress microbial growth in pure culture [8,9]. Diphenhydramine is also introduced to terrestrial environments through reclaimed water irrigation and biosolids fertilization of agricultural fields [10]. After introduction to soils, diphenhydramine can persist for months to years [5,10,11].

Municipal wastewater treatment was found to remove only 60% of the pharmaceutical, leading to wide dispersion in the environment [12,13]. Removal during wastewater treatment, however, does not mean that diphenhydramine is completely mineralized to CO_2. In addition to the detected diphenhydramine in the environment, it is possible that human and microbial metabolites of the compound are also present in aquatic systems, as occurs with nicotine, caffeine, digoxin, and other pharmaceutical products [14]. Microbial metabolites of pharmaceutical compounds comprise an important portion of emerging

contaminants in the environment. For example, in a survey of US streams, microbial metabolites of detergents account for almost 40% of total recovered contaminants (Kolpin et al., 2002). There is limited information concerning the microbial transformation of many emerging contaminants, including diphenhydramine. If diphenhydramine is not fully degraded, then its transformation products remaining after wastewater treatment also will be released to the environment with unknown effects.

This study investigates the ability of an anaerobic wastewater community to metabolize diphenhydramine. Biological wastewater treatment culminates with anaerobic digestion of sewage sludge, the longest solids retention time during wastewater treatment [15]. This relatively long retention time provides an opportunity for anaerobic microbes to metabolize diphenhydramine in wastewater. We report that the demethylation of diphenhydramine to N-desmethyl diphenhydramine is mediated by anaerobic sludge microbes and discuss its impact on the composition of the anaerobic community. To our knowledge this is the first report of diphenhydramine transformation by anaerobic microbes.

4.2. Methods

4.2.1 Wastewater Inocula Sources

Methanogenic bioactivity assays were established using anaerobic sludge from a digester of the Joint Meeting of Union and Essex Counties, Elizabeth, NJ. Inoculum for the anaerobic toxicity assay was obtained from a digester of the Rockland County Sewage Treatment Facility, Rockland, NY.

4.2.2 Bioactivity Assay

Anaerobic sludge was incubated at 35°C for 24 hours prior to culture establishment. A sludge slurry was established when 20% (vol/vol) sludge was added to defined methanogenic media and dispensed in 100mL aliquots under a 60mL 30% CO_2 : 70% N_2 headspace [16]. Bottles were sealed with butyl rubber stoppers and aluminum crimps. Enrichments received 1mM diphenhydramine (Sigma-Aldrich, St. Louis, MO) as the sole carbon source. Triplicate enrichment cultures were established with duplicate autoclaved sterile controls and duplicate background controls, which received no diphenhydramine.

Liquid samples from the bioactivity assay were taken periodically for HPLC analysis. Samples were extracted with equal volume methanol and filtered through a 0.22µm Spin-prep nylon filter. Diphenhydramine concentrations and intermediate metabolites were monitored on a Shimadzu LC-10AS HPLC equipped with a Phenomenex 5µm C-18 reverse phase column (Phenomenex, Torrance, CA) and UV-VIS detector. Aromatic analytes were detected at 254nm using a mobile phase of 70% methanol : 30% 20mM Na_2HPO_4 (pH 7) at a flow rate of 1mL/min.

The intermediate metabolite recovered in the bioactivity assay was identified using GC/MS. Samples were extracted using a ratio of 20:1 ethyl acetate:liquid culture. Extracts were dried under nitrogen using slight heat (40°C) for a prolonged time to ensure complete removal of water. The dried extracts were derivatized by addition of 200µL BSTFA/TMS-Cl in pyridine and heating the sealed vial to 75°C for 4 hours. N-desmethyl diphenhydramine analytical standard prepared in methanogenic media was extracted using the same procedure as described above. After cooling to room temperature, 1.0µL of derivatized extracts was injected into a Shimadzu TQ-1090 GC/MS with AOC6000 autosampler and the separation was performed on a RP-5SilMS

column using a 1:10 split ratio for injection. The injector was set to 280°C. The temperature program was: 80°C initial temperature, held for 1 minute, ramped linearly to 220°C at a rate of 10°C/min, held at 220°C for 3.5 minutes, and then linearly ramped to 310°C at a rate of 20°C/min, then held 15 minutes at 310°C.

4.2.3 Anaerobic Toxicity Assay (ATA)

The ATA [17] was carried out in 60mL serum bottles using anaerobic sludge inoculum obtained 24 hours prior to culturing. A sludge slurry was created as described above, except slurry volumes were 35mL under a 25mL 30% CO_2 : 70% N_2 atmosphere. Five sets of cultures were established: unamended controls, active controls, two sets of cultures amended with diphenhydramine, and one set spiked with N-desmethyl diphenhydramine. Unamended controls received inoculum and defined media to determine gas production from background carbon present in the sludge inoculum. Active controls received inoculum, media, and the addition of 18mM acetate and 5.5mM propionate. Diphenhydramine spiked assays received inoculum, media, acetate and propionate, and either 0.5mM or 1mM diphenhydramine. N-desmethyl diphenhydramine assays were established with inoculum, media, acetate and propionate, 14mL defined media, and 14mL filter-sterilized spent media from a diphenhydramine-transforming biodegradation assay for a final N-desmethyl diphenhydramine concentration of 0.1mM. No diphenhydramine was detected in the spent media.

Total biogas volume was measured using a degassed glass syringe fitted with an 18 gauge needle as described previously [17]. Headspace gas composition was measured using a Fischer Hamilton 1200 gas partitioner with a 3.35m x 4.76mm column packed with 80/100 mesh PoraPak Q (Supelco). 0.5mL headspace gas was injected using a Pressure-

Lok gas-tight syringe. CH_4 production was calculated by the percent CH_4 measured and headspace volume.

4.2.4 MiSeq Amplicon Sequencing

A MoBio PowerSoil DNA Isolation kit was used to extract genomic DNA from an actively transforming replicate and an unamended background control (MoBio Laboratories, CA, USA). Bacterial 16S rDNA was PCR amplified using 27F(5'-AGRGTTTGATCMTGGCTCAG-3')/530R(5'-CCGCNGCNGCTGGCAC-3') primers with barcode on the forward primer. MiSeq Illumina sequencing was performed by MrDNA (Shallowater, TX, USA) according to manufacturer's guidelines. Sequences were processed by the MrDNA pipeline. Sequences were joined. Barcodes, sequences less than 150bp, and those with ambiguous base calls were removed [18]. Data was denoised and chimera were removed. Operational Taxonomic Units were generated by clustering at 97% similarity and classified using BLASTn against the RDPII and NCBI databases.

4.3. Results and Discussion

4.3.1 Anaerobic Transformation

Anaerobic wastewater bioactivity assays showed diphenhydramine loss within 191 days (Figure 1). Though demethylation occurred slowly, the diphenhydramine concentration decreased by 20% in the first 29 days and continued to steadily decrease. HPLC analysis revealed the appearance of a transformation product beginning at day 94. The metabolite detected by HPLC was identified using GC/MS (Figure 2). The mass spectrum of the metabolite (Figure 2A) was consistent with the mass spectrum of known standard N-desmethyl diphenhydramine (Figure 2B), showing characteristic fragment ions at 183

m/z, corresponding to a loss of $CH_3NHCH_2CH_2$- fragment, and at 167 m/z, corresponding to the loss of $CH_3NHCH_2CH_2O$- fragment. N-desmethyl diphenhydramine was not detectable by HPLC until day 94, when it reached a concentration of 0.1mM. Following complete diphenhydramine disappearance, the demethylated metabolite accumulated to a concentration of 0.9mM. N-desmethyl diphenhydramine did not decrease in concentration at any point, even when incubations continued for an additional 120 days after diphenhydramine was consumed (data not shown). The near stoichiometric relationship with the parent compound indicates that all diphenhydramine was transformed via demethylation, and no secondary degradation pathways occurred.

There was no observed lag time, indicating anaerobic sludge contains microbes capable of demethylating diphenhydramine. Anaerobic sludge is continuously exposed to pharmaceutical compounds, so the indigenous sludge microbes have likely already been exposed to diphenhydramine [12]. Partial removal of diphenhydramine during wastewater treatment reported by Du. et al (2014) may be demethylation by anaerobic microorganisms. As demonstrated in this bioactivity assay, N-desmethyl diphenhydramine will accumulate in methanogenic environments, where it may exert adverse effects to organisms in anaerobic environments.

4.3.2 Anaerobic Toxicity Assay

The ATA evaluates the acute toxicity of a chemical to a heterotrophic methanogenic community (at day 2) and the potential of a methanogenic community to acclimate to the chemical (by day 30) [17,19]. Carbon substrates in the form of acetate and propionate are supplied in excess. Fermenting bacteria consume both substrates, producing CO_2. Methanogens, able to utilize acetate but not propionate, produce CO_2 and CH_4. A

decrease in total gas and methane generated by cultures amended with the test compounds relative to active controls indicates toxicity. The total gas produced at day 2, which includes both CO_2 and CH_4, determines the initial, acute impact of the test compound, while the end ratio at day 30 indicates the ability of the microbial community to acclimate to diphenhydramine or N-desmethyl diphenhydramine. Total gas ratios of <0.9 indicate inhibition to the overall community, while methane ratios of <1.0 indicate inhibition specifically to methanogens.

Diphenhydramine amended cultures showed decreased gas production and methane production throughout the duration of the ATA (Figure 3 A-B). Total gas and methane production were lower in diphenhydramine assays than the active controls at day 2, indicating a period of acute toxicity. Both total gas and methane production then rebounded between days 5 and 9, accumulating to volumes nearly equal to the active control. After this brief burst of activity, total gas and methane production lagged behind active controls for the remainder of the experiment. Diphenhydramine-exposed assays produced lower volumes of total gas and methane by the end of the assay, revealing the inhibitory effect diphenhydramine continued to exert on the acclimated microbial community. As shown in Table 1, the gas ratios reflect the total gas and methane suppression. In the first two days of incubation, the 0.5mM diphenhydramine treatment achieved a total gas ratio of 0.74 and methane ratio of 0.85. In the same time period, the 1mM diphenhydramine treatment exerted a greater inhibitory effect, with a total gas ratio of 0.47 and methane ratio of 0.49. By day 30, 0.5mM diphenhydramine cultures had a total gas ratio of 0.72 and methane ratio of 0.87, while 1mM incubations had a total gas ratio of 0.66 and methane ratio of 0.74. As seen in figure 3A, cumulative methane

produced by 1mM diphenhydramine cultures was less than that produced by 0.5mM cultures. There was no difference in cumulative total gas production between 1mM and 0.5mM diphenhydramine assays, as seen in figure 3B. Notably HPLC analysis indicated that diphenhydramine was not demethylated during the ATA (data not shown). This confirms that the methane and CO_2 generated during the ATA was derived from acetate and propionate metabolism, not diphenhydramine demethylation. Further, all inhibitory effects in the diphenhydramine ATA were from the parent compound, as there was no N-desmethyl diphenhydramine formed.

