Biodegradation of Trace-level Organic Contaminants by Rhizosphere Microbial Communities

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Abstract

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Wastewater treatment plants (WWTPs) remove simple organic compounds from wastewater but cannot fully degrade the thousands of trace-level organic contaminants (TOrCs) added to the waste stream each year. These TOrCs are released into the environment in WWTP effluent and can negatively impact aquatic ecosystems. One proposed method for treating TOrCs is rhizotreatment, which breaks down contaminants using the metabolic capability of the rhizosphere. In rhizotreatment, effluent is discharged onto a field of trees. As the water moves through the soil, it encounters rhizosphere microorganisms that degrade the TOrCs. Rhizotreatment is used at several WWTPs to treat nitrogen and phosphorus compounds, but little is known about the fate of TOrCs in the system, or about the changes to the soil microbial community induced by long-term rhizotreatment. This study examined the impact of rhizotreatment on the degradation of five TOrCs, as well as changes to the rhizosphere

community. Rhizosphere soil samples were collected from three WWTP effluent discharge fields planted with trees and exposed to effluent for at least ten years, and from nearby control plots that received no effluent. Short-term batch testing was conducted to compare the degradation of bisphenol A (BPA), carbamazepine, gemfibrozil, ibuprofen, and naproxen between effluent-exposed and control soils. Microbial community composition was also examined using 16S rRNA Illumina sequencing, and bacterial and archaeal biomass, along with the presence of a putative BPA degradation gene, were quantified via droplet digital PCR. Soils from all three sites exhibited intrinsic degradation of BPA and ibuprofen. Additionally, BPA degradation was enhanced in all treated soils, two of which also showed increased abundance of the BPA degradation gene. The other three TOrCs exhibited minimal degradation over the timeframe of the experiment. Principal component analysis of the sequencing results revealed distinct clustering of the treated and control communities at two of three sites where BPA degradation was enhanced, suggesting differences in microbial community composition between treated and control soils. In a correlation analysis, seven bacterial taxa were found to correlate with BPA degradation rate, and several additional taxa correlated with soil moisture. Together, these results suggest that long-term TOrC exposure may enhance BPA degradation and, in combination with increased soil moisture due to irrigation, alter rhizosphere microbial community composition. Further research is needed to examine the relationships between long-term TOrC exposure, enhanced TOrC degradation, and microbial community composition.

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1 Introduction and background

1.1 Literature Review

1.1.1 Trace-level organic contaminants

Trace-level organic contaminants (TOrCs) are a class of emerging chemicals that are increasingly detected in our water. They encompass several types of compounds, including pharmaceuticals, personal care products, fertilizers, pesticides, and industrial chemicals. TOrCs enter waterways with effluent from wastewater treatment plants and can impact organisms and ecosystems in a variety of ways, many of which are not yet understood. This study focuses on five TOrCs: bisphenol A, carbamazepine, gemfibrozil, ibuprofen, and naproxen. The compounds were chosen based on their prevalence in wastewater effluent and receiving waters (Table 1-1) and because analytical chemistry methods for their detection have previously been developed [1].

Bisphenol A (BPA) is an industrial precursor to plastics, epoxy resins, and some flame retardants [2]. It is estimated that 2.4 billion pounds of BPA are produced each year [2]. During manufacturing, trace levels of BPA are released into water and air [3]. Other sources of environmental release include inner coatings of metal cans [4] and thermal paper used for receipts [5]. The estimated yearly release of BPA into the environment is 1.1 million pounds [2].

Pharmaceuticals are also released into the environment in large quantities, often from hospitals and animal agriculture operations [6, 7]. Pharmaceuticals are classified by their use. Carbamazepine (CAR) is an anticonvulsant used to treat epilepsy. Gemfibrozil (GEM) is a medication for blood pressure control. Analgesics, such as ibuprofen (IBU) and naproxen (NAP), are pain relievers [6]. Trace amounts of these and many other pharmaceuticals, typically in the μg to ng/l range, are released into the environment in wastewater effluent [6].

TOrCs are beneficial when used in their proper context, but their unintended release into the environment can negatively impact aquatic ecosystems. The effects often depend on the nature of the compound. For example, BPA, a hormonally active chemical, can impact sexual development of aquatic organisms at concentrations as low as 1μg/L (Table 1-2). Antibiotics may select for antibiotic-resistant organisms in the environment, and pharmaceuticals can change the composition of natural microbial communities by selecting for organisms that otherwise would not be prevalent in the community [6]. Laboratory studies have tended to focus on the

effects of individual compounds on organisms, but this does not reflect the reality of nature. Most environments are polluted with a mixture of many TOrCs. These compounds may interact synergistically and behave in unpredictable ways [8-10].

Table 1-1: Concentrations of trace-level organic contaminants in receiving waters (μg/l).

Effluent source	**CAR**	**GEM**	**IBU**	**NAP**	**BPA**	**Ref.**
Various	0.1-0.5	0.010-1	0.001-1	0.08-0.3		[11]
Northern Scotland, near hospital	0.459 ± 0.133		0.073 ± 0.063			[7]
Gran Canaria, Spain (reverse osmosis)	0.00005	0.0001	0.00014	0.0001		[12]
Gran Canaria, Spain (electrodialysis)	0.0002	0.0007	0.00007	0.00012		[12]
Gran Canaria, Spain (wetland-based)	0.00006	0.0040	0.0098	0.0014		[12]
Samsun, Turkey	2.3 ± 0.27					[13]
Han river, S. Korea (fed by WWTP)	0.0688		0.327	0.079		[14]
Central Greece	0.0742					[15]
Clifynydd, Wales	2.499		0.263	0.370	86.0	[16]
Coslech, Wales	0.826		0.143	0.17	71.0	[16]
Zhejiang river, China (fed by WWTP)					471; 200	[17]
Dongjiang river, China (fed by WWTP)					406; 165	[17]
Seville, Spain	0.06; 0.15; 0.05; 0.14	3.07; 2.47; 1.52; 2.16	3.74; 1.05; 3.2; 8.0	2.58; 0.99; 0.15; 1.3		[18]
Baltimore, MD, United States		0.13	0.25	0.38		[19]
Basra, Israel (TWW-irrigated soil)	0.66	0.04	0.24	0.34		[20]
Mexico City		0.22	3.5	8.1		[21]
Mean concentrations (range)	0.506 ± 0.81 (0.00005-2.499)	0.885 ± 1.13 (0.0001-3.07)	1.36 ± 2.21 (0.00007-8.0)	0.989 ± 2.08 (0.0001-8.1)	233.17 ± 167.4 (71.0-471)	

CAR-carbamazepine; GEM- gemfibrozil; IBU- Ibuprofen; NAP- naproxen; BPA- bisphenol A

Table 1-2: Effects of Bisphenol A on select organisms.

Organism	Concentration	Effects	Ref
Ramshorn snail	1 μg/l	Super-feminization of female fish, overstimulation of egg clutch	[22]
Fathead minnow	0.16-1.28 mg/l	Male reproductive system deformation, decreased hatch rates	[23]
Three-spined stickleback	1, 10, 100 μg/l	Gonad deformation	[24]
Brown trout	1.75-5 μg/l	Complete loss of ovulation in females at 5 μg/l, decreased sperm quality in males at all concentrations	[25]
African frog tadpoles	10^{-7} M	Increased female to male ratio, feminization	[26]
African frog tadpoles	10-100 μM	Spinal malformations and apoptosis in CNS seen at 20 μM	[27]
Carp	1, 10, 100, 1000 μg/l	Changes to testes at all concentrations, 27% of fish became intersex	[28]
Carp	0.228-2.28 μg/l	Apoptosis induced in spleen lymphocytes	[29]
Zebra fish larva	1-12.5 mg/l	Exposed zebrafish embryos and saw a variety of negative effects	[30]
M. galloprovincialis	10 μg/l	Distinct differences between control and treated microbiomes	[31]
Young zebrafish	2-25 mg/l	Cardiac edema, disformed tails, and delayed hatch rate	[9]

1.1.2 TOrCs in wastewater treatment

Wastewater treatment plants protect our water by removing a variety of contaminants, including nutrients, suspended solids, organic compounds, and pathogens. They are not designed to treat the thousands of TOrCs that are increasingly common in our water [32]. Removal of these chemicals can vary from <1 percent to 99 percent, depending on the compound's structure and charge [11]. For example, 70 percent of BPA is degraded in some wastewater treatment plants, but in others as little as 10 percent is removed [33-35]. The fate of pharmaceuticals is similarly varied. Average ibuprofen and naproxen removal is around 80 percent but can vary from 0-99 percent and 0-95 percent respectively. Gemfibrozil and carbamazepine removal ranges from 0 to >95 percent, with averages of 50 percent and <20 percent, respectively [11].

TOrC degradation also correlates with the complexity of the wastewater treatment system. In treatment plants using only primary and secondary treatment, TOrCs are removed by biologic degradation, and by physical mechanisms including settling and sorption. Efficiencies of these systems are highly varied, with some TOrCs fully degraded and others barely removed [36]. Solids retention time is important for biologic degradation, and the optimal retention time can be difficult to determine. For example, a retention time of 2 to 5 days is needed for complete ibuprofen degradation [6], but over 20 days are required for complete carbamazepine removal [36].

Tertiary treatment steps further enhance the removal of TOrCs, but results vary by method and compound. Activated carbon can adsorb up to 99 percent of carbamazepine and 66 percent of BPA, but only 21 percent of ibuprofen [6, 33]. Oxidation with chlorine, UV light, or ozone can also enhance removal. Ozone appears to be the most effective method, with many common TOrCs removed almost completely during ozonation, including carbamazepine, ibuprofen, and BPA [6, 33]. Membrane bioreactors have shown some promise for TOrC treatment; both ibuprofen and BPA are almost completely removed in this process [33]. Finally, attached-growth bioreactors have the potential to remove many TOrCs, including over 90 percent of carbamazepine, gemfibrozil, ibuprofen, and naproxen [33].

Overall, TOrC removal in WWTPs correlates with solids retention time and complexity of the treatment process, with longer SRTs and more complex processes showing the greatest removal [33, 37]. Tertiary treatment methods, while highly effective, are often cost-prohibitive to

small wastewater treatment plants [38, 39]. A simple, affordable option for removing these contaminants is needed.

1.1.3 The rhizosphere in wastewater treatment

Wastewater treatment plants have recently begun to incorporate a new tertiary treatment method called rhizotreatment. This treatment harnesses the rhizosphere, which encompasses soils in the root zone and the microorganisms that dwell there, to degrade pollutants. The system was introduced to remove nitrogen and phosphorus from wastewater (Table 1-3). In rhizotreatment, a field of trees is planted near the wastewater treatment plant. Treated wastewater (TWW) is distributed onto the field via drip irrigation, where it moves through the soil and contacts rhizosphere microorganisms on the tree roots. These microorganisms and the trees that host them take up nitrogen and phosphorus and use them for growth [40].

The use of rhizotreatment has been shown to protect surface and groundwater from N and P runoff [40-42]. For example, in short rotation coppice systems planted with poplar and willow, the compounds are removed from primary effluent at rates up to 90 and 98 percent respectively [43, 44]. Most studies have not observed any negative impacts of TWW irrigation on tree health; in fact, the extra nutrient inputs often result in increased tree biomass [43-46].

The potential of rhizotreatment to remove compounds from wastewater extends beyond nitrogen and phosphorus. Studies have examined the fate of several TOrCs in rhizotreatment systems and observed varying levels of degradation. Removal rates range from <10 percent (carbamazepine) to complete removal (ibuprofen) [47]. More research is needed to investigate the efficiency of rhizotreatment in removing common TOrCs from treated wastewater.

Table 1-3: Nitrogen and phosphorus removal in vegetation filters.

Removal	Wastewater type	Ref
Nitrogen		
50%	After pre-treatment and OM degradation in stabilization ponds (29 kg N/ha)	[44]
90%	Primary effluent from small community (growing season)	[48]
45%	Primary effluent from small community (dormant season)	
90%	Municipal wastewater	[49]
80%	Primary effluent	[43]
Phosphorus		
70%	After pre-treatment and OM degradation in stabilization ponds (4 kg/ha)	[44]
98%	Primary effluent from small community	[48]
85%	Municipal wastewater	[49]
85%	Primary effluent	[43]

1.1.4 Microbial activity in the rhizosphere

The rhizosphere is home to one of the most diverse microbial communities on earth [50, 51]. Soil microbial activity is increased in the rhizosphere; bacterial populations can be up to two orders of magnitude higher compared to the bulk soil [50]. Rhizosphere microorganisms have the potential to use a wide variety of carbon and energy sources [52, 53], including environmental contaminants like pesticides, petroleum compounds, and micropollutants [54]. Microbial communities have high genetic diversity and short generation times, increasing the likelihood that genes to degrade TOrCs are present in the community or will arise with the appropriate selection pressure [55]. In addition, genes for degrading these contaminants are often contained on mobile elements, making it easier for them to spread through the community via conjugation [56].

1.1.5 Microbial degradation of TOrCs: Bisphenol A as an example

BPA-degrading microorganisms have been observed in many environments. Isolates from wastewater treatment plants and soil (Table 1-4) have demonstrated degradation capability, as have mixed environmental communities. In mixed communities, the presence of BPA may increase the abundance of the families *Pseudomonadaceae* and *Sphingomonadaceae* [57, 58]. For example, one study exposed sediment communities to BPA for several months. Over time, microbial community diversity decreased, but BPA degradation increased, and *Pseudomonas* and *Sphingomonas* were key genera in the process [59]. In another study, the genera *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* were enriched as BPA degradation increased from 28-29

percent to >90 percent in just two weeks [58]. Synergistic interactions also play a role in community BPA degradation [60].

Most experiments examining BPA degradation have used concentrations in the mg/L range, which are unlikely to be present in effluent (Table 1-1). Conversely, one strain of particular interest, *Sphingobium* BiD32, degraded BPA at environmentally relevant concentrations in the μg/l range [61]. A putative gene responsible for BPA degradation in BiD32 has been identified [62]. Further study is needed to determine its role and the extent to which the gene is present in the environment.

Table 1-4: Bacterial isolates capable of degrading bisphenol A.

Strain	**Source**	**BPA concentration**	**Ref**
Achromobacter xylosoxidans B16	Compost leachate	5-50 mg/L	[63]
Bacillus GZB	E-waste recycling site	10 mg/L	[64]
Sphingobium BiD32	Activated sludge	10 μg/L-100 μg/L	[61]
P. paucimobilis FJ-4	Activated sludge	0.1-1.0 mM	[65]
Pseudomonas MV1	Sludge from plastic production plant	1-1.5 mM	[66]
Sphingomonas AO1	Soil in vegetable-growing field	115 μg/ml	[67]
Arthrobacter sp. YC-RL1	Oil-contaminated soil	25 mg/L	[68]
Novosphingobium TYA-1	Reed plant rhizosphere	0.5 mM	[69]
Enterobacter gergoviae BYK-7	Petrochemical wastewater	200 mg/L	[70]
Bacillus megaserium ISO-2	Polycarbonate wastewater	5 mg/L	[71]
Shewanella haliotis MH 137742	Estuarine sediment	75 mg/L	[72]
Pseudomonas LBC1	Textile dye facility waste disposal site	160 μM	[73]
Nitrosomonas europaea	Activated sludge	0.2-1.6 mg/L	[74]
Pseudomonas aeruginosa PAb1	Effluent from thermal paper industry	10-40 mM	[75]

1.1.6 Droplet digital PCR

Droplet digital PCR (ddPCR) is a relatively new technique that can determine the exact concentration of target DNA in a sample, without the need for standards as in qPCR. The reaction is prepared using EvaGreen dsDNA binding dye, which is composed of two identical dye molecules connected by a flexible spacer. In the absence of double-stranded DNA, the two dye molecules interact with each other to form a loop structure that is weakly florescent. When double-stranded DNA is present, the dye molecules intercalate between the base pairs and separate, resulting in strong florescence [76, 77].

ddPCR is begun by loading reactants into a droplet generator, which partitions them into thousands of oil droplets. Then PCR is carried out as normal, and the PCR products remain separated within individual droplets. A droplet reader then quantifies the florescence of each droplet, and no-template controls are used to determine a minimum threshold for positive droplets. Based on the ratio of positive to negative droplets in the reaction, a Poisson distribution can be used to determine how many copies of the target DNA are present in the original sample. This method has higher reproducibility and precision than qPCR and can be up to ten times more sensitive [78, 79].

