Problem and Research Objectives

In the US, greater than one fifth of all homes depend on onsite systems, especially septic systems consisting of a septic tank followed by a drain field, to treat wastewater. While these onsite systems can be a cost- and energy-effective option for wastewater treatment, there is a strong need for improved understanding of the treatment processes and better management of such systems. The communities on Cape Cod are disproportionately served by on-site septic system with as many 97% of households employing them for wastewater treatment. The impact of on-site septic systems extends further to the entire New England region. According to the U.S. Census Bureau, the region has one of the highest percentages of homes with on-site wastewater treatment systems. When inadequately managed, septic systems can result in reduced performance and become non-point sources of environmental contamination.

On-site septic systems achieve poor nitrogen removal and are difficult to regulate. Only 1,800 of the 123,000 septic systems on Cape Cod are modern alternative systems that can accommodate some nitrogen removal. Nutrient release, particularly nitrogen, is a significant problem in the coastal estuaries and marine environments throughout Cape Cod , causing eutrophication (Cambareri et al., 2009). Eutrophication facilitates excessive algae growth and when the algae decay, oxygen is consumed, causing the death of indigenous plants and animals. The result is detrimental to the ecosystems and tourism industry on Cape Cod as it is not only odorous and ugly but significantly impacts the populations of mollusks and fish harvested for human consumption.

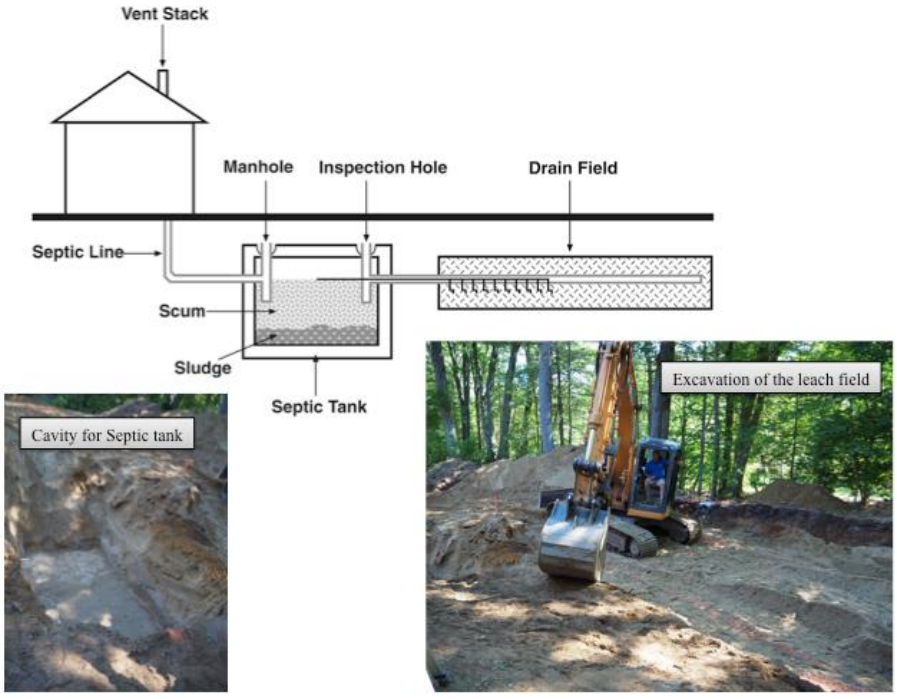

Figure 1. A layout of a domestic septic system and the site preparation for the installation of a septic system in Marlborough, MA

In addition to nutrient removal, the removal of pharmaceuticals and personal care products (PPCPs) has been a rising concern in water and wastewater treatment. A wide range of PPCPs are detected in domestic wastewater and are often not removed by centralized municipal wastewater treatment plants. PPCP removal in wastewater occurs mostly through microbial transformation and most of the known processes take place only under aerobic conditions. Anaerobic transformation and removal of PPCPs with the anaerobic septic system environment is largely understudied. A White Paper published by the Barnstable County Department of Health and Environment expressed concern of PPCPs discharge from septic system drain field leaching into groundwater used as drinking water. This is particularly concerning due to the U.S. Environmental

Protection Agency (EPA) designation of Cape Cod as having a sole-source aquifer, which indicates that all on-site wastewater discharge sites are hydraulically connected to drinking water supplies (Heufelder, 2012).