Diphenhydramine inhibited both total gas and methane production, exhibiting an effect on bacteria that ferment acetate and propionate and methanogens that utilize the acetate and fermentation products. At 1mM, inhibition was strongest at the onset of gas production. Following the acutely toxic stage, microbes were able to slightly acclimate to the presence of the xenobiotic, as demonstrated by the greater methane and gas ratios at day 30 compared with day 2 (Table 1). At 0.5mM, diphenhydramine exerted an inhibitory effect on both total gas and methane with little difference between acute and acclimated inhibition ratios. Throughout the ATA, 1mM diphenhydramine cultures produced less methane than the 0.5mM cultures, suggesting that methanogens are more sensitive to the higher concentration. There was no difference between the two concentrations in total gas production, suggesting that the fermentative community was equally suppressed at high and low concentrations.

A slightly different pattern was observed for the metabolite N-desmethyl diphenhydramine (Figure 4 A-B). Initial methane production was acutely inhibited at day 2, with a methane ratio of 0.57 (Table 1). Following this period of acute inhibition,

methane production increased, though it only achieved a methane ratio of 0.85 at day 30. Thus, after a period of acute toxicity, methanogens acclimated in the presence of N-desmethyl diphenhydramine but never recovered full methane production relative to active controls. Figure 4B also illustrates that at 0.1mM N-desmethyl diphenhydramine exerted no effect on total gas production. The volume of total gas produced in N-desmethyl diphenhydramine assays was equivalent to that in active controls at the acute impact stage as well as the cumulative end stage. Though less methane was produced, total gas volume was maintained because the fermenters utilized the portion of acetate not consumed by methanogens. The combination of decreased methane with equal volumes total gas demonstrates that fermenting bacteria were able to utilize propionate as well as remaining acetate, generating CO_2 and compensating for the lower volume of methane. With methanogens suppressed, the fermenting bacteria, unaffected by the dead-end metabolite, fermented the available acetate and propionate to CO_2.

The ATA of N-desmethyl diphenhydramine is, to our knowledge, the first report of the metabolite's toxicity to methanogens. Like the parent compound, N-desmethyl diphenhydramine maintains antihistaminic activity [3,4]. The ATA results suggest that the antimicrobial effects seen with diphenhydramine may remain in its demethylated metabolite.

Diphenhydramine and N-desmethyl diphenhydramine did not completely inhibit microbial activity. Microbes were still able to metabolize acetate and propionate into CO_2 and CH_4 in the presence of the compounds, though in lower amounts. Thus, while diphenhydramine and N-desmethyl diphenhydramine exert an overall inhibitory effect on

the microbial community, resilient members of the anaerobic sludge community could still thrive in the presence of high concentrations of these xenobiotic compounds.

4.3.3 Bacterial Diversity

16S rDNA bacterial community diversity in actively transforming diphenhydramine assays had notable differences from unamended background controls. The microbial families present in relative abundances greater than 1% of total sequences are displayed in Figure 5. Four families were present in greatly increased abundance relative to unamended background controls, including *Comamonadaceae, Symbiobacteriaceae, Anaerolineaceae,* and *Prevotellaceae*. *Comamonadaceae* are 3-fold more abundant in diphenhydramine cultures compared to the background. They are prevalent denitrifiers in activated sludge and are known to degrade complex organic compounds, such as bioplastics and aniline [20,21]. *Symbiobacteriaceae,* twice as abundant in diphenhydramine the bioactivity assay, is a newly reported family known to reduce nitrate [22]. *Anaerolineaceae* comprised almost twice as much total sequence in the diphenhydramine bioactivity assay compared to background. This family is frequently detected in diverse anaerobic hydrocarbon impacted sediments and have been found to be dominant in methanogenic alkane degrading communities [23]. *Anaerolineaceae* was also shown to perform aniline degradation under methanogenic conditions [24]. *Prevotellaceae* is abundant in human feces and rumen fluid [25]. Two families of bacteria, *Sphingobacteriaceae* and *Aquifaceae* were prevalent in unamended background controls yet were detected at only 0.2% and 0.02% of sequences in diphenhydramine transforming assays (included in “Other”, Figure 5). *Sphingobacteriaceae* account for 13% of the

background controls and *Aquifaceae* make up 19%. The loss of 32% of sequences indicates a direct impact of diphenhydramine on these community members.

Shifts in community structure reveal the impact of diphenhydramine demethylation on a community. Bacterial community shifts in response to pharmaceuticals have been demonstrated in wastewater sludge [26,27]. Changes in relative abundance of microbes in diphenhydramine bioactivity assays compared to background controls can be attributed to differing tolerance of and sensitivity to diphenhydramine and N-desmethyl diphenhydramine, as has been seen during polycyclic aromatic hydrocarbon contamination [28]. As organisms compete under organic contaminant stressors, tolerant taxa proliferated while sensitive groups decreased in relative abundance. Changes in the community profile can also reflect the enrichment of actively N-demethylating organisms. *Comamonadaceae, Symbiobacteriaceae, Anaerolineaceae,* and *Prevotellaceae* increased in the presence of diphenhydramine demethylation to N-desmethyl diphenhydramine, demonstrating tolerance to these chemical stressors. Two families abundant during diphenhydramine transformation, *Comamonadaceae* and *Anaerolineaceae,* contain known aniline degraders [20,24]. Aniline and diphenhydramine share the chemical features of an aromatic ring and an amine group, though aniline consists of a phenyl ring directly attached to the amine. *Anaerolineaceae* is also dominant in hydrocarbon degrading studies, indicating that its members can metabolize a range of aromatic organic contaminants. As a consequence, they are potential candidates able to metabolize diphenhydramine. Anaerobic N-demethylation, however, is poorly characterized, so it remains unclear which enriched taxa are responsible for diphenhydramine transformation. Though the 16S rDNA data suggests which organisms

are able to proliferate in the presence of diphenhydramine and its demethylated metabolite, it is insufficient to identify microorganisms responsible for the N-demethylation.

The absence of *Aquifaceae* and *Sphingobacteriaceae,* in contrast, indicates that these organisms cannot compete in a diphenhydramine transforming culture. This may be a result of sensitivity to diphenhydramine or N-desmethyl diphenhydramine. The ATA demonstrated that diphenhydramine is inhibitory to a subset of the bacterial community. Loss of two prevalent phylotypes provides further evidence of its toxicity. It is also possible that *Aquifaceae* and *Sphingobacteriaceae* are outcompeted by other taxa able to tolerate diphenhydramine and N-desmethyl diphenhydramine. Enriched organisms, tolerant to or metabolizing diphenhydramine, are likely to sequester nutrients more successfully and at the expense of those not participating in diphenhydramine transformation.

4.4. Conclusions

Under typical anaerobic wastewater treatment conditions, anaerobic sludge solids are retained in the digester for 25-35 days. In this time frame diphenhydramine can be demethylated to N-desmethyl diphenhydramine, as seen in Figure 1, even at these higher concentrations. Incomplete mineralization of diphenhydramine by wastewater microorganisms, however, indicates that the metabolite N-desmethyl diphenhydramine will enter the environment along with any unmetabolized diphenhydramine. Both the parent compound and metabolite can therefore be expected to be present in treated sewage biosolids and affiliated treated water.

Reports on common emerging contaminants in environmental samples have mainly targeted intact pharmaceutical compounds. Notably missing is the identification of the products of microbial metabolism from treatment processes or even metabolism by environmental microbes. N-desmethyl diphenhydramine is a metabolite produced by humans and anaerobic microorganisms. The demethylated byproduct of diphenhydramine metabolism maintains antihistaminic activity, and as demonstrated in this study, is inhibitory to the tested microbial community. Since it appears to be resistant to further biodegradation, measuring only the parent compound presents an incomplete picture of the environmental antihistamine load contributed by diphenhydramine. Its unintended effects to nontarget organisms may extend beyond microbes. This study demonstrates the need to also measure the anaerobic sludge dead-end metabolite, N-desmethyl diphenhydramine, in the environment.

4.5 Acknowledgements

This work was supported by the USDA National Institute of Food and Agriculture Hatch Multistate project 1007899 through the New Jersey Agricultural Experiment Station NJ07212. Sarah Wolfson was supported by a US National Science Foundation Fuels IGERT (NSF DGE 0903675) from Rutgers University.

	Biogas Ratio Day 2	Biogas Ratio Day 30	Methane Ratio Day 2	Methane Ratio Day 30
Diphen 1mM	0.47	0.66	0.48	0.74
Diphen 0.5mM	0.74	0.72	0.85	0.87
N-Desmethyl Diphen 0.1mM	1.035	0.977	0.57	0.85
Total Gas Ratio	$\frac{\text{test compound ATA headspace} - \text{unamended control headspace}}{\text{active control headspace} - \text{unamended control headspace}}$			< 0.9, inhibited
Methane Ratio	$\frac{\text{test compound ATA methane} - \text{unamended control methane}}{\text{active control methane} - \text{unamended control methane}}$			<1.0, inhibited

Table 1. Anaerobic Toxicity Assay total gas and methane ratios of diphenhydramine and N-desmethyl diphenhydramine. Red indicates inhibition.