1.2 Research Questions

This study investigated the impacts of long-term TWW exposure on the rhizotreatment system. Specifically, the research examined how exposure to wastewater effluent affects (1) the degradation rate of select trace-level organic contaminants, (2) the composition of the rhizosphere microbial community, and (3) the overall abundance of bacteria, archaea, and a putative BPA degradation gene in rhizosphere soils exposed to treated wastewater for at least 10 years. Samples were obtained from three rhizotreatment fields, and from nearby control plots at each site. Batch degradation tests, microbial community sequencing and analysis, and ddPCR were carried out to investigate these questions. It was hypothesized that TWW exposure would increase the degradation rates of target TOrCs, increase the abundance of the putative BPA degradation gene, and alter the composition of the rhizosphere microbial community compared to controls

2 Methods

2.1 General methods

2.1.1 Chemicals and reagents

Bisphenol A, carbamazepine, gemfibrozil, ibuprofen, and naproxen (>98% purity) were obtained from Sigma Aldrich (St. Louis, MO, USA). Deuterated standards for HPLC analysis were obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). All other chemicals were obtained from Fisher Scientific (Waltham, MA, USA) and Sigma Aldrich.

2.1.2 Study system

Soils were collected from three WWTPs in central Idaho by collaborators from the University of Idaho. These WWTPs have used rhizotreatment for at least 10 years, while also maintaining nearby control plots that were not exposed to effluent. Two sites, Ellisport and Garfield Bay, were dominated by Western red cedar, while the third, Heyburn, was dominated by Douglas fir and Ponderosa pine. Samples were taken from the control and treated rhizospheres in August 2020 and transported on ice to the University of Washington. Samples were stored at 4^{o}C upon arrival. Biodegradation tests and DNA extraction were conducted within one month.

2.1.3 Media preparation

Mineral medium was prepared as described in the *Manual of Environmental Microbiology* [80] with the modifications described in Zhou et al. [61]. In place of sodium bicarbonate, sodium carbonate was used as a buffer. Vitamin, mineral, and trace metal stock solutions were prepared at 1000x and filter sterilized. Buffer solutions were filter sterilized and added to medium at a final concentration of 2mM.

2.1.4 Soil characterization

Soil moisture was quantified by oven drying, and soil organic carbon by loss on ignition [81]. Soil texture was assessed using ASTM standard method D7928-17 [82, 83]. Briefly, 50-70 g of oven dry soil were added to a 1000 ml graduated cylinder along with 5 g of sodium hexametaphosphate as a dispersant. Deionized water was added to bring the total volume to 1000 ml. The solution was mixed thoroughly, and soil particles settled for 24 hours, with hydrometer readings taken at 1, 2, 4, 15, 30, 60, 240, and 1440 minutes. The readings were used to calculate the fractions of sand, silt, and clay in the soil samples [83].

2.2 Degradation experiments

2.2.1 Flask preparation

1000 mg/l stock solutions of bisphenol A, carbamazepine, gemfibrozil, ibuprofen, and naproxen were prepared in acetonitrile. To prepare flasks with 1 mg/l of the target TOrCs, 150 µl of each stock solution were added to a flask and acetonitrile was evaporated for 15 minutes under nitrogen gas. 140 ml of deionized water were added, and flasks were autoclaved to dissolve TOrCs. Mineral medium stock solutions were added, and final TOrC concentrations were measured to account for losses during autoclaving. Flasks were sealed with a rubber stopper and stored in the dark until use.

2.2.2 Experimental procedure

Short-term batch degradation experiments were conducted according to OECD protocol #307 [84] with slight modifications. Briefly, soils were screened to remove rocks and sticks, and to homogenize particle size. 50 g of dry soil were added to flasks of 150 ml of mineral medium in triplicate. Flasks were incubated at 20^{o}C in the dark to avoid photodegradation of TOrCs, and air was bubbled into flasks with a glass pipette to maintain an aerobic environment. Samples were collected daily in duplicate for 7 days. Controls consisted of parallel analyses with autoclaved soil from each sampling site.

2.2.3 Preparation of HPLC samples

Immediately after sampling, a solvent extraction was performed by adding 0.5 ml of acetonitrile (HPLC grade) to 0.5 ml of sample and vortexing. Samples were then centrifuged for 10 minutes at 10,000 rpm and the supernatant was transferred to HPLC vials and stored at 4^{o}C. Prior to HPLC analysis, samples were spiked with an internal standard solution in acetonitrile. Internal standards were prepared from acetonitrile stock solutions as follows: 250 µl of bisphenol A-d4 (200 mg/l stock), 50 µl of carbamazepine-d10 (100 mg/l stock), 250 µl of naproxen-d3 (100 mg/l stock), and 125 µl of ibuprofen-d3 (200 mg/l stock) were added to 50 ml of acetonitrile. Internal standards were spiked into each sample for final concentrations of 500 µg/l bisphenol A-d4, 50 µg/l carbamazepine-d10, and 250 µg/l naproxen-d3 and ibuprofen-d3.

2.2.4 HPLC analysis

Trace-level organic contaminants were separated and analyzed using an Agilent 1290 Infinity Ultra-High Performance Liquid Chromatography (UHPLC) paired with an Agilent 6460 triple

quadrupole dual mass spectrometer with electrospray ionization (Santa Clara, CA, USA). The column used was an Agilent Eclipse Plus C18 (21 mm x 100 mm, 3.5 μm particle size), with a Zorbax Eclipse XDB-C18 guard column (2.1 mm x 12.5 mm, 5 μm particle size) [1]. Samples were transported on ice to the University of Washington Tacoma laboratories at the Center for Urban Waters (Tacoma, WA, USA) and analyzed according to the method developed by James et al [1]. Briefly: 15 μl of sample was injected, and columns were held at 45°C. The gradient was as follows: started at 80% A (5 mM ammonium acetate) and 20% B (methanol:acetonitrile 1:1), 5% A and 95% B from 1.25 minutes to 4 minutes, 80% A and 20% B at 5 minutes. Mass spectrometer analysis was conducted according to the protocol in Hou et al [85]. Linear calibration curves were generated by plotting relative response (response area of compound divided by the peak area of the internal standard) against known concentration. Detection limits were established based on the concentrations used to generate the curve [1].

2.2.5 Data analysis

TOrC concentrations were plotted over time assuming first-order degradation kinetics. First-order degradation can be modeled by to the equation

$$C = C_0 e^{-kt}$$

Where C_0 is the initial concentration, k is the degradation constant, and t is time. To graphically determine the degradation constant, the equation was linearized to

$$\ln(C) = -kt + \ln(C_0)$$

Statistical analyses were then conducted in RStudio [86] to compare degradation rates between treated and control rhizosphere soils. Two-sided t-tests (α=0.05) were used to compare the degradation rate of each compound between treated and control soils. An analysis of variance (α=0.05) was conducted to test the effects of treatment, site, and treatment/site interactions on degradation rates of TOrCs. Code for these analyses can be found in Appendix A.

2.3 Microbial community analysis

2.3.1 Soil DNA extraction and sequencing

DNA was extracted from Heyburn soils using the FastDNA SPIN kit for soils (MP Biomedicals, Irvine, CA, USA) and from Ellisport and Garfield Bay soils using the Qiagen Spin Kit for Soils (Qiagen Sciences, Germantown, MD, USA). Extractions were performed according to respective

manufacturer protocols. Briefly: Cells were homogenized using a FastPrep-24 bead beater and lysis system (MP Biomedicals, Irvine, CA, USA) and centrifuged to remove cell debris. DNA was separated from the solution through a series of washing steps and eluted in the provided buffer solutions. DNA concentration and quality were determined using a Nanodrop One-C (Thermo Scientific, Waltham, MA, USA). Extracted DNA was normalized to 20 ng/µl and sent to Genewiz (South Plainfield, NJ, USA) for sequencing.

2.3.2 16S-EZ sequencing and QIIME data analysis

16S-EZ library preparation and next generation sequencing were performed by Genewiz (South Plainfield, NJ, USA). Briefly, sequencing libraries were prepared using a MetaVX 16S rDNA Library Preparation Kit (Genewiz) and loaded on an Illumina MiSeq instrument (Illumina, San Diego CA, US) according to manufacturer protocol. Sequencing was conducted using a 2x250 paired-end configuration. Base calls were made by internal Illumina software and sequence data was demultiplexed and converted to FASTQ format with Illumina's bcl2fastq 2.17 software. Data analysis was conducted using Qiime (version 1.9.1). Pair-end reads were joined, and barcode and primer sequences were removed. Chimeric sequences were removed and the remaining sequences were clustered into operational taxonomic units (OTUs) and assigned taxonomy based on comparison to the Greengenes database [87]. Alpha diversity was calculated using both Shannon and Chao1 indices. Community composition was examined across multiple taxonomic levels. Student's t-tests were conducted in RStudio to compare the abundance of select taxa between treated and control communities. A principal component analysis was also conducted. Code for these analyses can be found in Appendix A.

To examine potential relationships between the microbial community and other factors in the rhizotreatment system, correlation coefficients were generated using the equation

$$Correlation\ (X,Y) = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{\sum(x-\bar{x})^2}\sum(y-\bar{y})^2}$$

Pairs of factors with coefficients > 0.5 were considered to be correlated.

2.4 ddPCR

2.4.1 Primers

Amplification of bacterial and archaeal 16S rRNA was conducted using primers designed by Lane et al. [88] and Yu et al. [89], respectively (Table 2-1). Primers to quantify the putative BPA degradation gene N1MWA7, identified in Zhou et al. [62], were designed for this study using Primer3 (https://primer3.ut.ee/). Three primer sets were generated: 1079F/1468R, 2363F/3362R, and 490F/895R (Table 2-2). BiD32, the *Sphingobium* isolate in which the gene was first identified, was used to test the efficacy of the primers. Strain IBU4, which does not degrade BPA, was used as a negative control. Primer set 1079F/1468R was chosen for subsequent experiments.

Table 2-1: Primers used in ddPCR quantification experiments.

Target	Name	Annealing temp	FWD primer sequence	REV primer sequence	Ref
Bacteria	1114F/1275R	57°C	5'-CGG CAA CGA CGC CAA CCC-3'	5'- CCA TTG TAG CAG CAC GTG TGT AGC C-3'	[88]
Archaea	109F/348R	59°C	5'-ATTAG ATACC CSBGT AGTCC-3'	5'- GCCAT GCACC WCCTC T-3'	[89]
N1MWA7	1079F/1468R	51°C	5'- GCT TAA ATC TGG CAG CGT CG-3'	5'- GCA CGT CAT GCC GGA TAA AC-3'	This study

Table 2-2: Primer sets designed for amplification of the putative bisphenol A degradation gene N1MWA7.

Primer set	FWD primer sequence	REV primer sequence
1079F/1468R	5'-GCT TAA ATC TGG CAG CGT CG-3'	5'-GCA CGT CAT GCC GGA TAA AC-3'
2363F/3362R	5'-GGC AGC AAA GTT GTC CTG TG-3'	5'-TGA AAC TCT TCC TTC CGG GC-3'
490F/895R	5'-ACA ACA GGA GGT AAT GCG GG-3'	5'-CAT CGG GCC AAT CCT CTA CC-3'

2.4.2 ddPCR methods

ddPCR was used to quantify bacterial and archaeal 16S and abundance of the putative BPA degradation gene N1MWA7. All materials were obtained from BioRad (Hercules, CA, USA) unless otherwise noted. Each reaction contained 11 µl of QX200 EvaGreen 2x super mix, 5 µl of DNA, and forward and reverse primers at a concentration of 125 nM each. Reactions were prepared in 1.5 ml tubes and transferred to the QX200 Droplet Digital PCR Droplet Generator for partitioning. Droplets were transferred to a deep-well PCR plate and placed in a C1000 Touch Thermocycler for amplification. PCR was conducted according to the EvaGreen Supermix protocol with annealing temperatures optimized for each primer set. The following program was used: 5-minute activation at 95°C, 30 seconds at 95°C, 1 minute at 59°C (archaea), 57°C (bacteria) or 51°C (BPA gene), for 40 cycles, a 5-minute stabilization at 4°C, 5 minutes at 95°C, and an infinite hold at 12°C. Droplets were read with a QX200 Droplet Reader and interpreted by the accompanying Quantasoft analysis software. No-template controls (NTCs) were included in each run and used to calculate florescence thresholds for positive droplets.

2.4.3 Data analysis

Quantasoft Analysis Pro (version 1.0) was used to determine the raw number of positive and negative droplets in each well. Threshold values for positive droplets were determined based on the mean fluorescence in NTC wells. Total concentration in soil samples was determined using the following equation

$$\frac{x\ copies}{\mu l} \times \left(1 - \frac{NTC\ positives}{NTC\ total}\right) \times \frac{5000\mu l}{y\ grams\ dry\ soil} \times b$$

Where x represents the number of copies determined by the Quantasoft program, y represents the mass of dry soil used in the original extraction, and b is a constant accounting for the dilution of DNA when preparing ddPCR samples (b=440 for bacterial 16S, b=44 for archaeal 16S and N1MWA7). DNA was extracted quantitatively from Ellisport and Garfield Bay samples. To allow for comparison with the other soils, extraction efficiency for Heyburn was estimated by averaging the quantitatively obtained efficiencies.

Two-sided t-tests were conducted in RStudio to compare the abundance of all three targets between treated and control soils. An analysis of variance was also conducted to examine the effects of site and treatment on abundance. Code for these analyses can be found in Appendix A.

3 Results

3.1 Soil characteristics

Site characteristics and measured soil texture are shown in Table 3-1 and Table 3-2, respectively. Two of the three sites, Ellisport and Garfield Bay, had similar soil classifications and dominant tree species. Measured soil texture at these sites differed slightly from the reported texture, silt loam. Organic carbon content differed between treated and control soils at Garfield Bay. Soil moisture content, measured as received in the lab, was higher in treated soils compared to controls, potentially due to supplementation of water associated with TWW.

Table 3-1: Characteristics of Heyburn, Ellisport, and Garfield Bay sampling sites.

Site	Location	Soil Type (reported)	Dominant tree species	N (lb/ ac/yr)	P (lb/ ac/yr)	Irrigation duration	Treatment	Organic Carbon (%)	Moisture (%)*
Heyburn	Plummer, ID	Carlinton dry complex, Carlinton ashy silt loam	Ponderosa pine and Douglas fir	119	20	10 years	Treated	7.08	10.35
							Control	7.60	6.91
Ellisport	Hope, ID	Pend Oreille silt loam	Western Red Cedar, Conifers	87	20	20 years	Treated	11.24	32.57
							Control	11.79	13.15
Garfield Bay	Sagle, ID	Pend Orielle-Hoodoo silt loams	Western Red Cedar, Conifers	136	-	45 years	Treated	10.67	37.77
							Control	6.24	9.62

*Measured within one week of receiving soils

Table 3-2: Texture of Heyburn, Ellisport, and Garfield Bay soils.

Site	Treatment	% sand	% silt	% clay	Classification
Heyburn	Treated	50.8	46.2	3.0	Sandy loam
	Control	48.1	48.4	3.5	Sandy loam
Ellisport	Treated	60	38.6	1.4	Sandy loam
	Control	79.3	19.8	0.9	Loamy sand
Garfield Bay	Treated	25.1	71.6	3.3	Silt loam
	Control	38.1	58.7	3.2	Silt loam

3.2 TOrC degradation experiments

3.2.1 Degradation rates

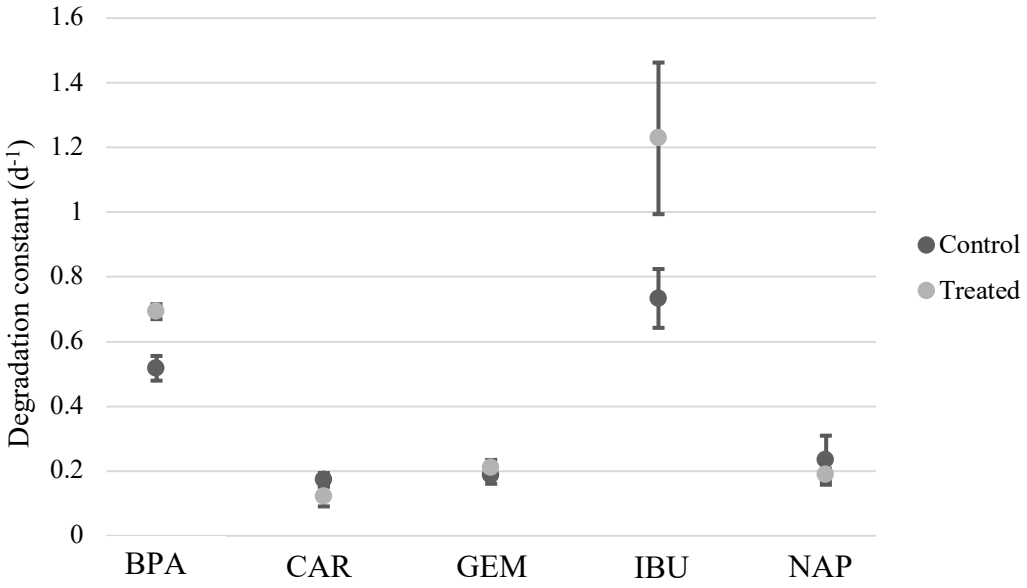
Short-term batch experiments were carried out to determine degradation rates of TOrCs by treated and control rhizosphere soils. Degradation was plotted over time (Figure A1) and degradation of all compounds approximately followed first order kinetics. Linearized data were then plotted to graphically determine degradation rate constants (Figure A2). Rate constants are presented in Table 3-3. In both treated and control soils, BPA and ibuprofen were degraded to below the limit of detection in all samples within 7 days. Autoclaved controls from these sites did not show degradation of either compound. This suggested that Heyburn, Ellisport, and Garfield Bay soils had an intrinsic ability to degrade ibuprofen and BPA. Additionally, BPA degradation was enhanced in treated soils from all three sites. This suggested that the gene or genes responsible for BPA degradation were present in higher proportions in treated soils compared to controls. Carbamazepine, gemfibrozil, and naproxen showed limited degradation over the one-week duration of the experiment. Slightly enhanced carbamazepine degradation was observed in Ellisport treated soils compared to controls.