Wastewater treatment, whether centralized or on-site, must be effective in both nutrient removal and overall reduction of potentially toxic PPCP-related compounds to ensure high quality of water resources. Even though a large number of houses in New England rely on septic systems, there is limited information on the performance of on-site septic systems with respect to both treatment goals. In particular, the role of microorganisms in the transformation of PPCPs in septic systems is largely unexplored. Additionally, the inhibiting effects of PPCPs on nitrifying microorganisms (Bolong et al., 2009) indicate another level of complication for achieving the two treatment goals simultaneously. Therefore, **the goal of this proposed work** was to better understand the fate of PPCPs within septic systems, which include septic tanks and drain fields, while also measuring nitrogen removal to determine whether both treatment goals are met. We have addressed this work with two objectives: **Objective #1:** *To assess the impacts of PPCP presence in septic tank effluents on the soil microbial community in drain fields* and **Objective #2:** *To determine the occurrence and fate of parent PPCP compounds and metabolites of microbial transformation as well as of nitrogen species in septic system drain field soils.*

Methodology:

Construction of Septic System

A lab-based septic system was constructed, consisting of a septic tank, distribution box and a drain field (Figure 2). The tank was fed continuously with effluent from the primary clarifier at the Amherst Wastewater Treatment Plant by peristaltic pump at a flowrate of 13 mL/min. The tank held 20 gallons (76 L) of wastewater with a hydraulic retention time (HRT) of 4 days. A 4 day HRT is consistent with the operation of many domestic septic tanks and compliant with the Massachusetts Department of Environmental Protection 310 CMR (Code of Massachusetts Regulations) 15.404, which specifies that the retention time of a septic system must be at least 24 hours. The effluent was discharged to the distribution box and then to a waste tank during the preliminary stabilization period. The distribution box was ultimately connected to 8 soil columns.

Eight soil columns were constructed in order to mimic the drain field of a septic system. The soil used in these columns was commercial grade sand from Home Depot chosen for its low organic content Additionally, sandy soils are representative of soil composition in Cape Cod. The columns had duplicates of four soil conditions chosen to explore the role of microorganisms in PPCP transformation: 1. unmodified soil, 2. soil inoculated with microorganism contained in primary effluent from a wastewater treatment plant, 3. autoclaved soil, and 4. autoclaved soil inoculated with microorganism from primary effluent. The soil columns were first subjected to a stabilization period for 10 days during which they supplied 16 mM phosphate buffered minimal growth media (Srinivasan et al., 2016) containing 1000 mg COD/L as acetate in a recirculated batch mode. During the batch recirculation operations, samples were collected at t= 0,1,2,4 hrs, then 1, 3, 7 and 10 days. After acetate was completely consumed, a second phase of batch operation was imposed. During this stabilization period, a growth media spiked with 100 µg/L of 4 representative PPCPs: ibuprofen, carbamazepine (antiepileptic drug), trimethoprim and sulfamethoxazole (antibiotics) was fed to each column at a flowrate of 1.64 mL/min. The sampling schedule after the stabilization period was hourly for the first day, daily for the first week and three times a week for the remaining weeks.