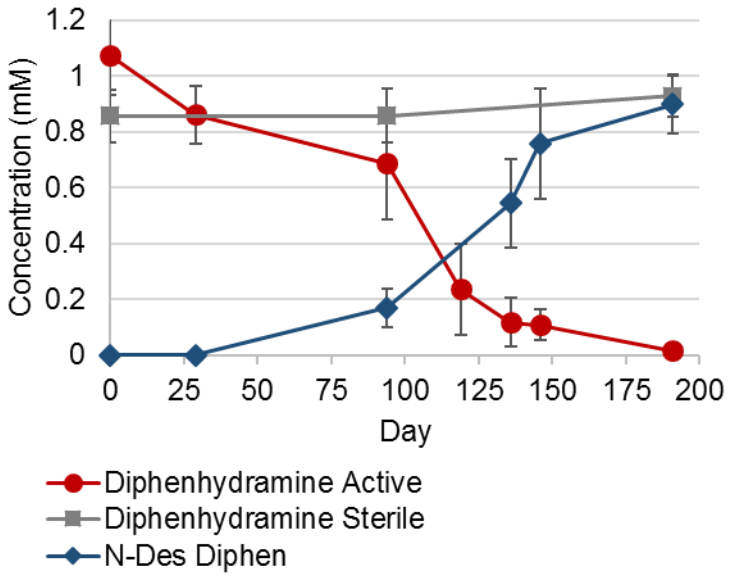

Figure 1. Transformation of diphenhydramine to N-desmethyl diphenhydramine (N-des Diphen) in Bioactivity Assays. (●) Diphenhydramine actives; (♦) N-desmethyl diphenhydramine in actives; (■) Diphenhydramine steriles.

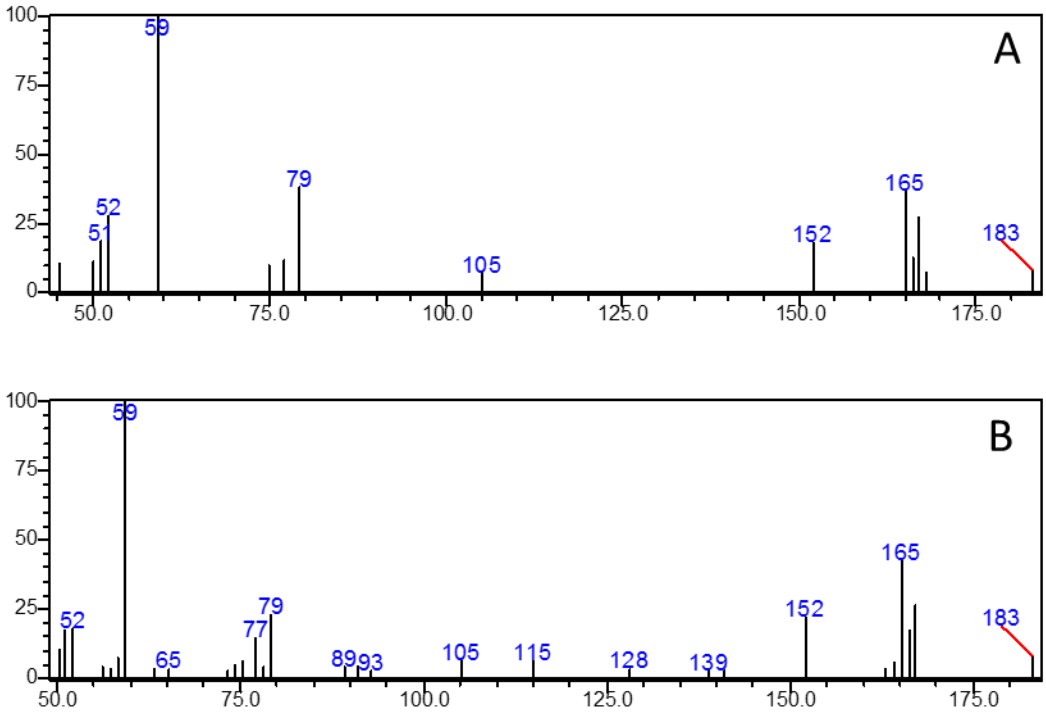

Figure 2. Mass Spectrum of the diphenhydramine metabolite compared to the analytical standard N-desmethyl diphenhydramine. A. Diphenhydramine metabolite in bioactivity assays B. N-desmethyl diphenhydramine analytical standard.

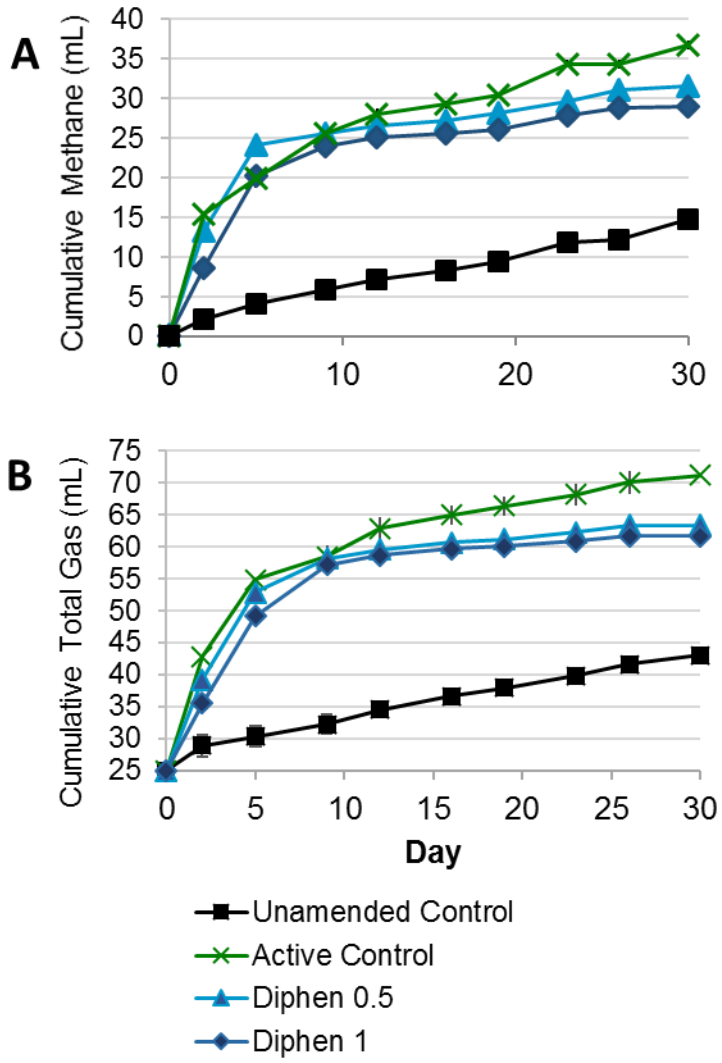

Figure 3. Anaerobic Toxicity Assay A) Diphenhydramine cumulative methane; B) Diphenhydramine cumulative total gas. (X) Active Control; (■) Unamended Control; (▲) Diphenhydramine 0.5mM; (♦) Diphenhydramine 1mM. Error bars are included for all data points. If they are not visible, they are within the size of the symbol.

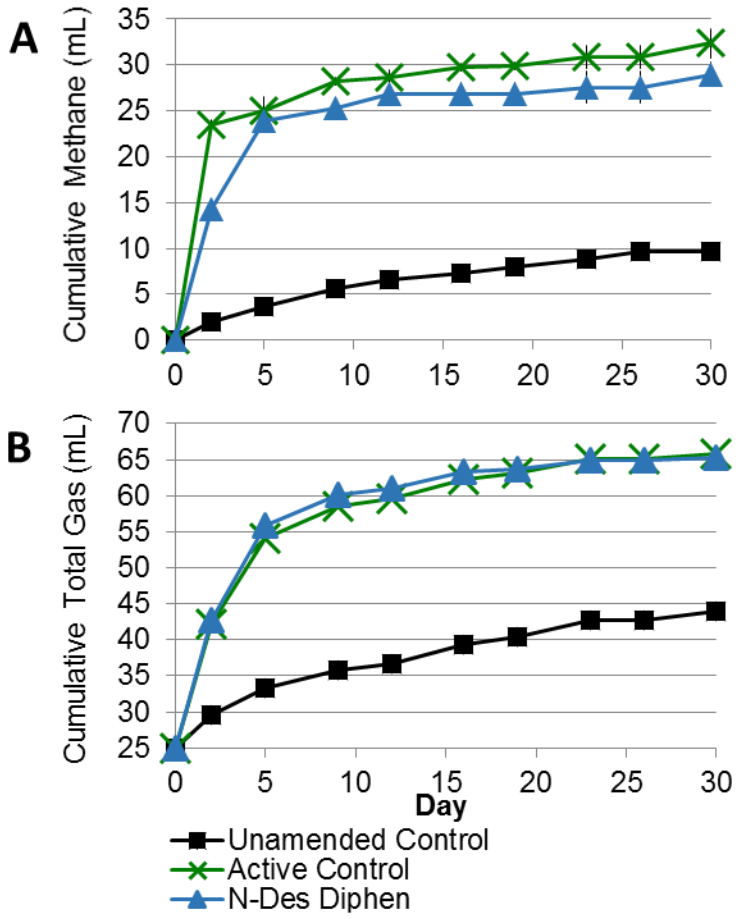

Figure 4. Anaerobic Toxicity Assay A) N-desmethyl diphenhydramine cumulative methane; B) N-desmethyl diphenhydramine cumulative total gas. (X) Active Control; (■) Unamended Control; (▲) N-desmethyl diphenhydramine, 0.1mM. Error bars are included for all data points. If they are not visible, they are within the size of the symbol.