Table 3-3: Degradation rates of trace-level organic contaminants (day^{-1}).

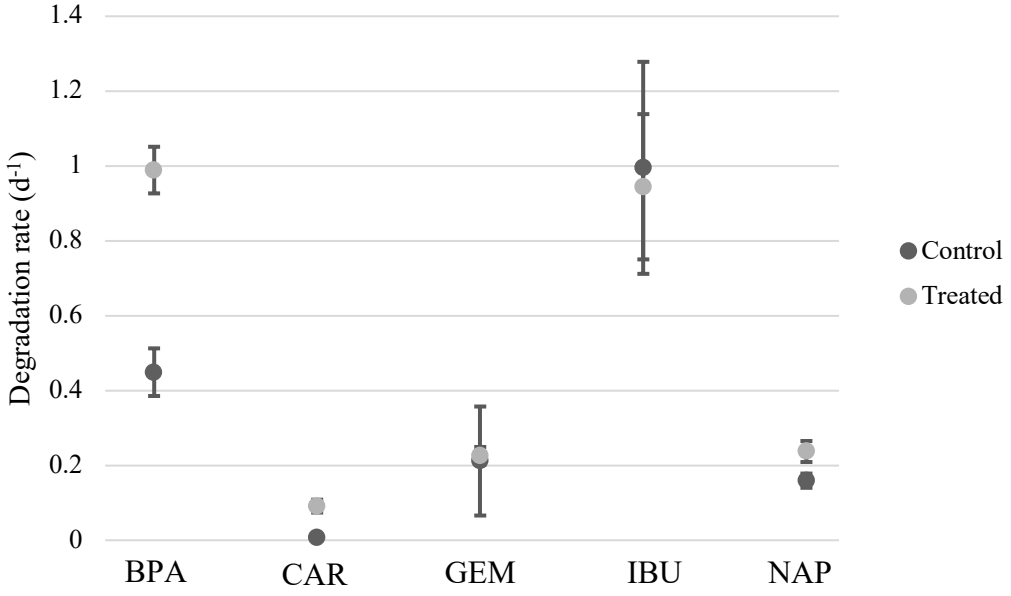
	BPA	**CAR**	**GEM**	**IBU**	**NAP**
Hey-T	0.692 (0.023)	0.121 (0.030)	0.210 (0.024)	1.23 (0.234)	0.188 (0.019)
Hey-C	0.518 (0.038)	0.173 (0.021)	0.187 (0.026)	0.734 (0.091)	0.234 (0.076)
Ell-T	0.989 (0.062)	0.091 (0.017)	0.225 (0.024)	0.945 (0.194)	0.237 (0.028)
Ell-C	0.449 (0.064)	0.008 (0.005)	0.212 (0.146)	0.995 (0.283)	0.159 (0.019)
Bay-T	0.783 (0.095)	0.043 (0.011)	0.126 (0.017)	1.047 (0.268)	0.173 (0.026)
Bay-C	0.472 (0.093)	0.063 (0.011)	0.136 (0.0002)	1.36 (0.229)	0.202 (0.016)

BPA- bisphenol A; CAR- carbamazepine; GEM- gemfibrozil; IBU- ibuprofen; NAP- naproxen
Hey- Heyburn; Ell- Ellisport; Bay- Garfield Bay
Average deviations are shown in parentheses (n=3)

a. Heyburn



b. Ellisport



c. Garfield Bay

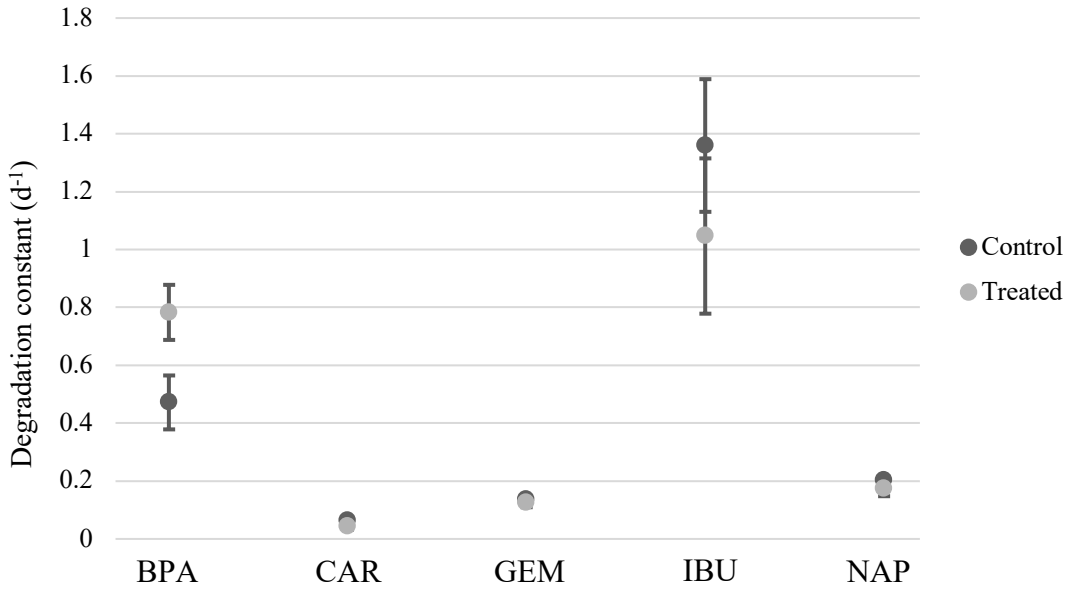


Figure 3-1: Degradation of trace-level organic contaminants by rhizosphere soils from (a) Heyburn (b) Ellisport and (c) Garfield Bay.
Error bars depict average deviation (n=3)
(BPA – bisphenol A; CAR – carbamazepine; GEM – gemfibrozil; IBU – ibuprofen; NAP – naproxen)

3.2.2 Statistical analysis

Two-sided Student's t-tests were conducted to test for significant differences in degradation rate between treated and control soils (Table 3-4). BPA degradation rates were significantly higher in treated soils from all three sites (Heyburn p=0.019; Ellisport p=0.001; Garfield Bay p=0.042). Carbamazepine degradation was also significantly higher in Ellisport treated soils (p=0.015).

Analysis of variance tests were conducted to determine the influence of treatment, site, and interaction effects on degradation rates (Table 3-5). A single factor did not emerge for the prediction of TOrC degradation rates. BPA degradation was significantly influenced by treatment (p=1.13e-5) and interactions between soil and treatment (p=0.028). Carbamazepine degradation was significantly influenced by soil ($p\approx0$) and interaction effects (p=0.004). Gemfibrozil, ibuprofen, and naproxen degradation were not influenced by soil, treatment, or interactions.

Table 3-4: Two-sided t-tests comparing degradation rates of trace-level organic contaminants between treated and control soils.

Site	Compound	p-value
Heyburn	BPA	0.019*
	CAR	0.118
	GEM	0.211
	IBU	0.097
	NAP	0.518
Ellisport	BPA	0.001*
	CAR	0.015*
	GEM	0.941
	IBU	0.858
	NAP	0.061
Garfield Bay	BPA	0.042*
	CAR	0.18
	GEM	0.545
	IBU	0.337
	NAP	0.335

BPA- bisphenol A; IBU- ibuprofen; GEM- gemfibrozil; CAR- carbamazepine; NAP- naproxen
*Denotes significant p-value (p<0.05)

Table 3-5: Analysis of variance comparing the effects of site and treatment on degradation of trace-level organic contaminants.

	DF	F value	Pr(>F)
BPA			
Soil	2	2.77	0.106
Treatment	1	57.02	1.13e-5*
Interaction	2	5.04	0.028*
Residuals	11		
CAR			
Soil	2	24.8	8.39e-5*
Treatment	1	0.239	0.635
Interaction	2	9.98	0.004*
Residuals	11		
GEM			
Soil	2	2.57	0.121
Treatment	1	0.172	0.686
Interaction	2	0.184	0.834
Residuals	11		
IBU			
Soil	2	1.103	0.363
Treatment	1	0.091	0.767
Interaction	2	2.702	0.107
Residuals	12		
NAP			
Soil	2	0.325	0.729
Treatment	1	0.003	0.955
Interaction	2	2.67	0.11
Residuals	12		

BPA- bisphenol A; IBU- ibuprofen; GEM- gemfibrozil; CAR- carbamazepine; NAP- naproxen
*Denotes significant p-value (p<0.05)

3.3 Microbial community analysis

3.3.1 DNA extraction and sequencing

Extracted soil DNA was quantified by nanodrop (Table 3-6). Extracted DNA was sent to Genewiz for sequencing and processed according to their standard NGS 16S-EZ pipeline. Merging the pair-end reads produced a total of 6,221,048 reads in 12 samples. OTU binning resulted in 9634 OTUs. 44 phyla, 120 classes, 157 orders, 168 families, and 201 genera were represented.

Table 3-6: DNA extraction yield and OTU diversity statistics.

Site	Treatment	dsDNA (ng/µl)	A260/280	A260/230	No. reads*	Richness (Chao1)	Diversity (Shannon)
Heyburn	Treated	22.6	1.70	0.23	210317 (64077)	6205.7 (30.1)	10.2 (0.0)
	Control	22.2	1.76	0.26	214058 (78333)	6319.9 (69.0)	10.28 (0.007)
Ellisport	Treated	32.3	1.85	1.99	233109 (18739)	6305.2 (16.1)	10.13 (0.014)
	Control	49.1	1.86	1.95	229420 (20239)	5890.0 (88.2)	9.94 (0.014)
Garfield Bay	Treated	34.7	1.84	1.91	204598 (38867)	6494.0 (76.6)	10.59 (0.28)
	Control	33.2	1.83	2.03	204017 (36912)	6294.0 (98.9)	10.22 (0.35)

Average deviations are shown in parentheses (n=2)

*Non-chimera pair-end reads in each sample

3.3.2 Microbial community diversity

At the phylum level, community composition was similar across all samples. The most common phyla were *Proteobacteria, Acidobacteria*, and *Actinobacteria* (Figure 3-2). These phyla were present in similar abundance across treatments apart from *Actinobacteria*, which was less abundant in Ellisport and Garfield Bay treated soils due to decreases in the orders *Solirubrobacterales* and *Gaiellales* (Table 3-7). In addition, *Bacteroides* were more abundant in Garfield Bay treated soils and less abundant in Ellisport treated and control soils. *Firmicutes* were more abundant in Heyburn treated soils, while phyla *Gemmatimonadetes* and *Nitrospirae* were higher in Ellisport treated soils.

The most abundant OTUs were visualized in a heatmap (Figure 3-3). OTUs 10 (genus *Bradyrhizobium*) and 61 (genus *Rhodoplanes*) were observed in all soils, and OTU 78 (phylum *Acidobacteria*) was abundant in all soils except for Ellisport control. Aside from these shared OTUs, the communities clustered in three distinct groups. Heyburn treated and control soils were most like each other and were notably different from the Ellisport and Garfield Bay sites. OTUs 197 (class *Acidobacteria*) and 4238 (class *Chloracidobacteria*) were particularly abundant in Heyburn soils, while OTUs 112 (order *Nitrospirales*) and 143 (class *Betaproteobacteria*) were absent in Heyburn and abundant in the other two soils. The Ellisport and Garfield Bay soils were grouped by treatment. Ellisport control soils were most like Garfield Bay control soils and the same was true of the treated soils. OTUs 183 and 68 (class *Gemmatimonadetes*) were abundant in the treated soils only, while OTUs 713 (class *Chloracidobacteria*), 57 (family *Sinobacteraceae*), and 111 (phylum *Acidobacteria*) were abundant only in control soils.

Table 3-7: Relative abundance of select taxa in Heyburn, Ellisport, and Garfield Bay treated and control soils.

	Heyburn		**Ellisport**		**Garfield Bay**	
	Treated	*Control*	*Treated*	*Control*	*Treated*	*Control*
Actinobacteria	16.6*	21.05	9.51*	20.11	7.74*	16.53
Solirubrobacterales	3.84*	6.35	2.27*	5.05	1.65*	3.95
Gaiellales	3.44	4.09	1.74*	2.42	1.46*	3.08
Bacteroidetes	6.63	5.83	4.41	4.29	9.39*	4.82
Flavobacterium	0.515*	0.305	0.54	0.30	2.19*	0.39
Firmicutes	2.28*	0.2	0.21	0.2	0.16	0.18
Bacilli	2.25*	0.18	0.18	0.175	0.135	0.165
Gemmatimonadetes	4.43	3.35	6.68*	3.35	4.35	4.18

*abundance in treated soils differs significantly from controls ($p<0.05$)

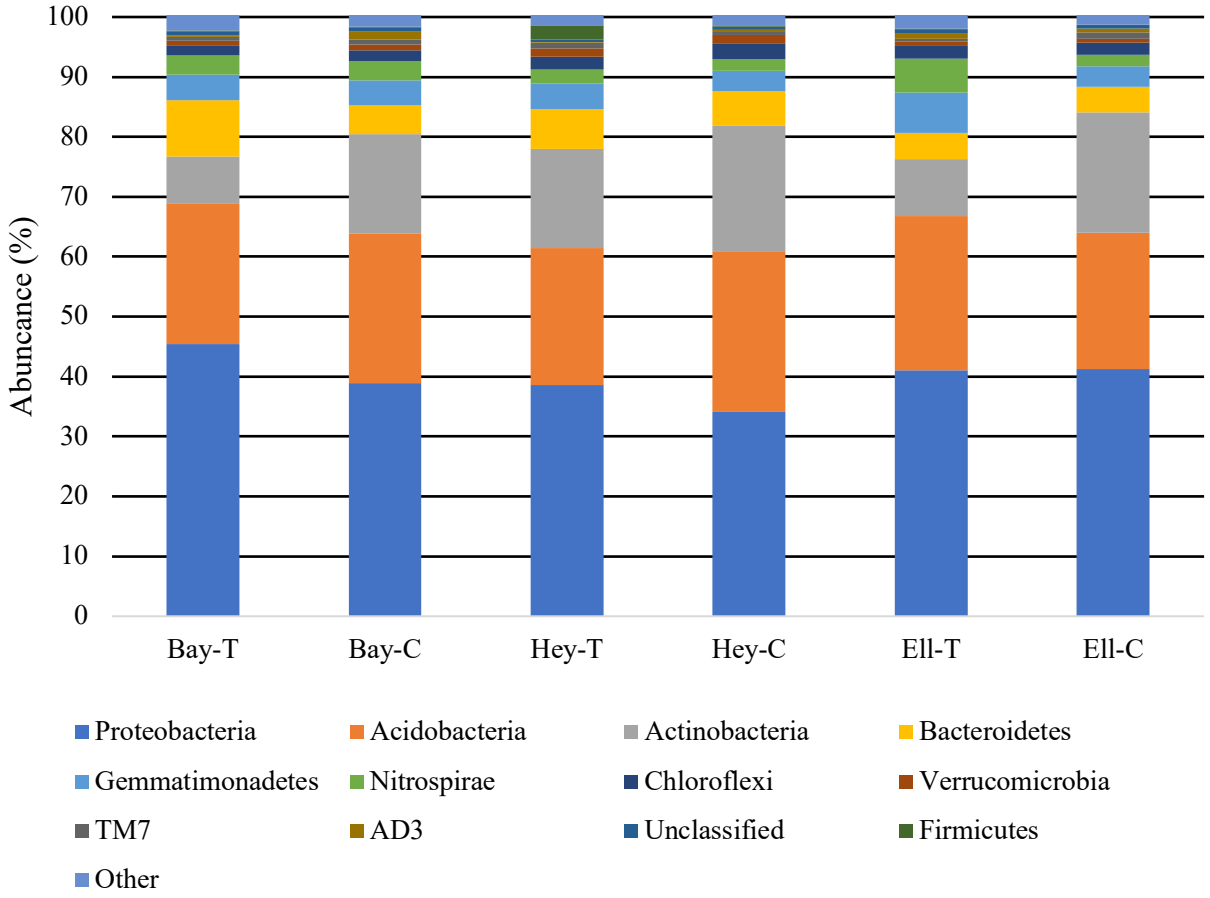


Figure 3-2: Most abundant phyla in DNA extracts from treated and control soils.
(Bay- Garfield Bay; Hey- Heyburn; Ell- Ellisport)