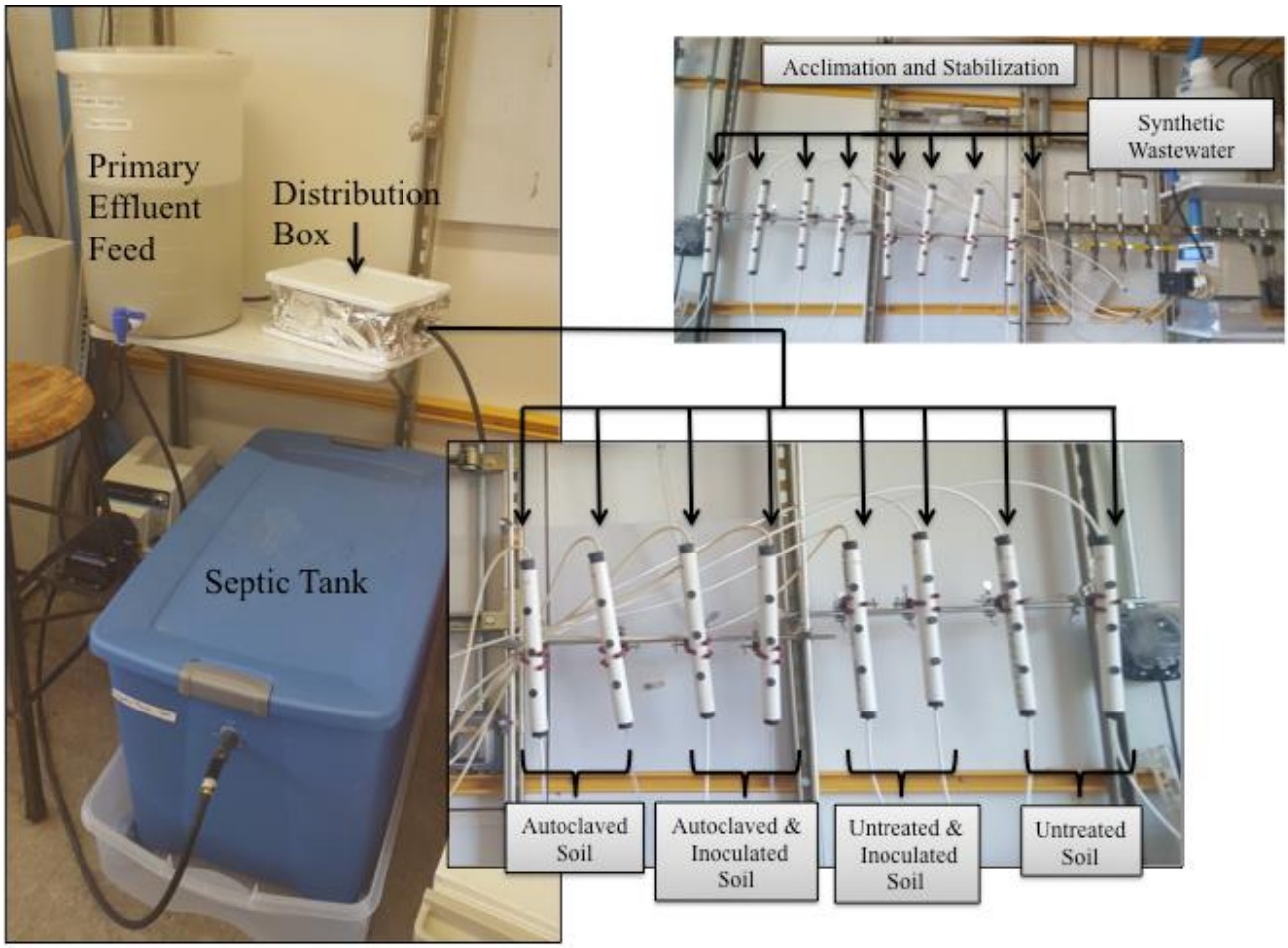

Figure 2. A picture of the septic system model in the lab. Column operation during acclimation and stabilization is presented in the inset figure.

Batch Studies

Batch studies mimicking the drain field soil environment were conducted in order to reinforce the biological impacts of pharmaceuticals in the soil in a more controlled environment. Soil conditions tested in duplicates were (i) autoclaved sand, (ii) autoclaved sand inoculated with microorganisms from primary effluent, (iii) autoclaved topsoil, (iv) autoclaved topsoil inoculated with microorganisms from primary effluent, (v) unmodified topsoil, and (vi) unmodified topsoil inoculated with microorganisms from primary effluent. Synthetic wastewater media comprising of 100 mg/L acetate and a 16 mM phosphate buffer was used in this two-week study. The media was spiked with 100 µg/L of three pharmaceuticals: sucralose (artificial sweetener), trimethoprim and sulfamethoxazole (antibiotics). These three compounds are being investigated since they are representative of diverse classes of pharmaceuticals present in the effluent of septic systems. A sample from each batch reactor was taken every 2-4 days over the course of the experiment.