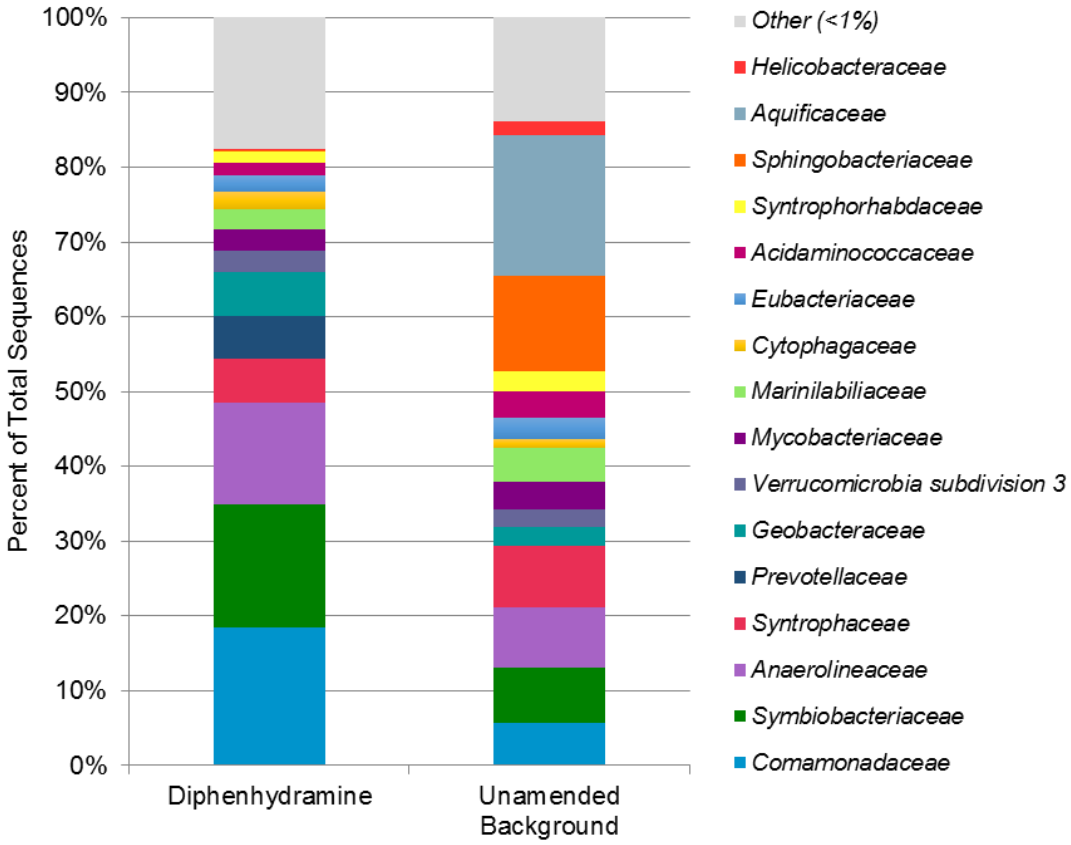

Figure 5. 16S rDNA family-level community composition of diphenhydramine biodegradation assay and unamended background control.

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Chapter 5

Anaerobic O-Demethylation of Pharmaceutical and Personal Care Products by Heterotrophic Microbial Communities

Abstract

The phenylmethyl ether chemical structure is shared by many pharmaceutical and personal care products (PPCPs). As described in Chapter 3, anaerobic wastewater microbes readily demethylate naproxen and the natural phenylmethyl ethers syringic acid and vanillic acid. A second naproxen transforming culture was enriched from anoxic marine sediment. The ability of both the NPX consortium, described in Chapter 3, and this marine culture, to demethylate a variety of phenylmethyl ether PPCPs was examined. Both cultures O-demethylated oxybenzone, guaifenesin, and methylparaben. No evidence of mineralization of the aromatic ring was observed in guaifenesin or oxybenzone demethylation. The demethylated metabolite of methylparaben, 4-hydroxybenzoate, however, did not accumulate in the NPX consortium, indicating complete mineralization of the aromatic structure to CO_2. In contrast, 4-hydroxybenzoate did accumulate in the marine culture, indicating no further metabolism. We thus propose that in anoxic environments, phenylmethyl ether contaminants will be demethylated, and the desmethyl metabolites will comprise a critical portion of total contaminants.

5.1 Introduction

Microbial metabolism of pharmaceuticals and personal care products (PPCPs) introduces metabolites to the environment in addition to the parent compounds.[1,2] These transformation products are newly recognized micropollutants, and as such have not yet

received the same attention as intact PPCPs.[3] The multitude and diversity of PPCP compounds, however, makes studying each individual transformation a monumental task. Understanding the magnitude and extent of PPCP contamination would benefit from a systematic approach to predicting their microbial transformation during wastewater treatment and in the environment. Recently, Gulde et al.[4] demonstrated this was possible in a comprehensive aerobic biotransformation predictor for aminated PPCPs.

A portion of PPCPs, however, also partition to anaerobic environments[5], which can occur in wastewater treatment as well as anaerobic sediments.[6] Once in anoxic environments, anaerobic microbes are important in transforming these emerging contaminants and altering their structure, fate, and transport[7] (Chapter 3, Chapter 4).

By altering the structure of PPCPs, microorganisms release byproducts related to the parent compound that may in many instances retain pharmaceutical activity. This phenomenon has been documented during anaerobic microbial transformation of the phenylmethyl ether venlafaxine, the active compound in the antidepressant Effexor. Microbes in both aerobic and anaerobic wastewater treatment processes O-demethylate venlafaxine to desvenlafaxine, which is itself the antidepressant pharmaceutical Pristiq[8]. Because the metabolite retains pharmaceutical activity, it may exert unforeseen effects to non-target organisms in ecosystems receiving treated wastewater[4]. Anaerobic O-demethylation of venlafaxine is the same biotransformation observed during our studies on naproxen metabolism (Chapter 3). It has been known for many years that naturally-derived phenylmethyl ethers can serve as a carbon source to anaerobic microorganisms[9], although this structural signature is frequently found in PPCPs as well. Acetogenic bacteria are able to cleave the ether linked methyl group, providing acetate to the

community and in the process produce a demethylated aromatic compound (Chapter 3). Because of the prevalence of the phenylmethyl ether structure in PPCPs, we propose that O-demethylation may be an important strategy of PPCP transformation during wastewater treatment and the environment. In this study, aromatic O-demethylation by two microbial communities was investigated. An established methanogenic culture enriched from wastewater treatment, and a sulfidic enrichment from estuarine sediment, both capable of demethylating naproxen, were tested for PPCP substrate diversity. Methanogenic wastewater is rich in nutrients and organic carbon, while anoxic marine sediment is highly sulfate-reducing and nutrient limiting. The two different inocula demonstrate phenylmethyl ether O-demethylation under contrasting anaerobic conditions. Three common phenylmethyl ether PPCPs, oxybenzone (UV absorber), methylparaben (preservative), and guaifenesin (expectorant, Mucinex®), were examined for microbial transformation. In addition to naproxen (Aleve®), the two communities also demethylated the other tested compounds. These findings are significant as the bioactivity of the demethylated byproducts produced toward nontarget organisms or to ecosystems in general remains unknown.

5.2 Methods

5.2.1 Enrichment Cultivation

Marine enrichments were established using anoxic subsurface sediment from Tuckerton, NJ USA. A 20% (vol/vol) sediment slurry was added to anaerobic marine minimal medium as described previously[10]. Naproxen was the sole added carbon source to primary enrichment cultures. 100mL of the inoculated media slurry was dispensed serum bottles and overlaid with a 70% N_2/30% CO_2 atmosphere. Naproxen was amended to

cultures at 1.5mM via coated silica grains as described previously in Chapter 3. Bottles were sealed with butyl rubber stoppers and aluminum crimps. Enrichments were established in triplicate with duplicate sterile controls, the latter were autoclaved three times on three consecutive days. All bottles were incubated statically at room temperature in the dark. Enrichments were re-amended when naproxen was no longer detected. Every fourth feeding, enrichments were transferred into 50% fresh media, resulting in a sediment-free enrichment culture.

The naproxen enriched consortium (NPX), as reported in Chapter 3, was enriched from anaerobic wastewater sludge. Briefly, an enrichment of anoxic sludge in methanogenic media was provided 1mM naproxen as the sole carbon source. Enrichments were transferred into 50% fresh media until a sediment free culture capable of rapidly transforming naproxen, known as NPX consortium, was established.

Samples were taken to monitor substrate loss and metabolite formation. Liquid samples were extracted with an equal volume of methanol and filtered through a 0.22µm Spin-X nylon filter (Corning Costar, Corning, NY). Samples were analyzed by HPLC equipped with a UV-VIS detector (Shimadzu, Columbia, MD) and a C-18 reverse phase column (Phenomenex, Torrance, CA). Naproxen and DMN (6-O-desmethylnaproxen) were detected using a mobile phase of 70% methanol : 28% water : 2% glacial acetic acid at 280nm with a flow rate of 1 ml min^{-1}.

5.2.2 16S MiSeq Amplicon Sequencing

Genomic DNA was extracted using MoBio PowerSoil DNA Isolation kit (MoBio Laboratories, CA, USA). Bacterial 16S rDNA PCR amplification and sesquencing was performed by MrDNA (Shallowater, TX) using 27F/530R primers with barcode on the forward primer. MiSeq Ilumina sequencing was completed according to manufacturer's guidelines. Sequences were processed using the MrDNA pipeline. Sequences were joined and barcodes, sequences <150bp, and sequences with ambiguous base calls were removed.[11] Sequences were denoised and chimera were removed. Operational Taxonomic Units were generated by clustering at 97% similarity and classified using BLASTn against the RDPII and NCBI databases.

5.2.3 Phenylmethyl Ether Substrate Diversity

Subcultures of sediment free enrichments were amended phenylmethyl ether substrates to determine the range of aromatic O-demethylation. At a concentration of 0.5mM, syringic acid, vanillic acid, methylparaben, oxybenzone, and guaifenesin were added to separate subcultures. Methylparaben, oxybenzone, and analytical reagents were purchased from Sigma (St. Louis, MO, USA). To deliver guaifenesin to cultures, generic tablets containing 400mg guaifenesin per 710mg tablet were crushed. To a 18mL culture, 6.6mg total tablet containing 3.7mg guaifenesin, was added per culture for a final concentration of 0.5mM.

Aqueous samples for HPLC analysis were performed as described above. All solvents were HPLC-grade and purchased from Thermo Fisher Scientific (Waltham, MA, USA). Oxybenzone was detected using a mobile phase of 70% methanol : 28% water : 2% glacial acetic acid. Liquid samples were extracted with an equal volume of ethanol and detected at 312nm. Methylparaben and guaifenesin were not extracted before HPLC

analysis. Methylparaben was detected at 280nm using a mobile phase of 70% methanol : 30% 20mM Na_2HPO_4 (pH 7). 4-hydroxybenzoate and guaifenesin were detected at 280nm with a mobile phase consisting of 64% 5mM formic acid in water : 30% methanol : 6% acetonitrile.