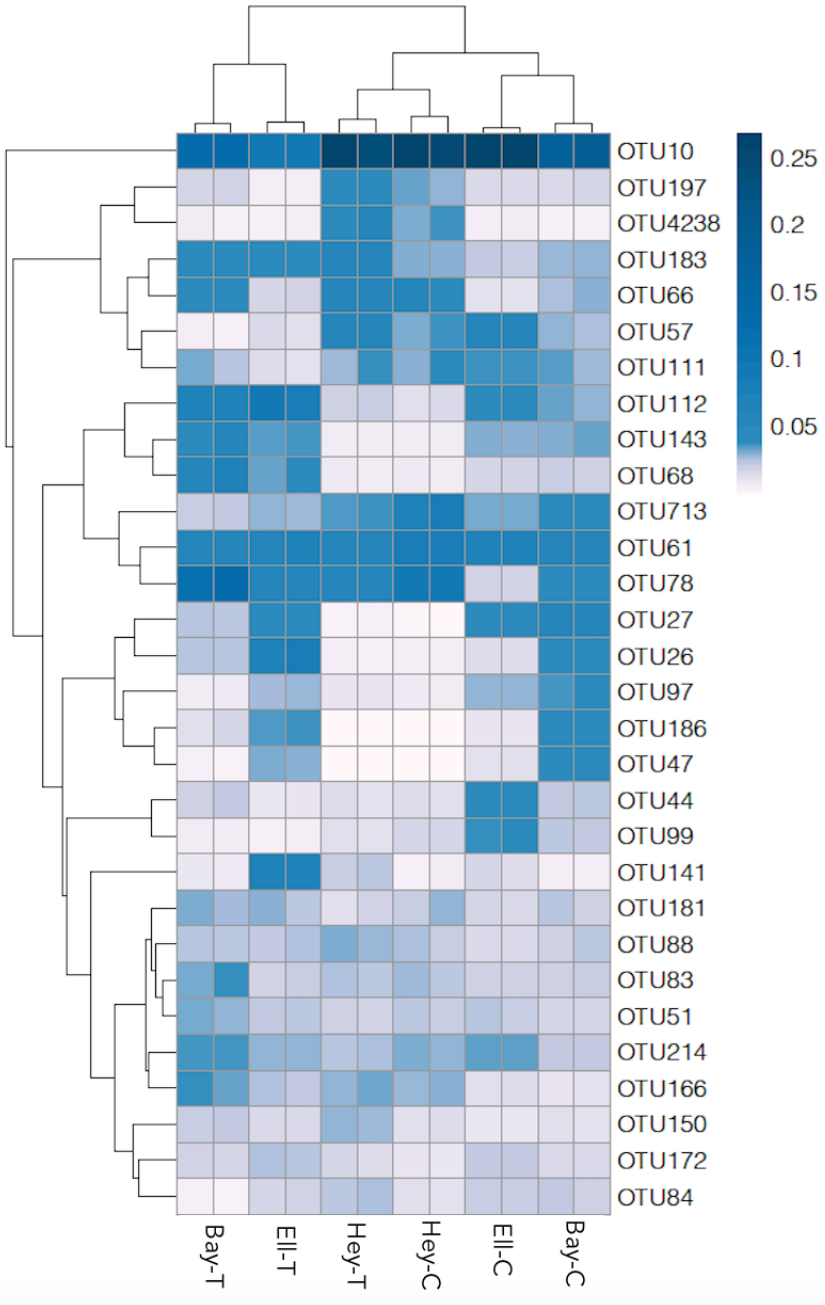


Figure 3-3: Heatmap of the most common OTUs in treated and control rhizosphere soils. Darker squares indicate greater abundance of OTUs.

3.3.3 Principal component analysis

A principal component analysis was conducted in RStudio (Figure 3-4) to examine differences between treated and control communities. PC1 explained 21.9% of the variation among communities and PC2 explained an additional 17.4%. As in the heatmap, Heyburn treated and control soils clustered together. Ellisport soils were separated mostly along PC1, and Garfield Bay soils were separated along both PC1 and PC2. This suggested that there were significant differences in microbial community composition between treated and control soils at both sites. The top OTUs driving the differences between microbial communities were OTU 10 (genus *Bradyrhizobium*), OTU 713 (class *Chloracidobacteria*), OTU 61 (genus *Rhodoplanes*), OTU 78 (class *Acidobacteri*a-6), OTU 112 (order *Nitrospirales*).

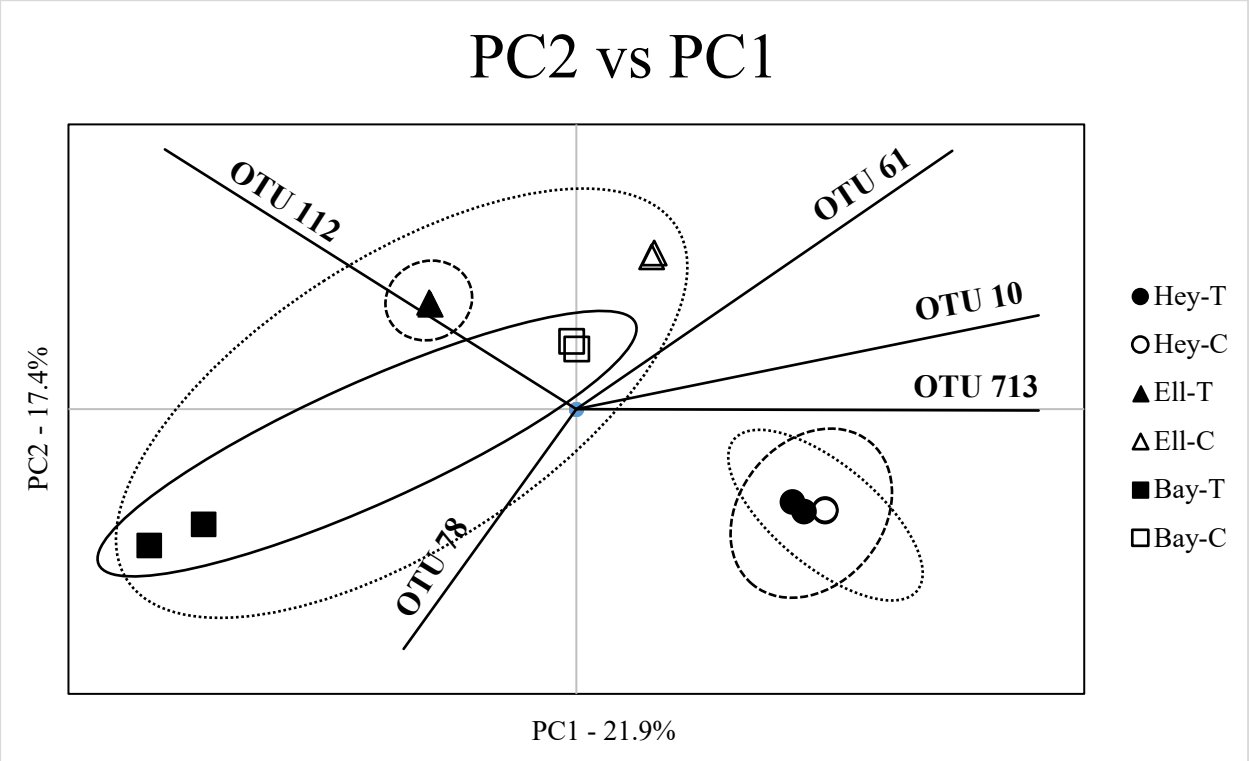


Figure 3-4: Principal component analysis comparing treated and control soils. PC1 accounted for 21.9 percent of the variation in the data, and PC2 accounted for an additional 17.4 percent. Solid line encircles silt loam soils and dotted lines encircle sandy loam soils. Ellisport control soil was classified as loamy sand. Dotted lines encircle soils with similar tree species composition.
(Hey- Heyburn; Ell- Ellisport; Bay- Garfield Bay)

3.3.4 Correlations

Correlation analysis was conducted in Excel to examine relationships between the presence of specific microbial groups and explanatory variables. Correlation coefficients are shown in Table 3-8. There were strong negative correlations between BPA degradation constant and both *Solirubrobacterales* and *Actinobacteria* (Figure 3-7a), and strong positive correlations between degradation constant and *Gemmatimonadetes* and *Nitrospirae.* BPA degradation was moderately positively correlated with OTU 713 and moderately negatively correlated with OTU 143 (Figure 3-7b). Finally, BPA degradation was moderately positively correlated with the ratios of bacteria and archaea to the BPA degradation gene.

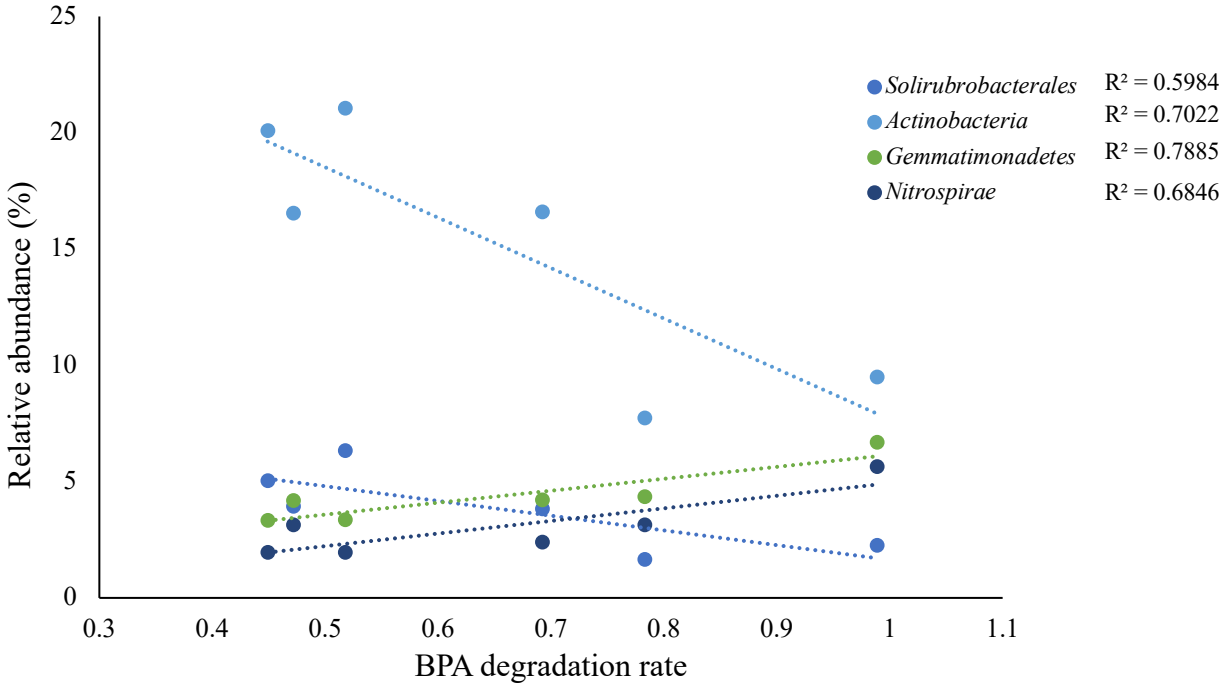
Soil moisture correlated positively with OTUs 143 and 68 (Figure 3-7c). It correlated negatively with OTUs 57, 112, 10, 713, and 197. The strongest positive correlation was between soil moisture and OTU 68 (0.933) while the strongest negative correlation was between moisture and OTU 10 (-0.839).

Table 3-8: Correlation coefficients relating differences in taxa abundance with BPA degradation and soil moisture.

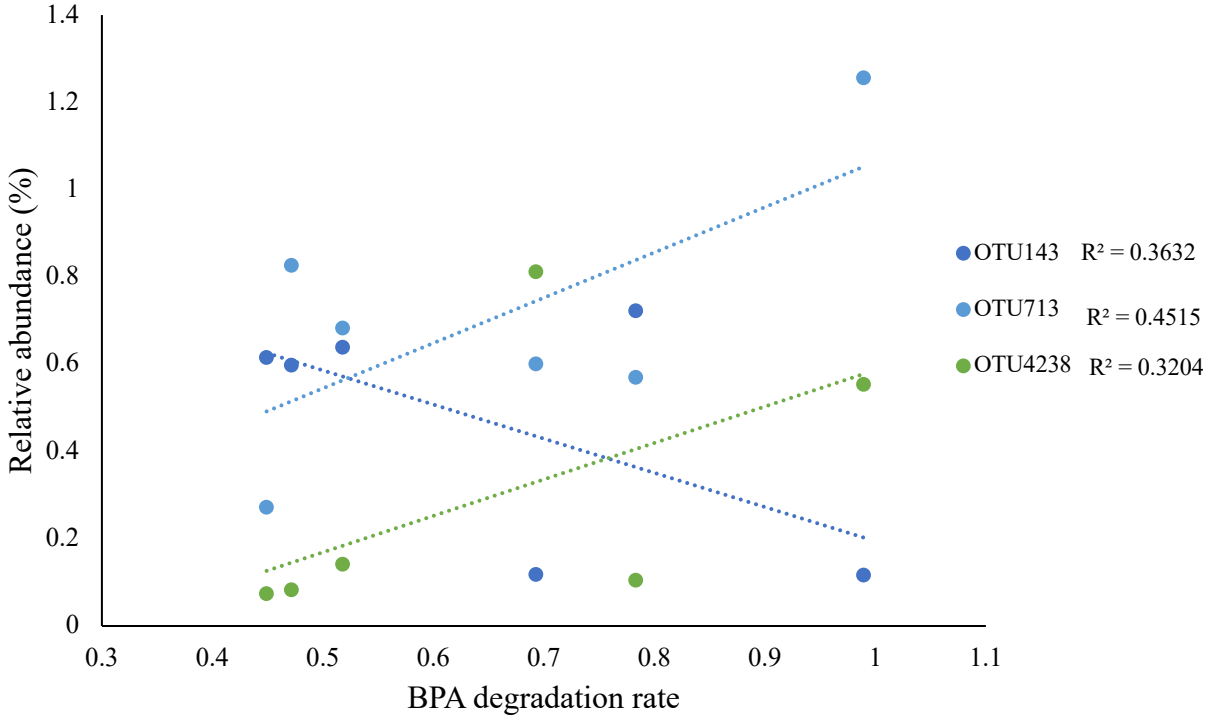
	Taxon	Correlation*
BPA degradation	*Solirubrobacterales*	-0.774
	Gaiellales	-0.608
	Actinobacteria	-0.838
	Gemmatimonadetes	0.888
	Nitrospira	0.827
	OTU 143	-0.603
	OTU 713	0.672
	OTU 4238	0.566
Soil moisture	OTU 10	-0.839
	OTU 112	0.745
	OTU 713	-0.772
	OTU 197	-0.750
	OTU 143	0.620
	OTU 68	0.933
	OTU 57	-0.740
	OTU 111	-0.803

*Variables with correlations >0.50 are shown here

a. Taxa abundance and BPA degradation rate



b. OTU abundance and BPA degradation rate



c. OTU abundance and soil moisture

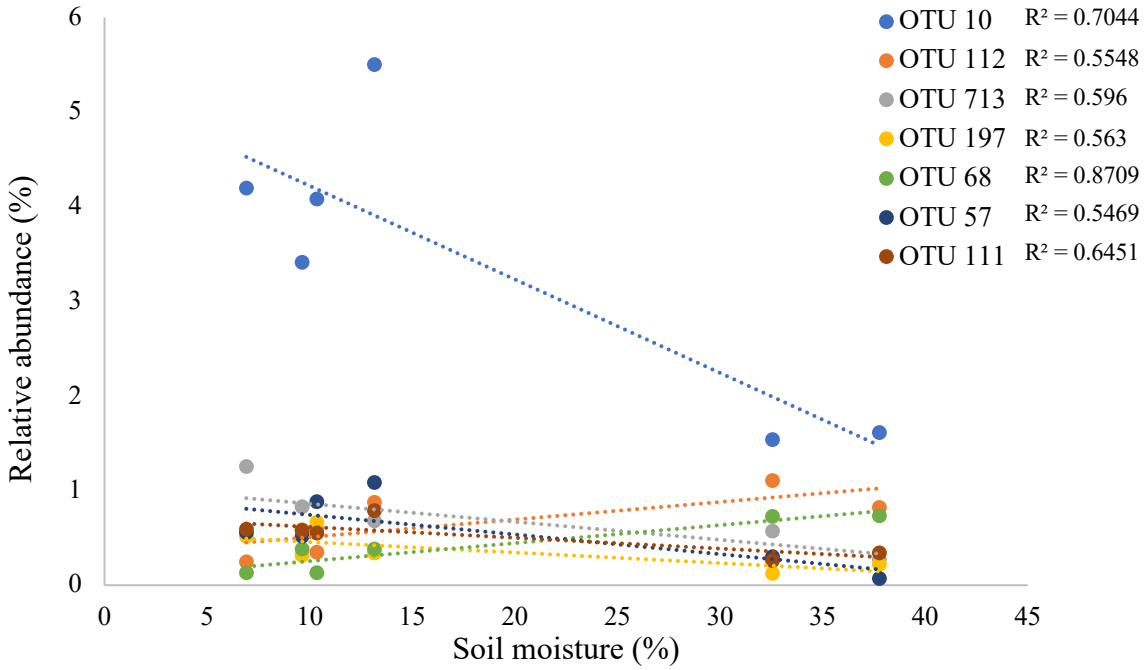


Figure 3-5: Significant correlations between: (a) taxa abundance and BPA degradation rate; (b) OTU abundance and BPA degradation rate; (c) OTU abundance and soil moisture.