Analytical Methods

Influent and effluent samples from the septic tank were collected weekly for a 6 month period. For the septic tank assessment, Total suspended solids (TSS), volatile suspended solids (VSS), and chemical

oxygen demand (COD) were measured for both influent and effluent samples following Standard Methods (Clesceri et. al, 1998). Dissolved oxygen (DO) concentrations were measured from influent and effluent as well as inside the tank with a Thermo Scientific Orion Rugged Dissolved Oxygen DO probe. Measurements of pH and temperature of the septic tank content were also conducted weekly.

Ibuprofen, sucralose, carbamazepine, trimethoprim and sulfamethoxazole were detected using Electrospray Ionization Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (ESI-UPLC/MS/MS) on a Waters Acquity UPLC connected to a Waters Quattro Premier MS instrument. Solvents A and B for the mobile phase of positive mode ESI that detected sucralose, carbamazepine, trimethoprim and sulfamethoxazole were 0.05% formic acid in 1 g/L ammonium formate solution and UPLC-grade methanol/acetonitrile (1:1) with 0.1% formic acid. Solvents A and B for the mobile phase of negative mode ESI that detected ibuprofen were 40 mg/L aqueous ammonium acetate and 100% UPLC grade methanol. The UPLC gradient elution and MS/MS parameters used were based on EPA Method 1694. Data collection was performed by multiple reaction monitoring (MRM) mode using Waters MassLynx software. Standard curves for the analytes were constructed using pure PPCP compounds and used for the quantification of unknown experimental samples.

DNA-based Molecular Methods

Composite soil samples from batch and column experiments were collected for DNA extraction and sequencing. Composite samples were prepared by consolidating multiple samples spatially distributed through the batch or column environment. DNA was also extracted from an operating septic system drain field in Marlborough, Ma and 'virgin' soil from a newly installed septic system drain field in Southampton, MA. DNA extractions were performed using MoBio PowerSoil DNA Extraction Kits following the manufacturer's guidelines. Presence of DNA was verified by agarose gel and the quality of the extracted genomic DNA was assessed using a NanoDrop measuring the A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} ratios. After genomic DNA quality was verified, samples were sent to Research and Testing Laboratory (Lubbock, TX) for Next Generation Sequencing of the 16S rDNA gene using the Illumina MiSeq platform.

Principal Findings and Significance:

Objective #1: *To assess the impacts of PPCP presence in septic tank effluents on the soil microbial community in drain fields.*

Working hypothesis: A subset of microorganisms within the indigenous soil microbial communities is responsible for the observed PPCP transformation in the septic system drain field. Observed concentrations of individual PPCPs are low enough to not result in significant shifts in the microbial community structure. However, a mixture of multiple PPCPs results in an increase in the overall toxicity to indigenous microbial populations and causes an enrichment of the microbial communities that can transform these complex compounds. Shifts in soil microbiota could have impacts on higher-order organisms within the ecosystem

Preliminary Batch Results – Removal of PPCPs in soil batch reactors was tested with or without prior inoculation with primary effluent-derived microorganisms. Over a 11-day period, there was little removal of sucralose (Figure 3a) in all soil types tested. Sucralose removal approached 20% after 11 days in reactors containing unmodified topsoil with and without inoculation with primary effluent. These results suggest that sucralose was largely recalcitrant and only slightly removed by soils with

higher contents of complex organic compounds as autoclaved soils tend to have more labile organic matter. This removal was likely due to adsorption rather than biodegradation.

Sulfamethoxazole was removed by up to 80% in 11 days in batches containing topsoil but not sand (Figure 3b). Prior inoculation with primary effluent appeared to not have a large effect on removal efficiency of sulfamethoxazole, suggesting that the primary mode of removal was physicochemical adsorption rather than biodegradation. The difference in sulfamethoxazole removal rates over the initial 7 days of the experiment between batches containing autoclaved and unmodified topsoil suggests that its adsorption may be dependent on the chemical nature of the organic matter present in the soil.