Metabolites were identified using GC/MS. Liquid samples of all subcultures were extracted using a ratio of 20:1 ethyl acetate:liquid culture. Extracts were dried under nitrogen and then derivatized by addition of 200µL BSTFA/TMS-Cl in pyridine. Derivatization occurred by heating the sealed vial to 75°C for 4 hours. Analytical standards prepared in water were extracted using the same procedure as described above. After cooling to room temperature, 1.0µL of derivatized extract was injected on a Shimadzu TQ-1090 GC/MS with AOC6000 autosampler. The separation was performed on a RP-5SilMS column using a 1:10 split ratio for injection. The injector was set to 280°C. The temperature program was: 80°C initial temperature, held for 1 minute, ramped linearly to 220°C at a rate of 10°C/min, held at 220°C for 3.5 minutes, and then linearly ramped to 310°C at a rate of 20°C/min, then held 15 minutes at 310°C. Compounds were identified by their characteristic fragmentation patterns by comparison to spectral databases such as NIST.

5.3 Results

5.3.1 Marine Naproxen Enrichment

Naproxen loss was observed within 59 days in primary sulfate-rich marine enrichments (Figure 1). No loss was observed in sterile controls. As the concentration of naproxen decreased, following 7 days of incubation, DMN (6-O-desmethylnaproxen) accumulation

was observed. Upon reamendment with naproxen, cultures transformed the substrate more quickly, completely demethylating 1mM naproxen within 26 days (Figure 1). DMN continued to accumulate with the transformation of reamended naproxen. Enrichments were reamended with naproxen every 4 weeks and transferred into 50% fresh media upon the 4th amendment. The rate of naproxen demethylation remained consistent following the second amendment. Enrichments consistently transformed naproxen between 3 and 4 weeks. In accord with previously described anaerobic naproxen transforming cultures, no loss of DMN was observed throughout the incubation. Concentrations only decreased upon dilution of the culture into fresh media. Though enrichment cultures were provided 20mM sulfate, no loss was observed during naproxen transformation (data not shown). If sulfate was not used as an electron acceptor, it is likely that organisms demethylated naproxen by acetogenic fermentation.

5.3.2 Community Composition

Figure 2 illustrates the family-level diversity of bacteria in marine enrichments compared with a background control after 104 weeks of incubation. Despite originating from marine sediment, the marine cultures were enriched in fermentative organisms common in mammalian gut and rumen, indicating the selection of a robust anaerobic fermenting community originating from marine sediment. *Prevotellaceae* dominated naproxen transforming enrichments, comprising 40% of sequences. Common in the mammalian gut, upon excretion host-derived *Prevotellaceae* are reported to survive in marine environments.[12] This family demonstrates wide fermenting substrate diversity, and as such are able to thrive in carbohydrate and amino acid limiting environments.[13] Their abundance in the marine enrichments, which are devoid of excess carbohydrates and

amino acids, indicate a tolerance to naproxen and DMN and involvement in naproxen demethylating carbon transfer. Whether they are responsible for O-demethylation, or consume acetate generated by O-demethylating organisms, is undetermined. An important gut acetogen, *Lachnospiraceae,* was also enriched in the naproxen enrichments (7%) compared to background controls (0.2%) (Gagen et al 2010). *Clostridiaceae* is another common acetogen, yet was approximately equally present in both naproxen transforming and background cultures. Its presence in both cultures indicates that naproxen does not exert selective pressure on *Clostridiaceae* in marine sediment. The potential for *Clostridiaceae* to acetogenically demethylate naproxen in these enrichments is thus inconclusive.

Campylobacteraceae and *Helicobacteraceae*, both members of the order *Campylobacterales,* are often found coexisting in both natural environments and laboratory enrichments.[14,15] *Porphyromonadaceae,* which comprise 6% of naproxen sequences, are virtually exclusively described in anoxic mammalian cavities.[16] This was not observed, however, in the marine enrichment culture. *Campylobacteraceae* comprised 13% of the naproxen enrichment sequences, yet was only 0.05% of background sequences. In contrast, *Helicobacteraceae* accounted for 53% of background sequences and only 0.1% of naproxen enrichments. Both of these taxa have been reported to be prevalent in sulfate reducing oil seeps.[14] Structural similarity between naproxen, a substituted naphthalene, and naturally occurring polycyclic aromatic hydrocarbons, may account for the prevalence of *Campylobacteraceae* in naproxen transforming enrichments. Its abundance may indicate tolerance to polyaromatic hydrocarbons. *Campylobacteraceae* and *Helicobacteraceae* are also dominant organisms in an anoxic

sewage treatment system.[15] Again, this suggests a link between *Campylobacteraceae* and complex organic contamination. In marine enrichments, the prevalence of *Camplyobacteraceae*, combined with the absence of *Helicobacteraceae*, may indicate sensitivity of the latter organism to naproxen or DMN in marine environments.

Enrichment of *Campylobacteraceae* and absence of *Helicobacteraceae* may also be a factor of available organic carbon for heterotrophy in naproxen enrichments. *Helicobacteraceae* are able to metabolize chemolithoautotrophically. This may account for its prevalence in background controls, in which no organic carbon was added. In the absence of organic carbon and sunlight, sulfide and CO_2 may select for chemolithoautotrophic *Helicobacteraceae*. In general, the enrichment data show that the marine cultures consist of a fermenting community, while the background controls, devoid of organic carbon, are more representative of a traditional marine chemolithotrophic community.

5.3.3 Aromatic O-Demethylation

In this study, the ability of NPX consortium and the marine enrichment to demethylate a variety of phenylmethyl ethers was tested. Figure 3 illustrates the structures of the phenylmethyl ether substrates used in the study and their demethylation in the NPX consortium and marine enrichments. Both cultures transformed natural and anthropogenic phenylmethyl ethers, demonstrating broad substrate diversity formerly observed only with natural compounds.[17]

We demonstrate that the NPX consortium can demethylate the plant methoxyaromatic compounds syringic acid and vanillic acid (Chapter 3). As seen in Figure 3, the marine

enrichments completely transformed vanillic acid to protocatechuic acid within 37, while only 60% was transformed by the NPX consortium in 31 days. Syringic acid, which contains two-methyl groups, was rapidly and completely demethylated to gallic acid by both cultures. Marine enrichments, however, removed both methyl groups within 17 days, while the NPX consortium completed the transformation within 31 days. The same demethylated metabolites of both plant phenolics accumulated in the NPX consortium and the marine enrichment.

Oxybenzone, a UV-absorber, was completely demethylated by the NPX consortia within 120 days (Figure 3). The marine enrichment transformed 93% of oxybenzone in the same time period. Both cultures demethylated oxybenzone to 2,4-dihydroxybenzophenone (BP-1). The mass spectrum was consistent with that of the known BP-1 standard (Figure 4) (will add the spec). The characteristic fragment ion at 343m/z corresponds to a loss of an O-TMS fragment. The demethylated aromatic ring corresponds to fragment ion 164m/z, with a loss of the O-TMS fragment in the 2 position.

In a previous study, oxybenzone was found to be demethylated to BP-1 under nitrate, iron, and sulfate reducing conditions by a wastewater community.[6] Here, we also report oxybenzone demethylation under methanogenic conditions in anaerobic wastewater sludge and by anaerobic marine sediment microorganisms. Such comprehensive evidence of anaerobic oxybenzone demethylation to BP-1 indicates that the opportunity for BP-1 generation from oxybenzone is prevalent. It should be noted that this metabolite, BP-1 is a UV stabilizer and recognized environmental contaminant.[18] Ready demethylation of oxybenozone to BP-1 is of concern because the demethylated compound exerts estrogenic activity.[19] Notably, BP-1 elicits a stronger response in tested human and fish

estrogen receptors than the parent compound oxybenzone.[18,20] Microbial transformation of oxybenzone to BP-1 thus introduces a new source of xenoestrogens to the environment.

Similar to oxybenzone, Guaifenesin, the active ingredient in Mucinex®, was transformed by both the NPX consortium and the marine enrichment (Figure 3). Complete demethylation was observed within 120 days in the marine enrichment and 180 days in the methanogenic NPX consortium. The metabolite present in both cultures was identified as 3-(2-hydroxyphenoxy) propane-1,2 diol (O-desmethyl guaifenesin) using GC/MS (Figure 5). Because no analytical standard was available, the metabolite was identified by its mass spectrum compared with that of the parent compound guaifenesin. The mass spectrum of the metabolite, derivatized with trimethyl silyl (TMS), is displayed in Figure 5A. It was hypothesized that the molecular ion of 400m/z matches that to 3-(2-hydroxyphenoxy)-propane-1,2-diol, with all 3 hydroxyl groups derivatized with TMS. This is consistent with metabolic loss of the methyl group, which freed the -OH moiety, which was then derivatized by trimethylsilylation. The fragment ion at 245m/z corresponds to the loss of -OTMS from the compound. The fragment ion at 166m/z corresponds to the demethylated aromatic ring with a loss of the TMS-OCH_2CH(O-TMS)CH_2-O- fragment. The peak at 103 is consistent with -CH_2-OTMS fragment, wherein the propane-diol chain was fragmented between the 2 and 3 carbon. Comparison with the mass spectrum of the guaifenesin parent compound (Figure 5B). This shows the molecular ion at 342m/z, corresponding to di-O-trimethylsilylated guaifenesin. One can clearly see similarities in fragments below 200m/z, but no evidence of the characteristic fragments (400m/z, 254m/z) used to identify O-desmethyl guaifenesin. There were a

number of shared ions between the unknown compound and known guaifenesin (147m/z, 129m/z, 103m/z, 73m/z), suggesting similar carbon skeletons. Based on these results, the unknown metabolite was determined to be O-desmethyl guaifenesin

O-desmethylguaifenesin has been detected as a trace impurity in guaifenesin.[21] The mammilian liver also metabolizes a minor portion of ingested guaifenesin to 3-2hydroxyphenoxy propane-1,2 diol.[22] The presence of guaifenesin or its metabolite have not yet been determined in wastewater or the environment. Demethyation by the NPX consortium indicates that O-desmethyl guaifenesin is likely to be produced during the anaerobic digestion process in wastewater treatment. Parallel metabolism in the marine enrichments indicates that guaifenesin demethylation will also occur in anaerobic sediments, therefore, there is a strong likelihood that O-desmethyl guaifenesin may be present in the environment.