3.4 ddPCR

3.4.1 Methods development and optimization

Temperature-gradient PCR was conducted to determine the ideal annealing temperatures for 16S bacterial and archaeal primers. Bacterial primers were tested with temperatures from 50-60ºC, and the optimal annealing temperature was 57ºC. Archaeal primers were tested in a range of 53-63ºC, and the optimal temperature was found to be 59ºC.

3.4.2 Primer design for BPA degradation gene

Primers for N1MWA7 were designed and tested. Strain IBU4, which is not active on BPA and presumably does not possess the gene, was used as a negative control. All three primer sets were specific to the BPA degradation gene, showing no amplification of IBU4. The primers 1079F/1468R were chosen for use in quantification experiments. A temperature gradient PCR reaction was then conducted with temperatures ranging from 45-55ºC, and the optimal annealing temperature was found to be 51ºC.

3.4.3 Determination of bacterial and archaeal 16S

ddPCR was conducted to test for total bacterial and archaeal 16S abundance in soils (Figure 3-6). These measures served as a proxy for total biomass. Based on negative controls, threshold values for positive droplets were set at 4675 for bacteria and 4645 for archaea. Bacterial 16S was significantly higher in Heyburn (p=0.001) and Garfield Bay (p=0.038), while the opposite was true in Ellisport soils (p=0.037) (Table 3-9). Archaeal 16S was higher Heyburn and Garfield Bay treated soils as well (p=0.017 and 0.002 respectively). Finally, the ratio of bacteria to archaea was examined (Table 3-10). The ratio was significantly altered in Garfield Bay soils (p=0.0003), with more archaea relative to bacteria in treated soils.

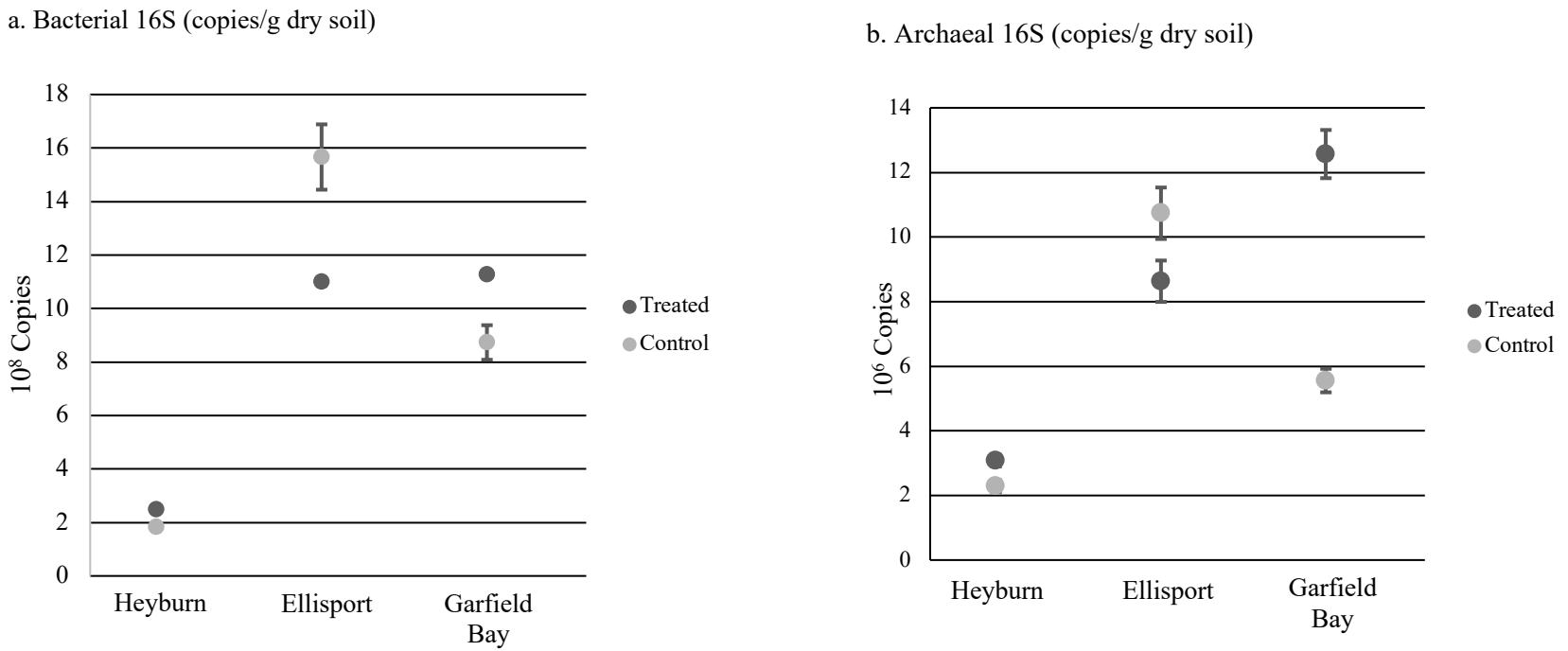


Figure 3-6: Copies of bacterial and archaeal 16S per dry gram of rhizosphere soil, measured by droplet digital PCR. Error bars depict average deviation of ddPCR replicates (n=3).

3.4.4 Determination of putative BPA-degradation gene abundance

ddPCR was conducted to determine the abundance of the putative BPA degradation gene in treated and control soils (Figure 3-7). The gene was more abundant in Heyburn (p=0.013) and Garfield Bay (p≈0) treated soils compared to their respective controls. There was no statistically significant difference in gene abundance between Ellisport treated and control soils (p=0.095).

The ratio of bacteria to the BPA gene was lower in Garfield Bay treated soils compared to controls (Table 3-10), which suggested that a greater proportion of the bacterial population possessed the BPA degradation gene in treated communities. Ratios were not significantly different in Heyburn or Ellisport soils.

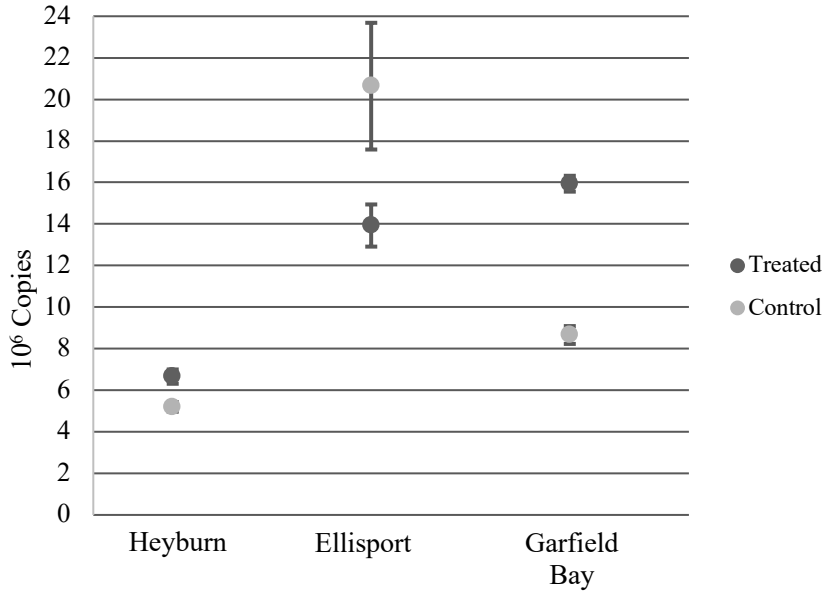


Figure 3-7: Copies of the putative BPA degradation gene (N1MWA7) per dry gram of rhizosphere soil, measured by droplet digital PCR. Error bars depict average deviation (n=3).

Table 3-9: Abundance of bacterial 16S, archaeal 16S, and putative bisphenol A degradation gene N1MWA7 in rhizosphere microbial communities (100,000 copies/g dry soil).

Sample		Bacteria	p-value	Archaea	p-value	N1MWA7	p-value
Heyburn	Treated	2470 (61.3)	0.001*	30.8 (1.84)	0.017*	66.6 (3.44)	0.0134*
	Control	1820 (32.9)		22.9 (1.88)		52.0 (2.30)	
Ellisport	Treated	11000 (163)	0.037*	86.3 (6.41)	0.068	139 (10.1)	0.0954
	Control	15700 (1220)		107 (7.97)		206 (30.5)	
Garfield Bay	Treated	11300 (84.1)	0.038*	126 (7.46)	0.002*	159 (3.82)	0.0001*
	Control	8730 (646)		55.6 (3.65)		86.6 (4.29)	

Average deviations are shown in parentheses (n=3)
*Denotes significant p-value (p<0.05)

Table 3-10: Ratios of bacteria, archaea, and putative bisphenol A degradation gene N1MWA7 in rhizosphere microbial communities.

Sample		Bac:Arc	*p*-value	Bac: N1MWA7	*p*-value	Arc: N1MWA7	*p*-value
Heyburn	Treated	80.39 (2.71)	0.958	37.16 (1.52)	0.242	0.46 (0.028)	0.407
	Control	80.10 (5.86)		35.09 (1.11)		0.44 (0.017)	
Ellisport	Treated	127.96 (8.06)	0.365	79.44 (6.39)	0.991	0.63 (0.074)	0.263
	Control	147.74 (22.38)		79.25 (19.778)		0.53 (0.048)	
Garfield Bay	Treated	89.93 (5.13)	0.0003*	70.69 (2.26)	0.023*	0.79 (0.036)	0.019*
	Control	156.94 (3.94)		100.87 (7.31)		0.64 (0.030)	

Average deviations are shown in parentheses (n=3)
*Denotes significant p-value (p<0.05)

4 Discussion

This study was a first look at the intrinsic and adaptive degradation of trace-level organic contaminants by rhizosphere soils exposed to treated wastewater. It also examined the impacts of long-term effluent exposure on microbial communities. Intrinsic degradation of both BPA and ibuprofen was observed in all soils, and BPA degradation was enhanced in all treated soils. This may be attributed to microbial community shifts in response to TOrC exposure, an overall increase in biomass due to higher nutrient inputs, and changes to various soil properties.

The increased BPA degradation in effluent-exposed soils may indicate a response by the rhizotreatment system to long-term TOrC exposure. While the composition of the effluent applied to these sites is unknown, trace levels of BPA are found in nearly all effluent samples [33, 34], so it is likely that some amount of BPA was present. Thus, it is possible that exposure to BPA in the effluent caused changes to the microbial community, increasing the abundance of BPA-degrading microorganisms in the soil, and enhancing degradation. Indeed, several taxa were found to correlate positively with BPA degradation rate, including the phyla *Gemmatimonadetes* and *Nitrospirae*, as well as OTUs 713 and 4238, both of which are members of the *Acidobacteria*. This finding has important implications for rhizotreatment, as it suggests that the system can adapt to the presence of some TOrCs and will remove them more effectively from effluent over time.

Enhanced BPA degradation could also be attributed to the changes in total microbial biomass in treated communities. Increases in the abundance of bacterial and archaeal 16S were observed in Heyburn and Garfield Bay treated soils. This is in line with other studies that have found an increase in overall biomass in communities exposed to TWW compared to control communities [90-93]. It is believed that this increase is due to the influx of nutrients and organic carbon brought in with the effluent [94]. Thus, the enhanced degradation observed in these two soils compared to controls could be due in part to the higher bacterial and archaeal populations found there.

It is also possible that shifts in the microbial community were caused by the differences in soil moisture induced by unequal irrigation regimes. Treated trees were supplemented with effluent, while control trees received no additional water. This resulted in significantly higher water content in treated soils. Soil moisture can profoundly alter the rhizosphere community by

changing water potential, which in turn affects gas exchange and can shift the ratio of aerobic to anaerobic niches [95]. This may explain some of the changes observed in treated microbial communities. Several OTUs correlated negatively with soil moisture including three Proteobacteria and two Acidobacteria. Both of these phyla are known to contain anaerobic organisms. Other soil properties may also explain the observed differences. Exposure to effluent has been shown to affect several soil properties that were not characterized in this study including pH, salinity, and electrical conductivity [93]. These changes in turn affect the types of microorganisms that a rhizosphere can support.

Finally, observed differences could be based on duration of the rhizotreatment, with treated and control communities diverging over time. Garfield Bay has been conducting rhizotreatment since 1975 and showed the greatest separation between treated and control communities in the PCA. Ellisport has been operational for 20 years and showed moderate separation, while Heyburn has used rhizotreatment for 10 years and showed almost no separation between control and treated communities. These results may reflect the impact of long-term adaptation of the rhizosphere to effluent exposure. To test this idea, the treated and control communities at Heyburn could be monitored over the next decade to see if they diverge over time as Ellisport and Garfield Bay communities did.

One might expect that at sites with enhanced BPA degradation, there would also be an increased prevalence of the putative BPA degradation gene. This gene is hypothesized to be active on BPA at low concentrations [62] like those used in this study, and it is the first gene identified that can degrade BPA in the μg/l range. An increase in this gene was observed in treated soils at Garfield Bay, but not Ellisport. At the Ellisport site, the control soil had more copies of the putative gene, despite showing lower BPA degradation rates than treated soils. There are two possible explanations for this seemingly contradictory finding. The first is that a second, currently uncharacterized gene responsible for BPA degradation at low concentrations may be present in soils. This gene would degrade additional BPA without being detected by ddPCR. To identify the gene, a combined metabolomics and metaproteomics study could be conducted. This approach has previously been used to examine the degradation potential of microbial communities for environmental pollutants [62, 96-98]. These methods could help identify candidate genes that were upregulated in the presence of BPA, and thus may be involved in BPA degradation.

The second explanation for the mismatch between gene abundance and degradation is the primers used. The BPA degradation gene primers were designed to be specific to one putative BPA degradation gene, studied in a single isolate. In the organism IBU4, which is confirmed to be incapable of BPA degradation, the gene was not detected. However, the behavior of the primers may be different in mixed communities, where a variety of genes with similar sequences to the BPA gene could be present, than in a pure culture. It is therefore possible that, in the control community, genes were amplified that did not actually have BPA degradation ability but were structurally similar to the putative gene. This could have artificially inflated the degradation rate of control soils, making the gene abundance appear higher than treated soils despite a lower degradation rate. Conversely, it is also possible that a functional BPA degradation gene was present and active in treated communities, but due to random mutations in the primer binding region, some versions of the gene were not amplified in ddPCR. Repeating ddPCR with additional BPA degradation gene primers would test this hypothesis. If similar results were confirmed with multiple primer sets targeting different sections of the gene, it would confirm that the abundance of the putative BPA degradation gene was truly lower in Ellisport treated soils compared to controls.