Trimethoprim was rapidly and completely removed within 1 day in all batches containing topsoil (Figure 3c). In batches with unmodified topsoil, the complete removal of trimethoprim was observed within 30 minutes of PPCP addition. Trimethoprim was also removed by up to 97% in 11 days in batches containing sand. Primary effluent-inoculated sand samples resulted in a lower trimethoprim removal (80%), possibly due to changes in the surface chemistry of sand particles by the colonization of microorganisms. These results suggest that trimethoprim was removed via physicochemical mechanisms (e.g., adsorption) rather than biodegradation. While these results indicate that physicochemical removal is more likely than biodegradation, these trends were likely due to the chemical characteristics of the three PPCPs tested and the microbial community of the inoculum. The subsequent lab-scale septic system experiments were designed to determine the importance of the microbial community for the fate and transport of a wider variety of PPCPs.

Microbial Ecology of Soil Communities – DNA was extracted from batch microbial communities leach fields and the quality was verified (Table 1). DNA will also be extracted for soil from new and existing septic systems and the column experiments described below. For cost conscientiousness (the greater the number of samples processed, the lower the cost per sample), we are aggregating the DNA extracted from this study from both batch and column experiments and field data to be sequenced one time. At the time this report was written, the raw data results of the sequencing analysis were pending.