Methylparaben was also demethylated by both enrichments (Figures 3, 6). In marine enrichments, complete loss was observed after 51 days (Figure 6A). Its demethylated metabolite, 4-hdroxybenzoate, was detected by HPLC. The metabolite accumulated in the marine enrichment and did not decrease in concentration by day 59. More 4-hydroxybenzoate was recovered than methylparaben amended, which can be attributed to greater water solubility, and thus detection, of 4-hydroxybenzoate compared to methylparaben. Methylparaben was metabolized differently in the methanogenic NPX consortium. As seen in Figure 3B, loss of the substrate occurred more slowly, with only 80% of methylparaben metabolized by day 60. The metabolite 4-hydroxybenzoate, however, accumulated to a maximum concentration of 25µM, 6% of the amended methylparaben, at day 51. By day 60 12µM of 4-hydroxybenzoate remained.

The low recovery and subsequent decrease in 4-hydroxybenzoate concentration indicate that following demethylation, the methanogenic enrichment cleaved the aromatic ring and completely degraded methylparaben. Under anaerobic conditions, benzoate is degraded via the benzoyl-CoA pathway, the central anaerobic pathway used to degrade naturally occurring and xenobiotic aromatics (Porter & Young 2013).[23] As such, benzoate is utilized as a carbon source by anaerobic organisms. Anaerobes are able to directly metabolize 4-hydroxybenzoate, or dehydroxylate the compound to benzoyl-CoA before further degradation.[23,24] By demethylating methylparaben to a common metabolite, it follows that 4-hydroxybenzoate is the only metabolite further degraded in the NPX enrichments.

Mineralization of 4-hydroxybenzoate did not, however, occur in our marine enrichments. This is contrary to the well documented, complete mineralization of 4-hydroxybenzoate by marine anaerobes.[25] Because degradation did not occur in the marine enrichments, however, it is evident that microorganisms in this marine enrichment are not primed for aromatic ring cleavage. In the absence of monoaromatic substrates during previous enrichment on naproxen, it is likely that microbes enriched for naproxen demethylation outcompeted those able to mineralize benzoates.

The extensive use of methylparaben as a fungicide and preservative has led to ubiquitous dispersal in the environment (Haman 2015).[26] Methylparaben is a naturally occurring compound, however, and undergoes microbial degradation.[27] Despite aerobic metabolism, intact methylparaben resists wastewater treatment and is released in treated effluent (Hernandez Leal 2010). To our knowledge, this is the first report of methylparaben metabolism under sulfate reducing and methanogenic conditions. Madsen

et al. (2001) reported biogas production during an anaerobic respirometry assay spiked with methylparaben, indicating possible biodegradation.[26,28] Recently, methylparaben loss was observed under nitrate reducing conditions.[27] Combined with this reporting of methylparaben metabolism in methanogenic and marine enrichments, it is expected that methylparaben will eventually degrade in anaerobic environments. Demethylation by the NPX consortium and marine enrichment to our knowledge is the first demonstrated utilization of methylbaraben as a carbon substrate by anaerobic microorganisms.

In addition to the compounds included in this study, the phenylmethyl ether antidepressant venlafaxine is also O-demethylated by anaerobic wastewater microbes.[8] In agreement with naproxen, oxybenzone, and guaifenesin metabolism, wastewater microbes cleave the ether linkage of venlafaxine, leaving a demethylated aromatic metabolite that resisted further anaerobic degradation. Systematic phenylmethyl ether O-demethylation demonstrated in this study can be extrapolated to a variety of PPCPs in wastewater treatment and anaerobic environments, with unknown ecosystem impacts. Anaerobic O-demethylation of the numerous phenylmethyl ether PPCPs may support niche heterotrophic communities in anoxic environments.

Though only a small portion of PPCPs have been investigated for presence in the environment, evidence indicates that many escape wastewater treatment.[1,2] Further, absence in the environment does not mean complete removal during wastewater treatment. Instead, it presents the possibility that microbes transformed the parent compound to an altered metabolite that has not yet been surveyed.

5.4 Conclusions

To the authors' knowledge, this is the first reporting of microbial metabolism of guaifenesin and the first demonstrated methanogenic methylparaben biodegradation. Individually studying the microbial transformation of all PPCPs in both wastewater treatment and the environment will take monumental effort. In this study, a systematic approach to assessing biotransformation potential was demonstrated with aromatic O-demethylation. The NPX consortium and marine enrichments demethylated phenylmethyl ethers of both natural and anthropogenic origin. It can therefore be expected that O-methyl aromatic PPCPs will be demethylated under anaerobic conditions. As previously demonstrated, the NPX consortium conserves energy through acetogenic O-demethylation. In the absence of readily available organic carbon, the NPX consortium utilizes the cleaved methyl substituent as carbon substrate, transferring this -CH_3 group through the acetogenic/methanogenic food web. The fermentative community supported by naproxen demethylation in marine enrichments indicates these heterotrophs also conserve energy through O-demethylation. In both cultures, phenylmethyl ether O-demethylation supported robust heterotrophic communities. Energy conserved and acetate produced by aromatic PPCP O-demethylation may drive microhabitats within anaerobic environments.

With the exception of common intermediate metabolites, such as 4-hydroxybenzoate, the demethylated transformation products in this study were not further degraded by the same communities responsible for demethylation. Demethylation of phenylmethyl ethers without aromatic ring cleavage will lead to accumulation of the metabolites. As demonstrated by oxybenzone demethylation to BP-1, these demethylated metabolites continue to be bioactive. If further degradation does not occur, these metabolites can

accumulate to levels that can be disruptive to the environment and ecosystem, ultimately disrupting human and environmental health.

5.5 Acknowledgements

GC/MS analysis was completed with the help of Tom S. Villani and Dr. James Simon.

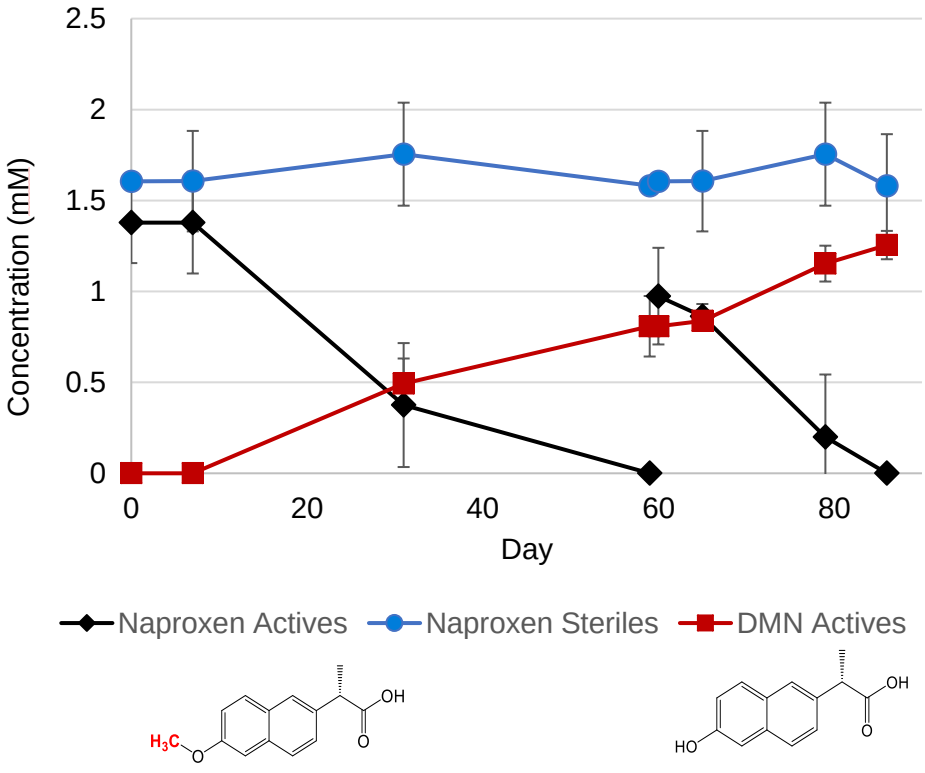


Figure 1. Naproxen loss in primary enrichment cultures. Enrichments were reamended with 1mM naproxen on day 60, indicated by the arrow. (♦) Naproxen in actives, (●) naproxen in steriles, (■) cumulative DMN in active cultures. The structures of naproxen and DMN are included in the legend.

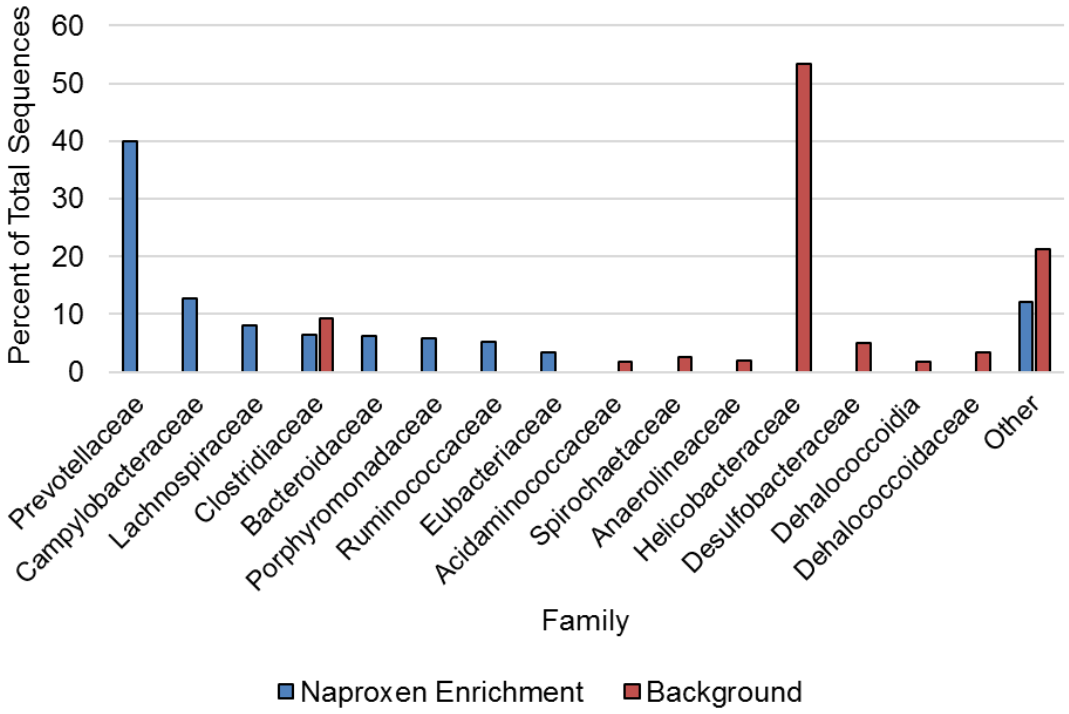


Figure 2. 16S rDNA family level community composition of sulfidic naproxen enrichment and corresponding background control. Phylogenetic distribution is displayed as percent of total sequences. Blue bars, sulfidic naproxen enrichment; red bars, background control.