Finally, it is possible that in some organisms, the putative BPA degradation gene was present in the genome and thus detected in ddPCR but was not expressed or not functional due to one or more missing cofactors. If so, then the difference in gene abundance between Ellisport treated and control soils would be accounted for by higher prevalence of the gene in the control community in an inexpressible form. This study utilized minimal medium, which may have lacked some of the molecules necessary for transcription or translation of the gene, or for activation of the synthesized enzyme. Additionally, it is suspected that some TOrCs are degraded by co-metabolism, a process in which microorganisms break down a difficult to degrade TOrC simultaneously with another compound like a sugar that is easier to degrade [99]. For example, naproxen is more readily degraded in the presence of phenol or glucose than alone [100]. The minimal medium used in this experiment was prepared without carbon sources aside from the TOrCs, which may have prevented co-metabolic degradation.

It may seem counterintuitive that microorganisms would evolve genes to degrade TOrCs in a carbon- and nutrient-rich environment like the rhizosphere. One hypothesis to explain this is that many TOrCs are structurally similar to compounds that microorganisms naturally produce in

the soil environment as anti-microbials. Ten percent of all bioactive compounds discovered to date, including some TOrCs, were isolated from microorganisms, and many of these microorganisms are soil dwellers [101]. One purpose of these compounds in nature is hypothesized to be self-defense in the rhizosphere environment. Microorganisms compete for resources by releasing antimicrobial molecules, some of which resemble TOrCs, to eliminate nearby organisms. Those target organisms have in turn evolved protective mechanisms against anti-microbial compounds. It is hypothesized that these protective enzymes, which degrade antimicrobial compounds in the soil, will be active on structurally similar TOrCs [50]. For compounds with no natural analog, degradation is more difficult but by no means impossible. Because of their high genetic diversity and short generation times, it is believed that microbial communities can degrade any compound, including a wide variety of TOrCs, if given the appropriate habitat and selective pressure. It is therefore possible that degradation genes for these compounds will arise in the microbial communities of rhizotreatment systems under appropriate selective pressure [55].

When genes with activity on TOrCs do arise in a community, they often appear in a single organism and are then passed to others via lateral gene transfer. This may be the case for the putative BPA degradation gene examined in this study. In Bay-T soils, the ratio of copies of bacterial 16S to copies of the gene was increased, implying that a higher proportion of the population possessed the gene. This may mean that one of the microbial groups enriched in Bay-T possessed the BPA degradation gene and was selected for by TOrC exposure. The altered ratio could also indicate that the BPA degradation gene is found on a plasmid, and was spread laterally between members of the community [56]. Preliminary research suggests that in BiD32, the gene is on a mobile element and not part of the main chromosome (unpublished data). This has important implications for BPA degradation. If the gene can easily be transferred between microorganisms, then degradation is less dependent on specific members of the community and is instead controlled by the presence or absence of the gene in the population. Sequencing or conjugation experiments could confirm that the gene is in fact on a plasmid.

Exposure to TWW has highly varied impacts on microbial communities, but a few common changes have been observed. Decreases in *Actinobacteria* have been seen, as have increases in abundance of *Proteobacteria,* particularly *Betaproteobacteria* and *Gammaproteobacteria* [92, 93, 102]. A decrease in *Actinobacteria* was observed in Ellisport and

Garfield Bay treated soils and was accounted for by a decrease in the orders *Solirubrobacterales* and *Gaiellales*. Little is known about the specific ecological roles of these bacteria [103], although some research has associated *Solirubrobacterales* with the presence of ants [104] and epigeic earthworms [105]. Thus, the observed decrease in this microbial order could correspond to a decrease in microfauna populations in treated soils, due to exposure to compounds in the effluent. This is further supported by the strong negative correlation observed between BPA degradation rate and *Solirubrobacterales* abundance. Decreased abundance could also be due to drowning of burrows from increased irrigation, leading to a reduction in microfauna populations.

The microbial communities at the Heyburn site did not follow the patterns observed at Ellisport or Garfield Bay. This may be attributed in part to tree species at the sites. Heyburn is dominated by Douglas fir and Ponderosa pine species, while Ellisport and Garfield Bay are dominated by Western red cedar. Tree species directly influences the microbial community by releasing exudates into the soil and attracting different organisms [93]. Thus, the difference in dominant trees may partially explain why Heyburn treated and control soils differ more from other sites than from each other. Studies characterizing rhizosphere microbiomes of individual species are limited, and the above species have not been examined. Further research is needed to understand the contributions of tree species to the observed differences in microbial community composition.

In contrast to BPA and ibuprofen, all three soils showed very little intrinsic or adaptive degradation of carbamazepine, gemfibrozil, and naproxen over the one-week experiment. Slight removal was observed, but in both treated and control communities, degradation was lower than the rate at which the compounds would enter a rhizotreatment system with frequent effluent application (Table 1-1). This means that even with some degradation occurring, a constant influx of carbamazepine, gemfibrozil, and naproxen in effluent could lead to a buildup of the compounds in soil.

There are several explanations for this limited degradation. One possibility is that the effluent did not contain these particular TOrCs, so the communities had never been exposed to them. Although the three compounds are frequently detected in receiving water, the composition of the effluent applied to these rhizotreatment sites is unknown. Additionally, some research suggests that degraders of these compounds are rare in the environment. So far, only one gemfibrozil degrader [61] and a few naproxen degraders [100, 106] have been isolated and

characterized. While several carbamazepine degraders have been identified [107, 108], the compound is known to be extremely resistant to degradation in the environment, exhibiting a half-life of several years in certain systems [109]. This finding has important implications for rhizotreatment, as it suggests that the system may not be suitable for some recalcitrant compounds. However, research has shown that endophytes within tree roots are able to degrade carbamazepine within a much more reasonable timeframe of 30 days [107]. Conducting degradation experiments in a more realistic system that can account for the contributions of endophytes and trees to TOrC removal would give a clearer picture of the degradation of these compounds in rhizotreatment.

The experimental set-up may also have impacted degradation rates of carbamazepine, gemfibrozil, and naproxen. In this experiment, all five target TOrCs were combined in the same medium to simulate real-world conditions. Research has suggested that the presence of one TOrC may influence the degradation of other compounds. For example, the presence of gemfibrozil may decrease naproxen degradation and vice versa [10, 106]. A long-term degradation study of mixed contaminants would help determine the degradation rates of these compounds in rhizotreatment systems, as well as increasing understanding of the often-neglected synergistic interactions between TOrCs in the environment. Finally, the medium used in the experiment might have impacted TOrC degradation rates. Naproxen degradation has been shown to increase in the presence of phenol [100, 110], and gemfibrozil degradation rates varied significantly depending on the source of organic carbon used [111]. This study used a minimal medium, which may have lacked the diverse carbon sources needed for maximum TOrC degradation.

The intrinsic degradation rates observed in this study, even in compounds that saw limited degradation, are higher than rates reported elsewhere [112-116]. Previous studies examining the degradation of these compounds either used agricultural soil or did not specify whether their samples were taken from the rhizosphere or the bulk soil. The soils used in this study were taken from the rhizosphere, which tends to have a larger and more diverse microbial community than bulk soil [56]. Thus, the higher baseline degradation rates might be explained by a higher microbial population.

Overall, the data suggest that rhizosphere microorganisms have an intrinsic ability to degrade ibuprofen and BPA, and that BPA degradation may be enhanced by exposure to treated

effluent. One of the inherent challenges in this work was the inability to control all variables in the rhizotreatment system, which limits the extent to which the results can be attributed directly to long-term effluent exposure. Future work should focus on controlling external variables to isolate the effects of TWW exposure on TOrC degradation and microbial community composition.

5 Conclusion

The rhizotreatment system has been used by WWTPs for many years to remove nitrogen and phosphorus from wastewater, but the fate of TOrCs in the system is not fully understood. This work aimed to improve the understanding of rhizotreatment by quantifying the degradation of five TOrCs by rhizosphere soils and by examining changes to the rhizosphere microbial community at rhizotreatment sites.

Rhizosphere microbial communities showed an intrinsic ability to degrade BPA and ibuprofen. Additionally, BPA degradation was enhanced in all treated soils, suggesting that exposure to treated wastewater may impact the degradation of select TOrCs. There were also significant differences between treated and control microbial communities at these sites, which may be attributed to a combination of TOrC exposure, increased soil moisture in treated soils, and other environmental factors.

To continue studying the rhizotreatment system, the Gough lab has constructed reactors to simulate the process in a controlled environment. The reactors will allow for detailed study of the impacts of treated wastewater on microbial communities and help gain a clearer understanding of the relationship between effluent exposure and degradation of trace-level organic contaminants in a rhizotreatment system. Future experiments with these reactors will include the following:

- Application of simulated treated wastewater to the rhizotreatment systems. Reactors will be irrigated with a mixture of trace-level organic contaminants and water will be collected as it leaves the system to test for the removal of TOrCs. Changes to the microbial community will also be tracked.
- Application of supplemental clean water to control reactors, to test the effects of increased moisture on the microbial community in the absence of TOrCs.
- Monitoring the health of trees in the rhizotreatment system.
- Observing the impacts of simulated wastewater on abundance of soil microfauna, including ants and earthworms.
- Confirming that the BPA degradation gene targeted in this study is found on a plasmid and identifying additional BPA degradation genes in the environment.

6 Bibliography

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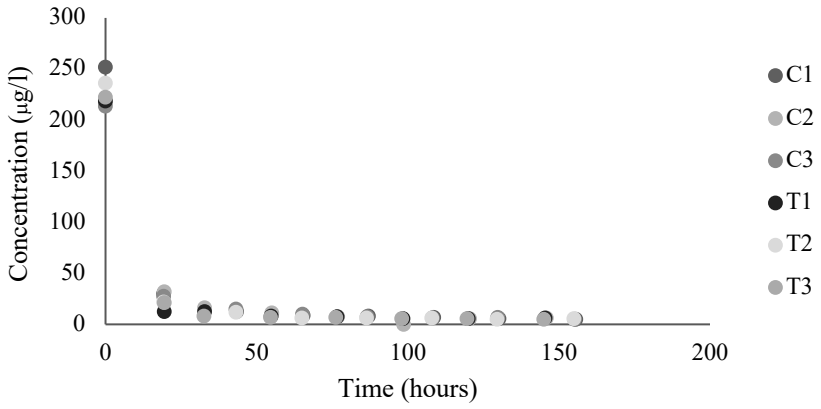
7 Appendix

a. Heyburn BPA



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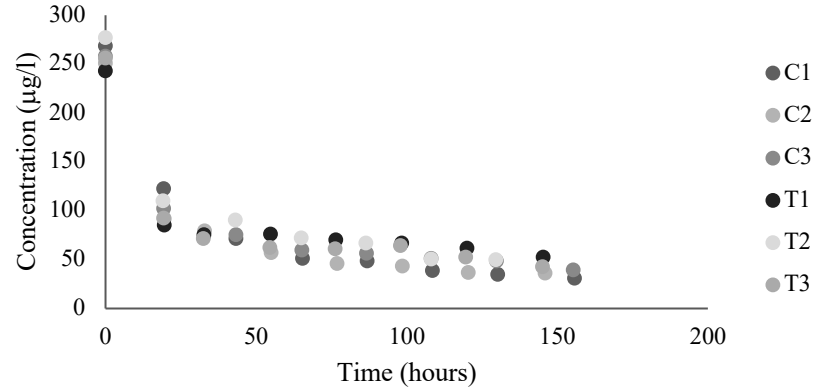
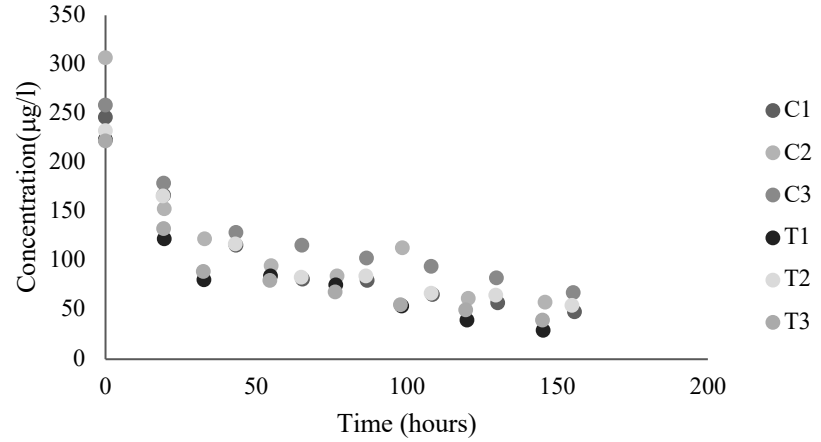
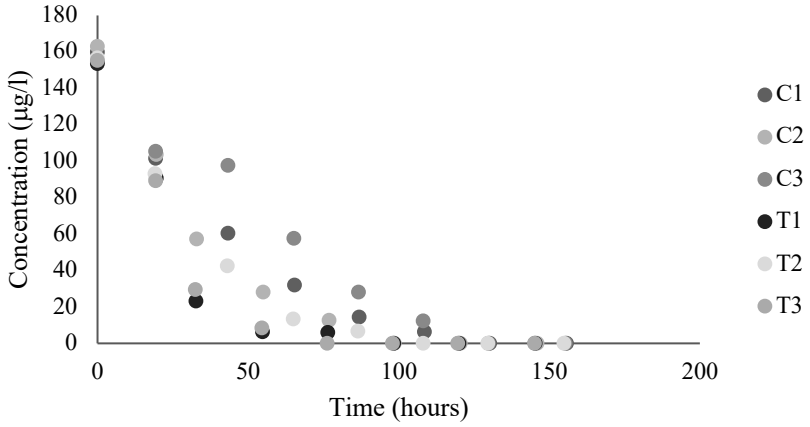