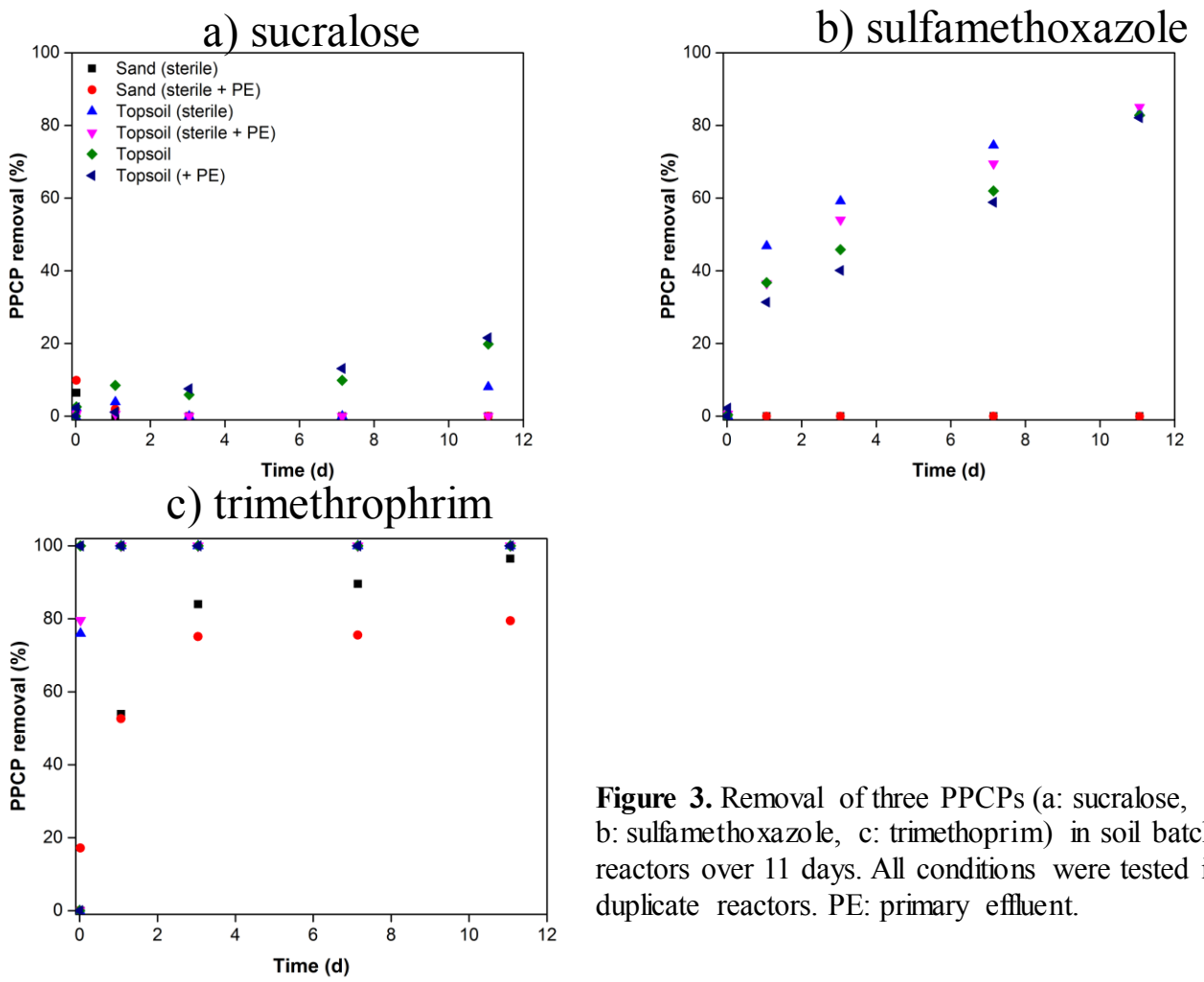

Figure 3. Removal of three PPCPs (a: sucralose, b: sulfamethoxazole, c: trimethoprim) in soil batch reactors over 11 days. All conditions were tested in duplicate reactors. PE: primary effluent.

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Table 1. A Subset of the Quantity and Quality of Extracted Genomic DNA

Sample	**DNA Concentration (ng/µL)**	**A_{260nm}/A_{280nm} (Target: >1.8)**
Autoclaved Sand (1)	1.9	2
Autoclaved Sand (2)	1.4	1.9
Autoclaved & Inoculated (1)	1.4	4.4
Autoclaved & Inoculated (2)	2.6	2.1
Untreated Sand (1)	2.5	2.7
Untreated Sand (2)	1.7	2.7
Untreated and Inoculated (1)	4.5	1.7
Untreated and Inoculated (2)	2.3	1.8

Objective #2: *To determine the occurrence and fate of parent PPCP compounds and metabolites of microbial transformation as well as of nitrogen species in septic system drain field soils.*

Working hypotheses: Microbial transformation of PPCPs is not synonymous with removal or detoxification, and metabolites of microbial PPCPs transformation may persevere beyond the drain field. The majority of nitrogen removal occurs via nitrification (biological conversion of ammonia to nitrite and nitrate) in the drain field instead of in the septic tank; the nitrogen removal in drain field soils is not negatively affected by simultaneous PPCP transformation.

Stabilization of Septic Tank Operation- To better understand and confirm transformation of PPCPs in the septic system drain fields, we must first establish the role of the individual components of the septic systems. A lab-based model septic system was constructed including a septic tank, distribution box and 'drain field' soil columns (Figure 2). The septic tank portion of this set-up has been in operation for 6 months. After 4 weeks of stabilization, the septic tank achieved steady-state performance. The primary effluent used as influent for the septic tank contained 80-125 mg VSS/L, 80-150 mg COD/L and a 1-6 mg DO/L dissolved oxygen (DO). The septic tank achieved 49% reduction in VSS and 20% reduction in COD (Figure 4). DO was significant reduced in the septic tank as expected and remained less than 0.2 mg DO/L, near the detection limited of the DO probe.