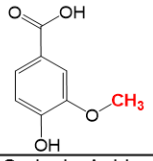
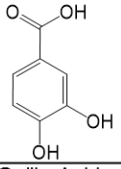
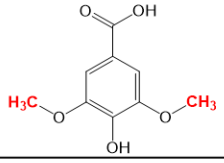
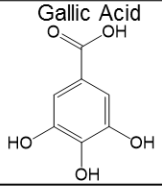
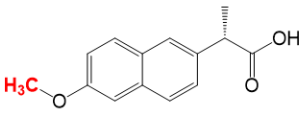
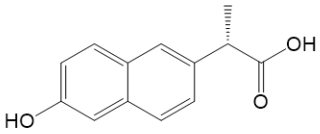
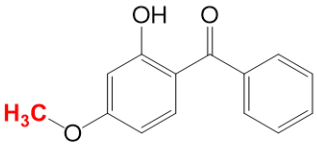
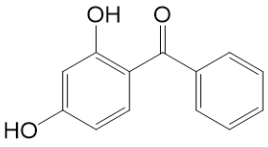
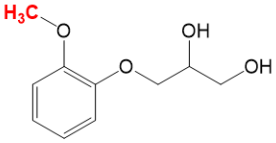
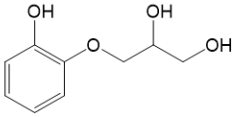
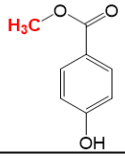
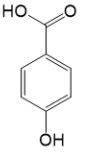
	Parent Compound	% Loss NPX	% Loss Marine	Metabolite
Plant Phenolics	Ħ Vanillic Acid 	60% loss within 31 days	100% within 37 days	Protocatechuic Acid 
	Ħ Syringic Acid 	100% loss within 31 days	100% within 17 days	Gallic Acid 
PPCPs	Ħ Naproxen 	100% within 22 days	100% within 59 days	DMN 
	Oxybenzone 	100% within 120 days	93% within 120 days	BP-1 
	Guaifenesin 	100% within 180 days	100% within 120 days	3-(2-hydroxyphenoxy) propane-1,2-diol 
	Methylparaben 	80% within 60 days	100% within 51 days	4-Hydroxybenzoic acid 

Figure 3. Substrate Loss in Methanogenic (NPX consortium) and Marine enrichments. All phenylmethyl ether substrates were amended to the cultures at 0.5mM. Left column is substrate (methyl group in red) and the far right column is the demethylated metabolite recovered following incubation. The removal of the ether linked methyl group results in a hydroxylated aromatic compound.

Ħ NPX data adapted from Chapter 3.

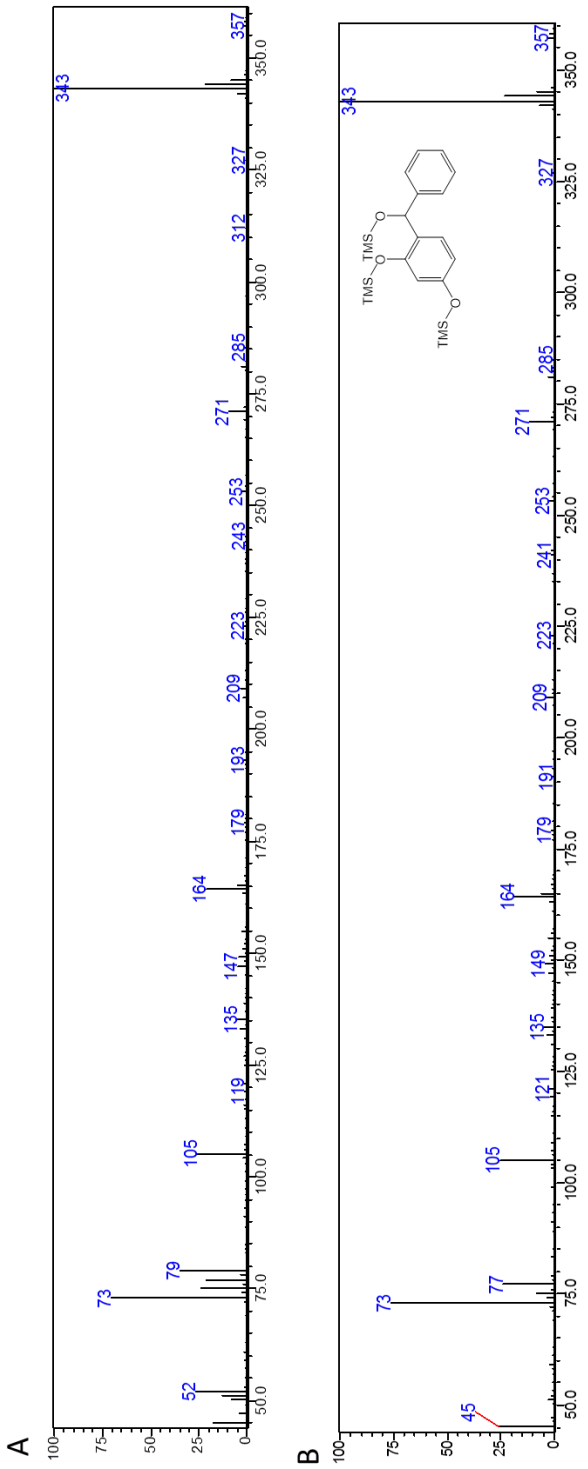

A
B
TMS
TMS
TMS

Figure 4. GC/MS spectrum of the oxybenzone metabolite recovered from both the marine enrichment and the NPX consortium. A. Intermediate metabolite recovered from the two cultures. B. Analytical standard of 2,4-dihydroxybenzophenone (BP-1). Both the metabolite and standard are derivatized with TMS. The characteristic fragment ion at 343m/z corresponds to the loss of an O-TMS fragment. The derivatizing agent, TMS, is seen at m/z 73.

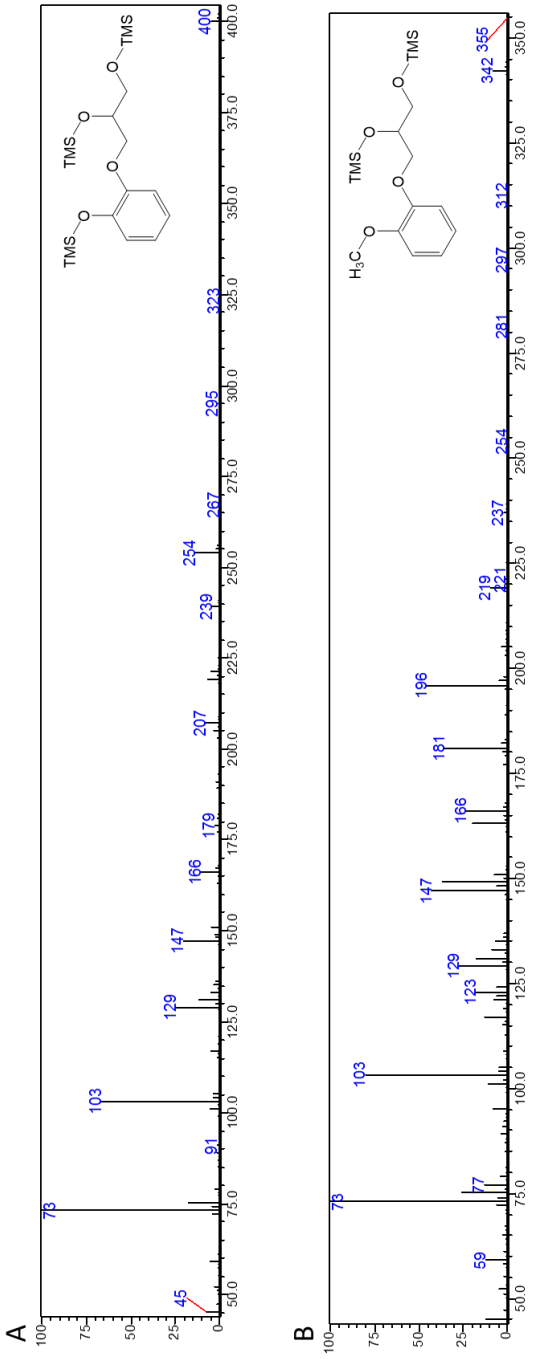


Figure 5. Mass spectrum of the trimethylsilyl derivatized guaifenesin metabolite (A) compared with guaifenesin parent compound (B). The molecular ion at 400 m/z matches that of the hypothesized metabolite 3-(2-hydroxyphenoxy)-propane-1,2-diol (A). The di-O-trimethylsilylated guaifenesin molecular ion is seen at 342m/z (B).