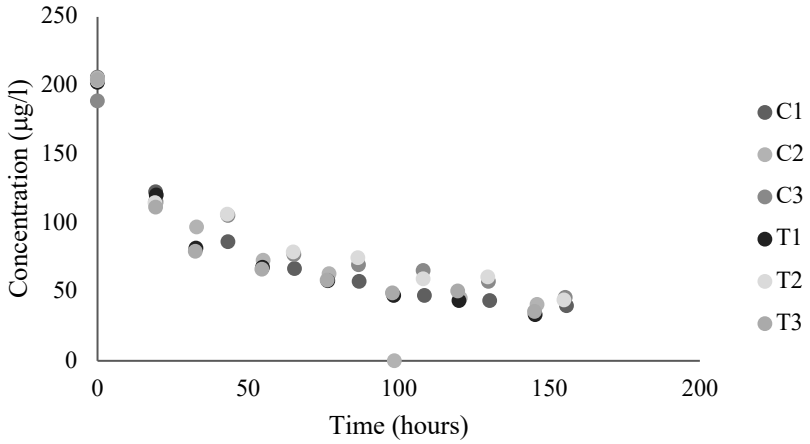
c. Heyburn GEM



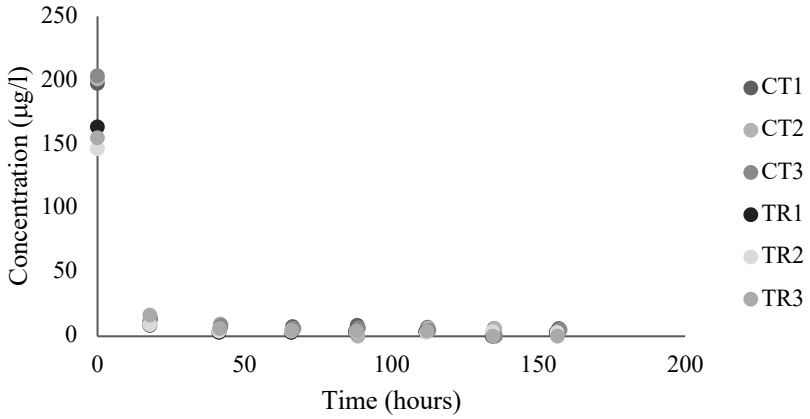
d. Heyburn IBU



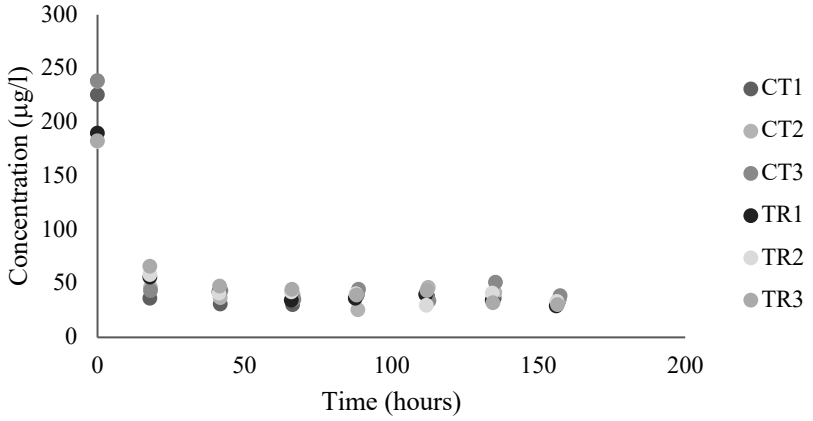
e. Heyburn NAP



f. Ellisport BPA



g. Ellisport CAR



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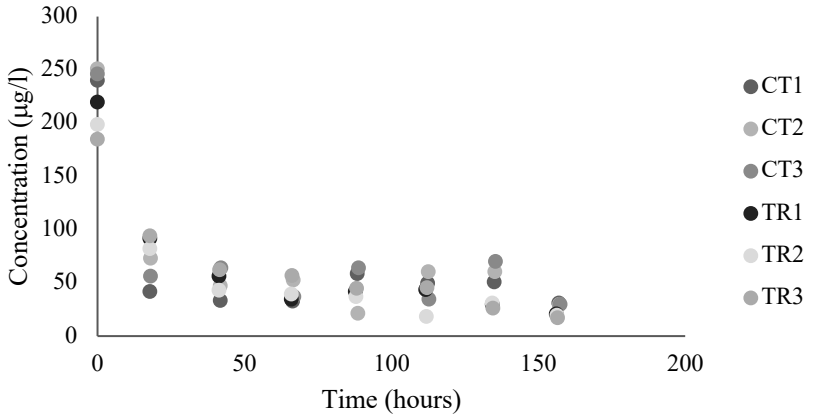
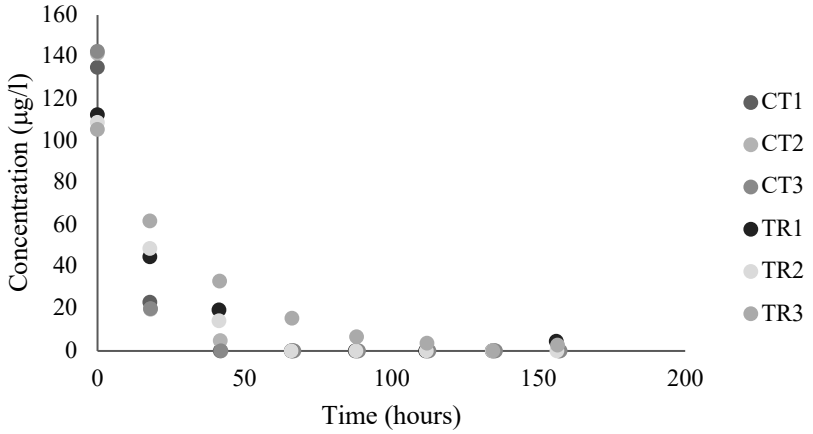
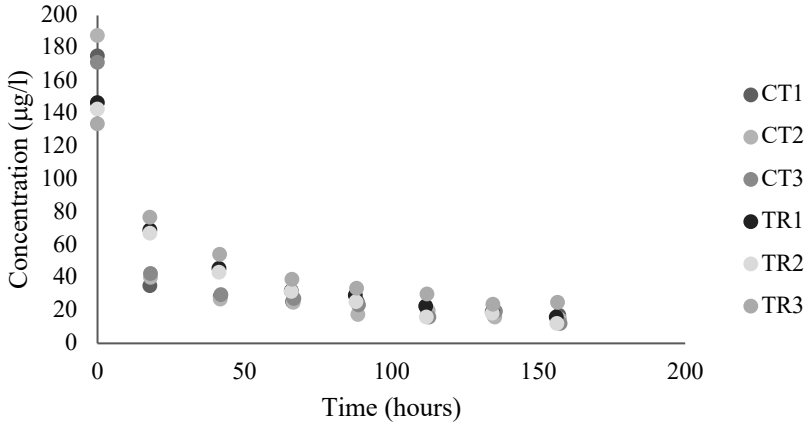


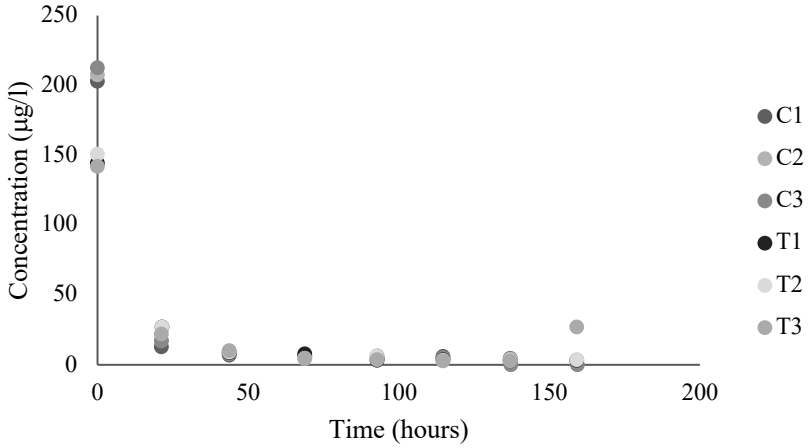
i. Ellisport IBU



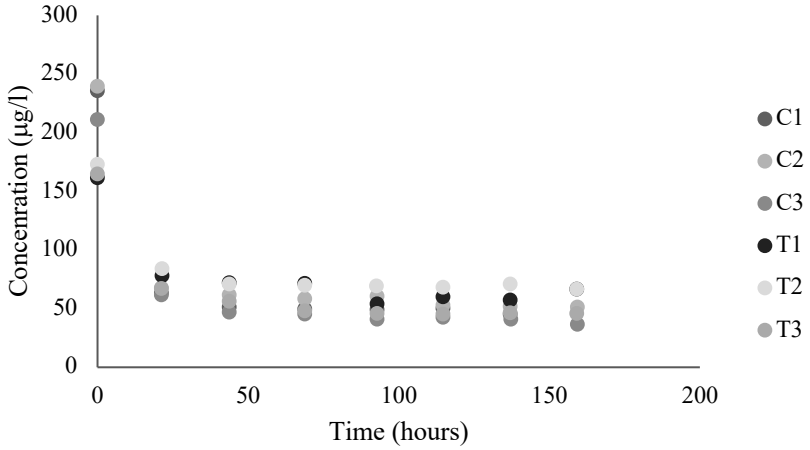
j. Ellisport NAP



k. Garfield Bay BPA



l. Garfield Bay CAR



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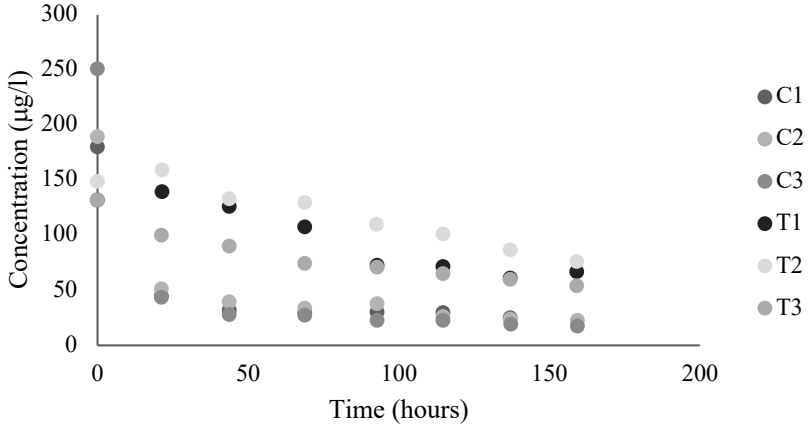

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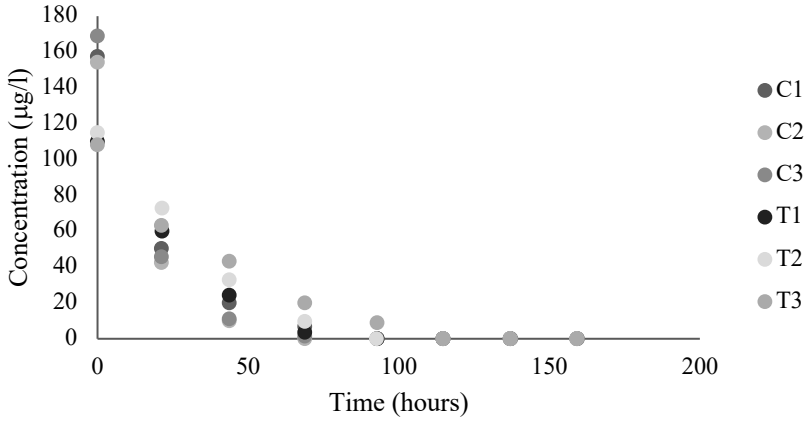


o. Garfield Bay NAP

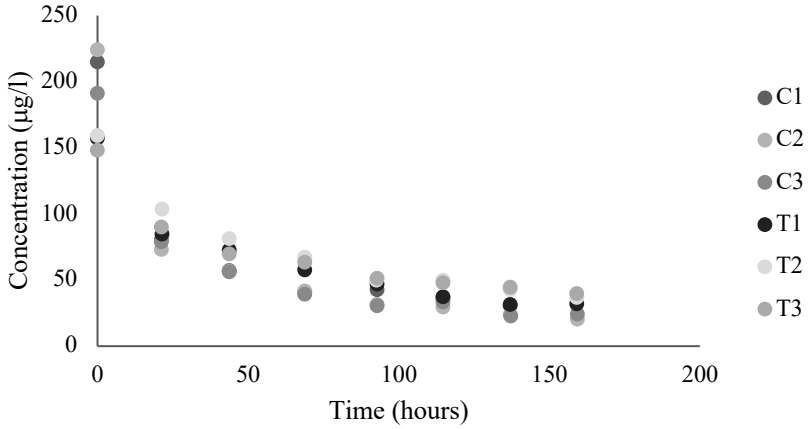
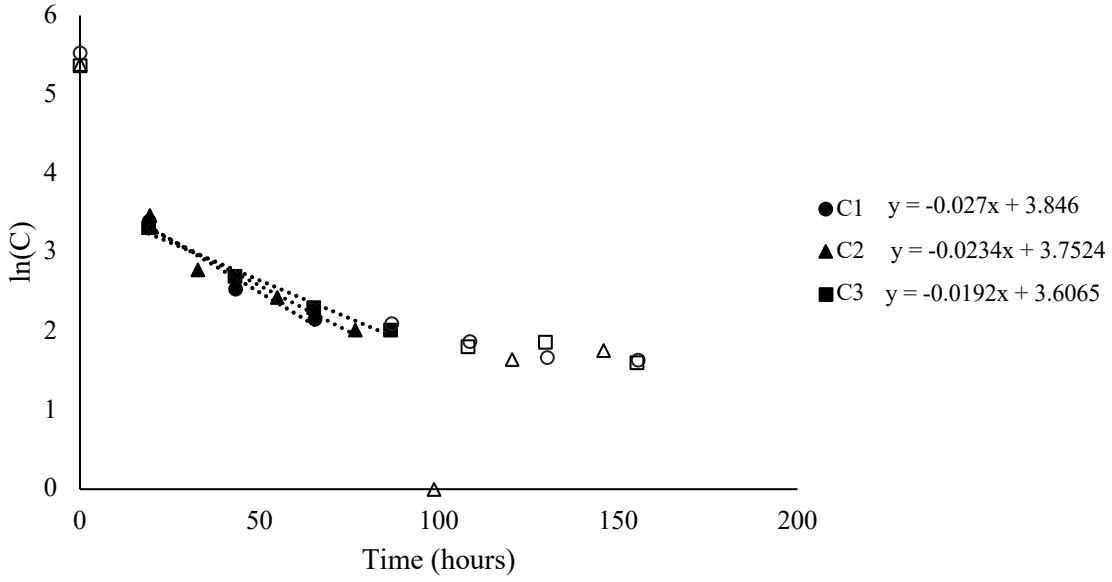


Figure A1: Degradation of trace-level organic contaminats by rhizosphere microbial communities from Heyburn (a-e), Ellisport (f-j), and Garfield Bay (k-o).

a. BPA Control



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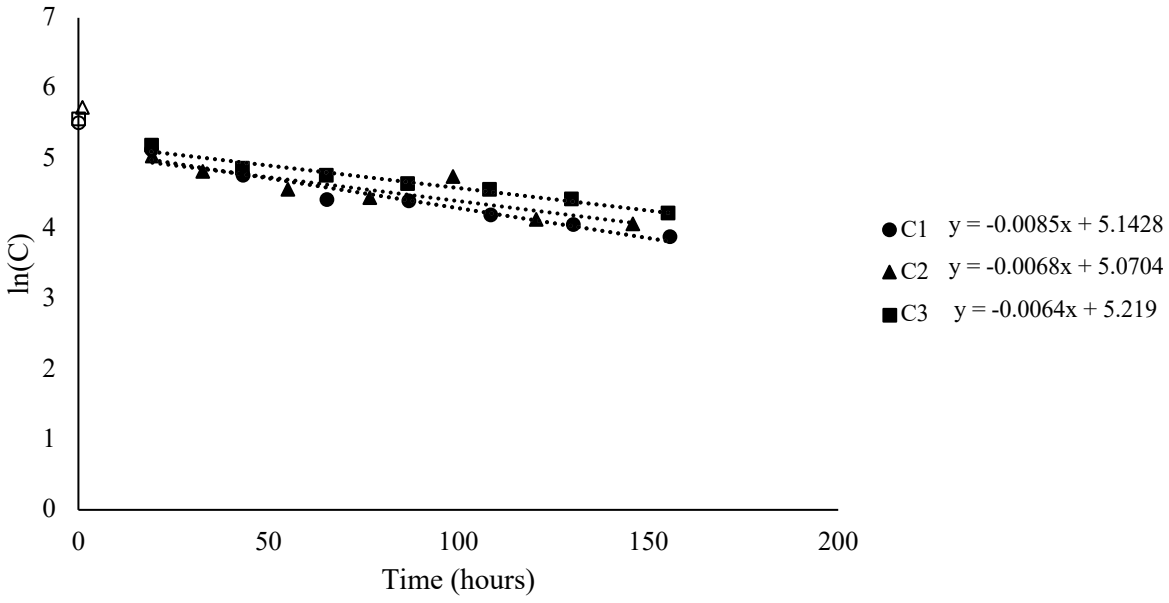
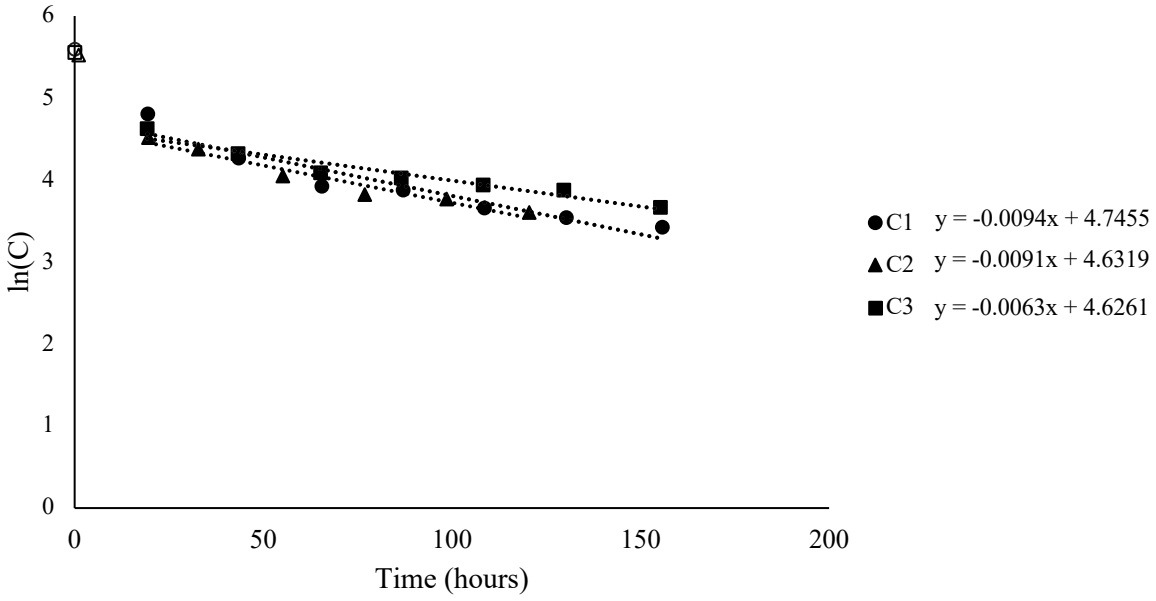
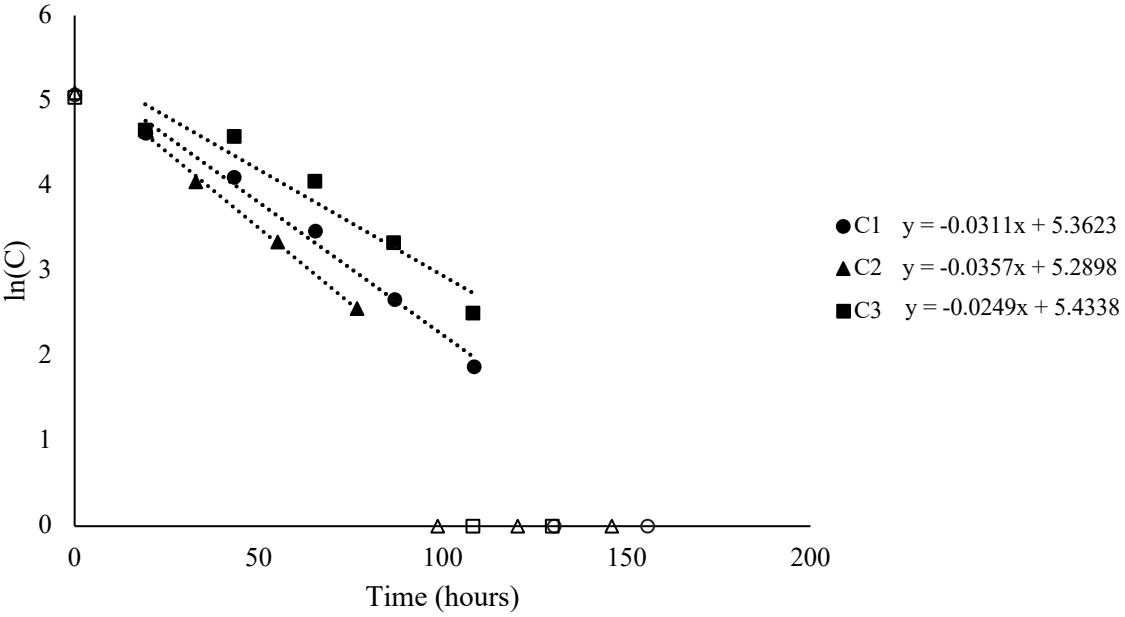