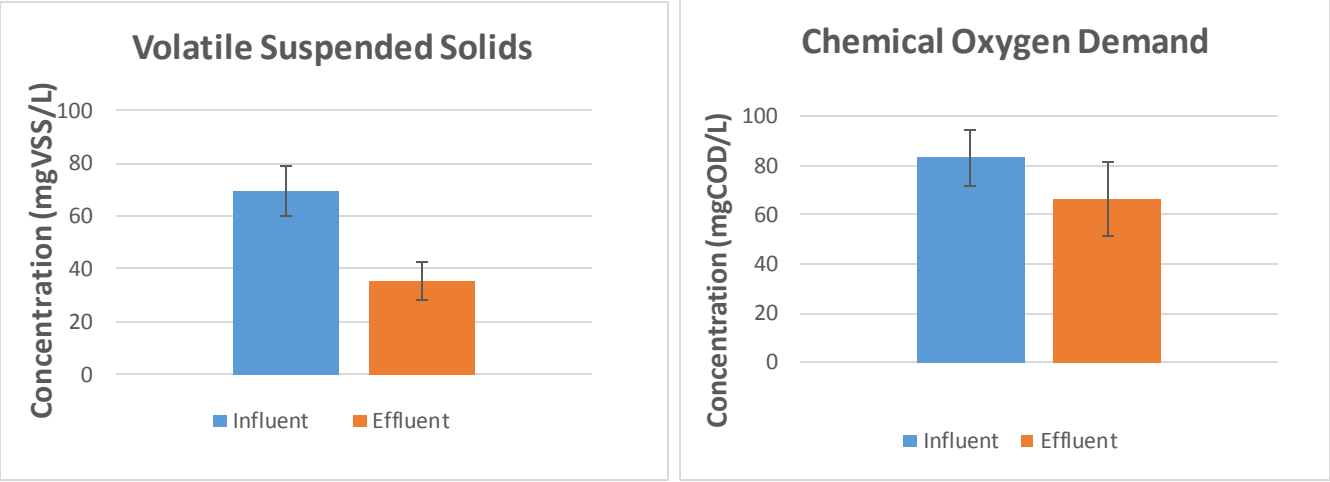

Figure 4. Average volatile suspended solids and COD removal in the septic tank collected over a 6 month period.

Transformation of PPCPs in Leach field soil – Acetate was completely consumed within 10 days of start-up in each of the 4 styles of column configurations each operated in duplicate – 1. autoclaved sand, 2. autoclaved sand inoculated with microorganisms from primary effluent of the Amherst Wastewater Treatment Plant, 3. untreated sand and 4. untreated sand inoculated with primary effluent. As an initial assessment of PPCP transformation potential by the soil and inoculated microorganisms, the soil columns were fed with a synthetic growth media spiked with 100 µg/L of 4 representative PPCPs: ibuprofen, carbamazepine (antiepileptic drug), trimethoprim and sulfamethoxazole (antibiotics). These experiments are currently ongoing. Throughout these experiments, soil samples will be collected and the genomic DNA will be extracted for microbial community analysis. After 2 months of operation with the PPCP-spiked synthetic growth media, the columns will be connected to the lab-based septic tank and fed with tank effluent. The septic tank will be supplied with primary effluent from the Amherst Wastewater Treatment Plant spiked with 100 µg/L of the 4 previously described PPCPs.

Challenges

This project has faced a number of challenges since the award period started in March 2015. The PI, Dr. Butler was on parental leave during the Spring 2015 semester. Co-PI Dr. Ikuma transitioned to a new position at Iowa State University in July 2015. Ms. Washington, the graduate research assistant, struggled academically since she started in June 2015. She was encouraged by the PIs to focus on succeeding in her courses and as a result was less productive with her research. Additionally, a key piece of equipment the UPLC was out-of-service for a period during the Fall 2015 semester and we had unanticipated challenges with DNA extraction procedures in January and February 2016. Had this award been eligible, it would have benefitted from a no-cost extension. We continue to pursue the research objectives outlined in this work and Drs. Butler and Ikuma plan to seek further funding to support effort to explore microbial transformations of PPCPs in septic systems.

Conclusions and Significance

The goal of this work is to demonstrate the impact of the microbial communities within the drain field in remediating nutrients and detoxifying PPCPs in on-site septic systems. Early data suggests microorganisms play a role in the PPCP transformation in soils associated with septic system leach fields. On-going efforts will elucidate the key microbial communities involved. Non-point nitrogen and PPCP release from antiquated septic systems is not unique to Cape Cod. Seasonal, coastal communities from Maine to South Carolina with a high septic system usage jeopardize sensitive coastal ecosystems vulnerable to eutrophication and PPCPs effects. Other rural communities relying on septic systems that discharge into potential drinking water sources also pose an elevated risk for both ecosystem and human health. Our long-term research goal is to provide sufficient information on nutrient removal and PPCP detoxification within on-site septic systems. This information could motivate and provide a scientific basis for replacing or repairing antiquated septic systems. Upgrading septic systems in areas that cannot adequately support centralized treatment systems could significantly improve the water quality.

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