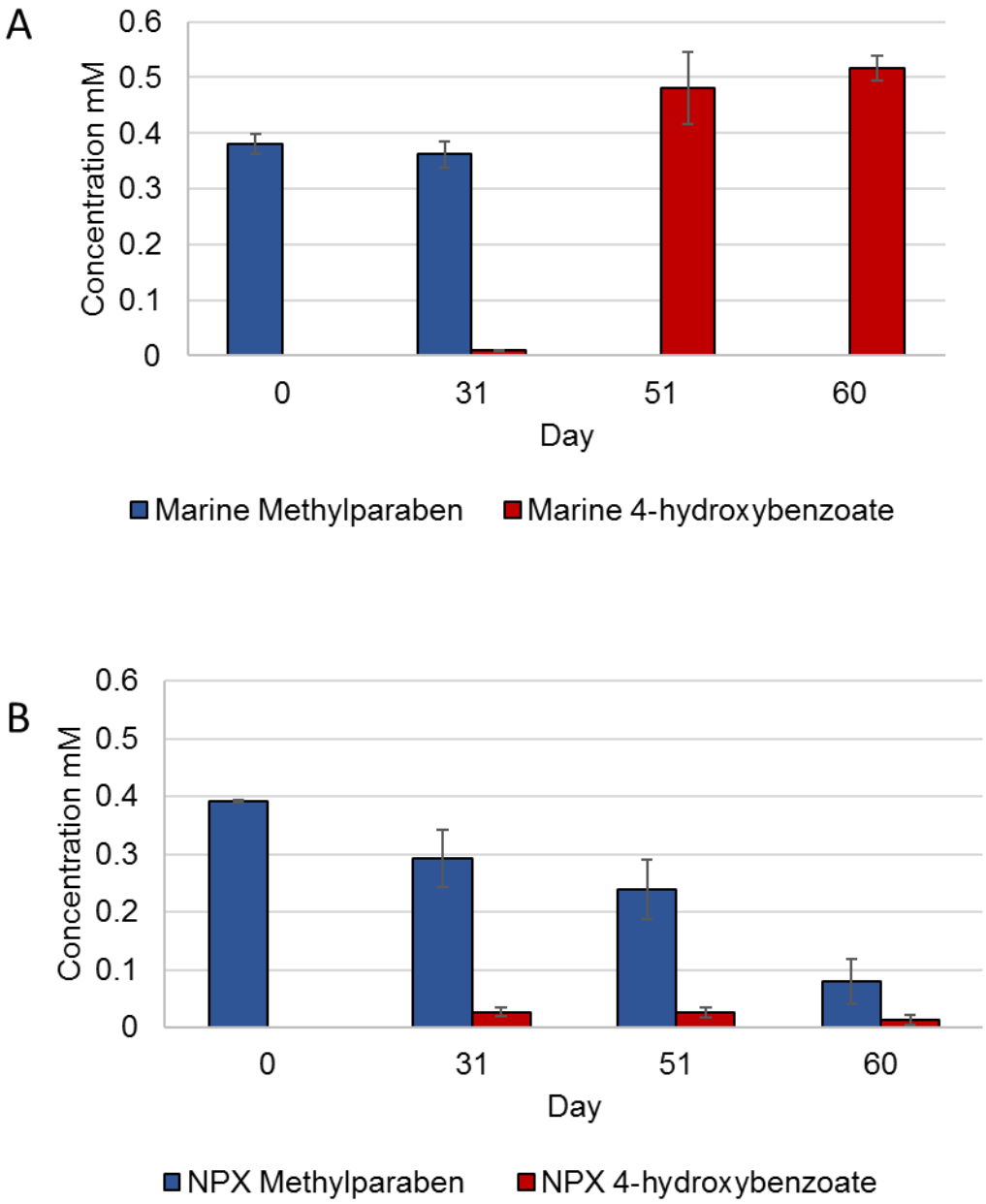


Figure 6. Methylparaben loss and 4-hydroxybenzoate appearance in A. Marine enrichments and B. NPX consortium. Blue bars, methylparaben. Red bars, 4-hydroxybenzoate.

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Chapter 6

Conclusions

6.1 Conclusions and Future Directions

Chapter 2 characterizes the microbes responsible for naphthalene degradation enriched from unpolluted marine sediment. Within the complex microbial community of pristine estuarine sediment, organisms exist that specialize in metabolizing naphthalene as a carbon source. The enrichment culture described in Chapter 2 is the first demonstrated anaerobic naphthalene degradation from inoculum that has not been exposed to high levels of anthropogenic hydrocarbon pollution. Additionally, the inoculum source in Tuckerton, NJ receives little natural hydrocarbon input. Despite minimal exposure to hydrocarbons, organisms capable of utilizing naphthalene as a food source were present and identified. Stable isotope probing revealed three primary naphthalene degraders. Two were identified as closely related to the two currently known naphthalene degrading isolates, NaphS2 and NaphS3.[1,2] The third organism is a member of a poorly characterized taxa, sharing only 86% identity with its most closely related known relative, *Desulfomicrobium thermophilum*. Though its identity was not confirmed, this new taxon represents the first non-NaphS2/S3 organism identified to anaerobically degrade naphthalene.

It is noteworthy that in addition to naphthalene, the only other component in Alaska North Slope and Alba crude oils utilized by this culture within a 16 week incubation was 2-methylnapthalene. All other polycyclic aromatics, monoaromatics, and alkanes

remained unmetabolized. Such selective metabolism suggests that the naphthalene degraders identified in this study have a very limited metabolism.

The chemical and community data linked in this study provide the basis for future avenues of research. Because two of the three naphthalene degraders enriched in this study are closely related to NaphS2 and NaphS3, it would be useful to determine the substrate diversity of these two isolates. If their metabolism is limited to naphthalene and 2-methylnaphthalene, as is the case with my enrichment culture, PAH degradation in the environment would require a suite of organisms. If, on the other hand, the two isolates are capable of degrading a variety of petroleum components, then there is a crucial functional difference between the isolates and organisms enriched in this study, despite a close phylogenetic relationship. Further information regarding the third, uncharacterized naphthalene degrader could be revealed through metagenomic study. This work is being streamlined and put together for publication.

In Chapter 3, I describe the transfer of naproxen carbon through an anaerobic wastewater consortium. Chemical analysis of the enrichment culture revealed rapid naproxen demethylation, forming 6-O-desmethyl naproxen. This metabolite accumulated in the culture, indicating that it is not further degraded by the methanogenic naproxen transforming (NPX) consortia. Upon transformation of 1mM naproxen, 0.083mM methane was generated.

High throughput sequencing of bacteria revealed a community enriched in acetogenic organisms. The NPX consortium was dominated by *Eubacteriaceae* and *Thermoanaerobacteriaceae*, both of which are known to demethylate phenylmethyl via acetogenesis.[3] Surprisingly, the archaeal community in the NPX consortia unexpectedly was dominated by non-acetoclastic methanogens. Instead, the H_2/CO_2 methanogens *Methanomicrobiaceae* dominated the archaeal community. To obtain sufficient reducing power to perform methanogenesis, a source of H_2 within the naproxen transforming community would be necessary.

Therefore, the resulting community interactions can be explained as follows. The dominant acetogenic bacteria in the NPX consortia enriched in this study is responsible for cleaving the ether linked methyl substituent. Acetate, a byproduct of this energy-conserving naproxen demethylation, is then utilized by syntrophic acetate oxidizers. Namely, syntrophic bacteria oxidize the acetate and form $4H_2$ in the process. This is an energetically unfavorable step, however, so the microbes live in tight syntrophy with methanogens, which utilize the $4H_2$ to generate methane. This latter reaction is energetically favorable, thus providing sufficient energy for both groups. In summary, the methyl group of naproxen is transformed to methane via a three step process – demethylation to acetate, syntrophic acetate oxidation, and CO_2 reduction. This study links the transformation of naproxen with the biochemistry and microbial ecology of a methanogenic consortium from a wastewater digester.

In Chapter 4, I describe an anaerobic wastewater community capable of transforming diphenhydramine to the demethylated metabolite N-desmethyl diphenhydramine. In the

unenriched primary culture, partial transformation was observed after 29 days. This is within the typical solids retention time of an anaerobic digester, indicating that diphenhydramine transformation can occur during wastewater treatment. Incomplete metabolism of diphenhydramine by wastewater microorganisms also indicates that the metabolite will enter the environment along with any unmetabolized parent diphenhydramine.[4] Both the parent compound and metabolite can therefore be expected to be present in treated sewage biosolids and affiliated treated water. No further degradation of N-desmethyl diphenhydramine was carried out by the anaerobic community in this study. Because it will be released to the environment, the ultimate fate of this metabolite and its potential pharmacological effect on the environment is an important future research direction.

In the anaerobic toxicity assay, diphenhydramine and N-desmethyl diphenhydramine both reduced the gas generation compared to active controls with acetate and propionate by the anaerobic community. Diphenhydramine suppressed both methane and carbon dioxide, indicating toxicity to both fermenters and methanogens. N-desmethyl diphenhydramine was only inhibitory to methanogens. The differing toxicity to the two functional groups in the anaerobic community demonstrate a broader unpredictability of impact to nontarget organisms. As demonstrated, this inhibition to microbes sets the stage for future research on the toxicity of diphenhydramine and its metabolite N-desmethyl diphenhydramine to other organisms in environments receiving wastewater effluent and sludge biosolids. The research described in Chapter 4 has been submitted to *Water Research.*

The final research chapter, Chapter 5, explored the phenylmethyl ether substrate diversity of the NPX consortium from Chapter 2 and an additional naproxen degrading marine enrichment. As was observed in the NPX consortium, the marine enrichment rapidly transformed naproxen. Primary cultures demethylated naproxen to 6-O-desmethyl naproxen within 59 days, and further enrichment completed this transformation within 26 days.

Once a consistent, sediment-free culture was established, both methanogenic and marine cultures were amended with vanillic acid, syringic acid, oxybenzone, guaifenesin, and methylparaben. The first two compounds are natural lignoaromatics of plant origin and were demethylated to their respective hydroxylated metabolites. The latter three compounds, all pharmaceuticals and personal care products, were also demethylated by the two cultures. Demethylation of all methoxyaromatic compounds amended, regardless of natural or anthropogenic origin, demonstrates the broad substrate diversity of these cultures. It also suggests a systematic aromatic O-demethylation of a variety of diverse chemical structures found in pharmaceutical and personal care products introduced to wastewater and the environment. The multitude and magnitude of PPCPs continually entering the environment makes studying each individual chemical difficult. Thus, the systematic approach used in this study can help predict the behavior of similar chemicals. Broad substrates demethylated in Chapter 5 can be understood within the context of the syntrophy model proposed in Chapter 2. The transfer of methyl carbon through the anaerobic wastewater food web elucidates community level metabolism of aromatic O-methyl pharmaceuticals and personal care products.

With the exception of naphthalene, the anaerobic communities described in chapters 3, 4 and 5 did not cleave the aromatic ring. Instead, microorganisms partially metabolized the parent compound, leaving a demethylated aromatic compound that may still exert effects to nontarget organisms. Surveys that have been taken of emerging contaminants in the environment focus on intact pharmaceutical and personal care compounds.[5] Microbial metabolites as illustrated here are mostly overlooked. One very telling reason is that in many cases they are not yet known or described. The systematic demethylation of aromatic O-methyl compounds creates a predictive model for understanding the microbial transformation in anoxic environments of other similar compounds containing an O-methyl substituent. This is demonstrated by the demethylation of the 5 compounds in this chapter, as well as that of diphenhydramine in chapter 3 support that demethylation is a common first step of anaerobic microbial transformation.

6.2 References

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