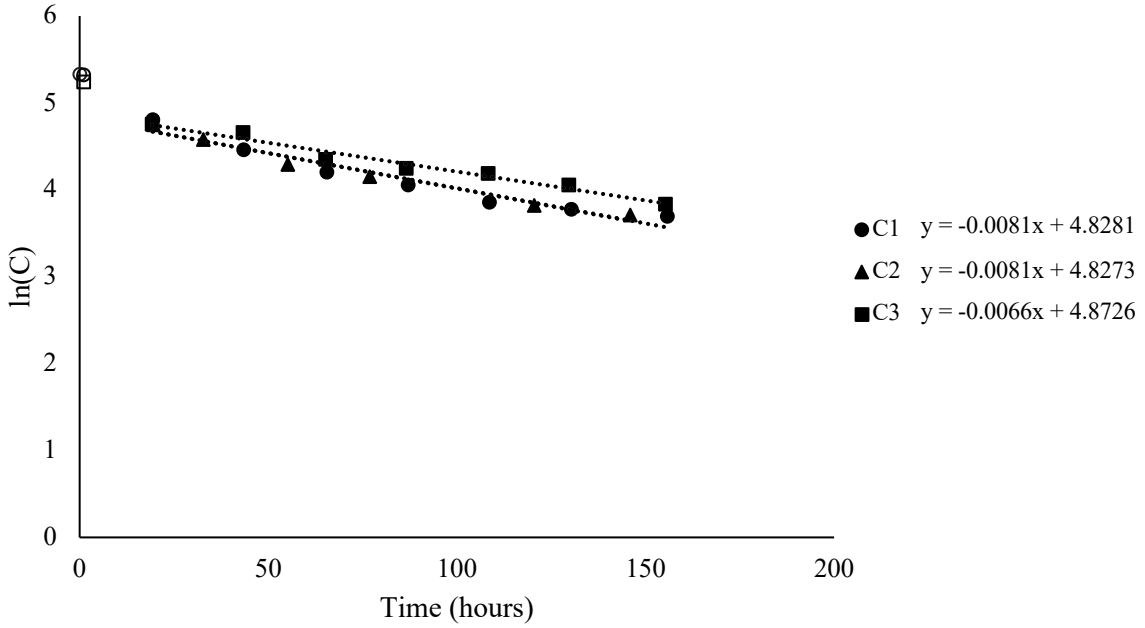
c. CAR Control



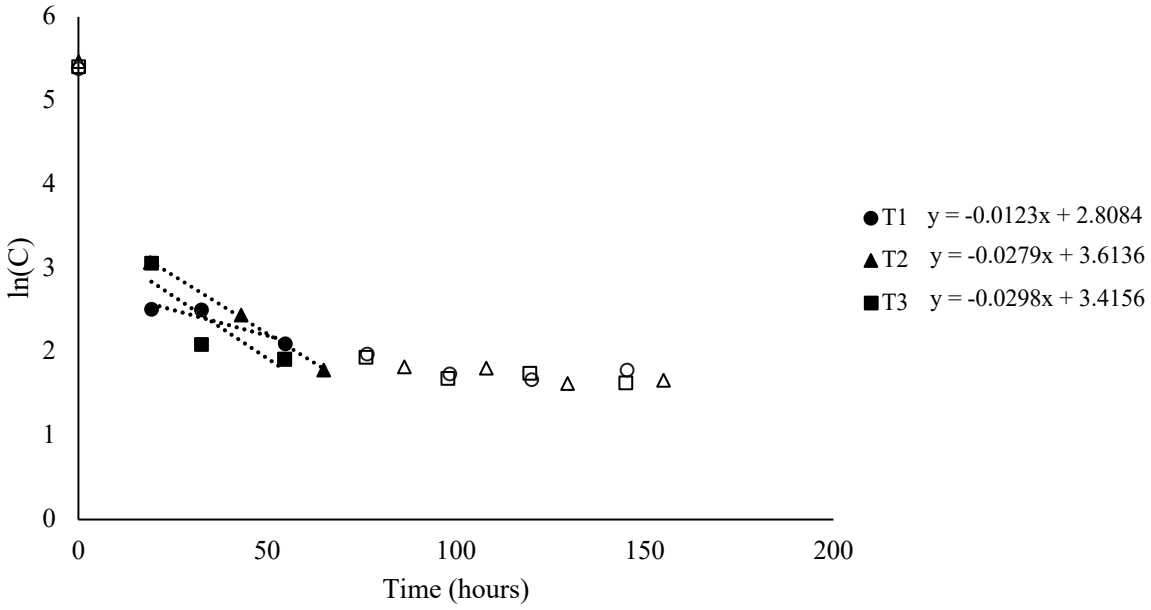
d. IBU Control



e. NAP Control



f. BPA Treated



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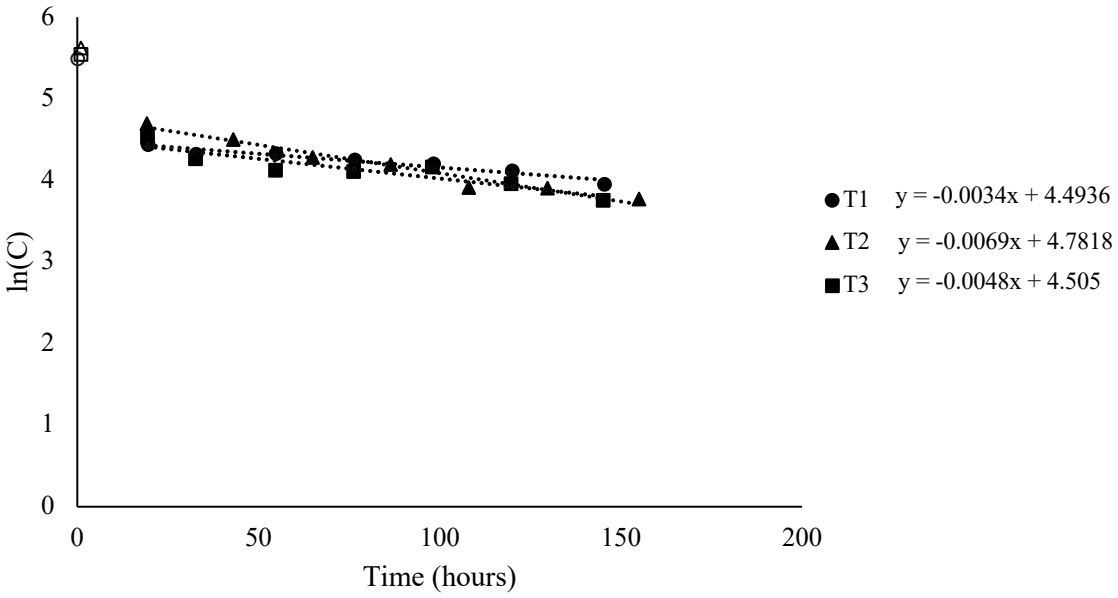
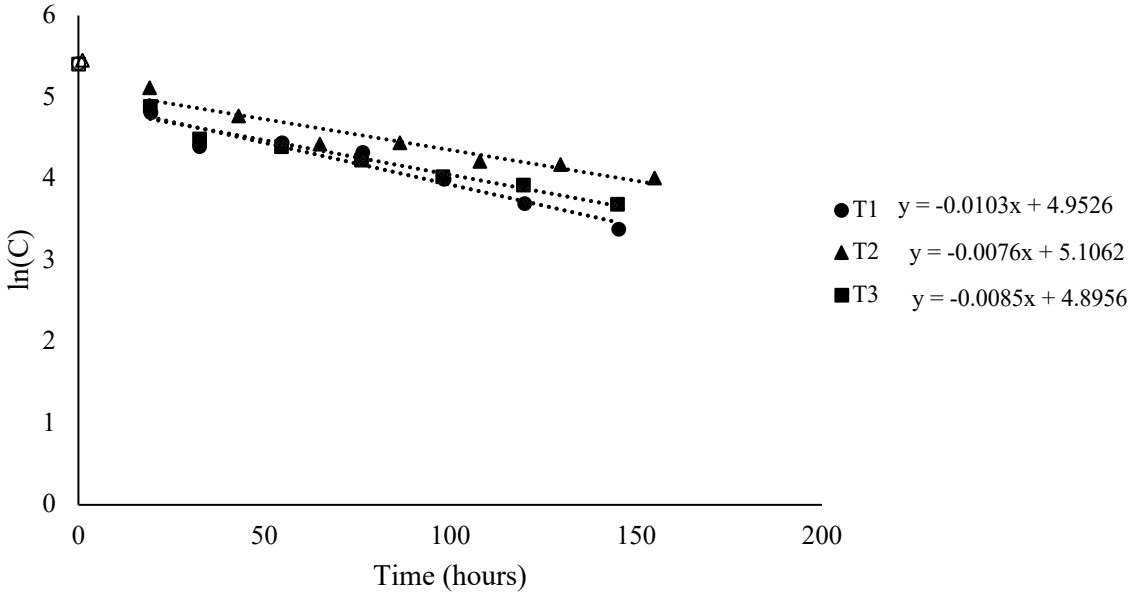
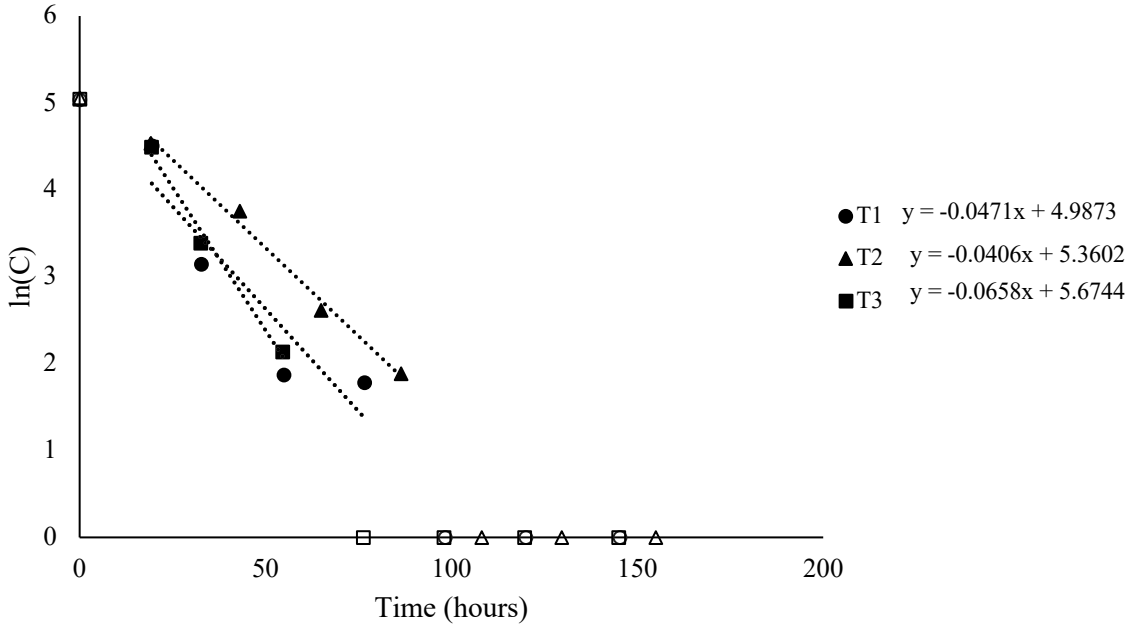


h. GEM Treated



i. IBU Treated



j. NAP Treated

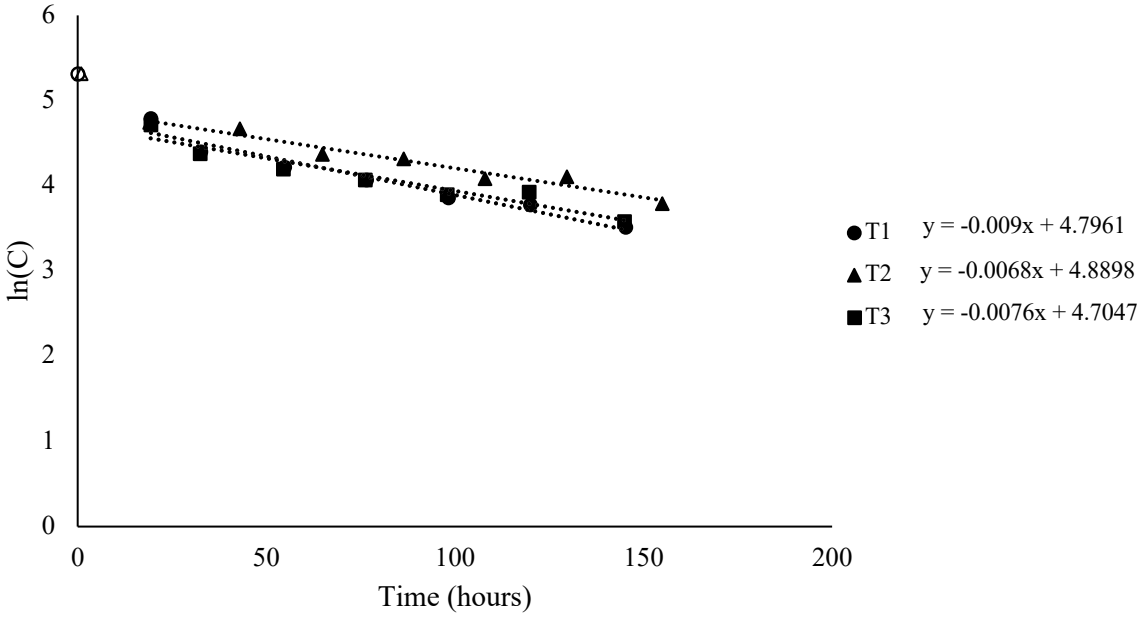
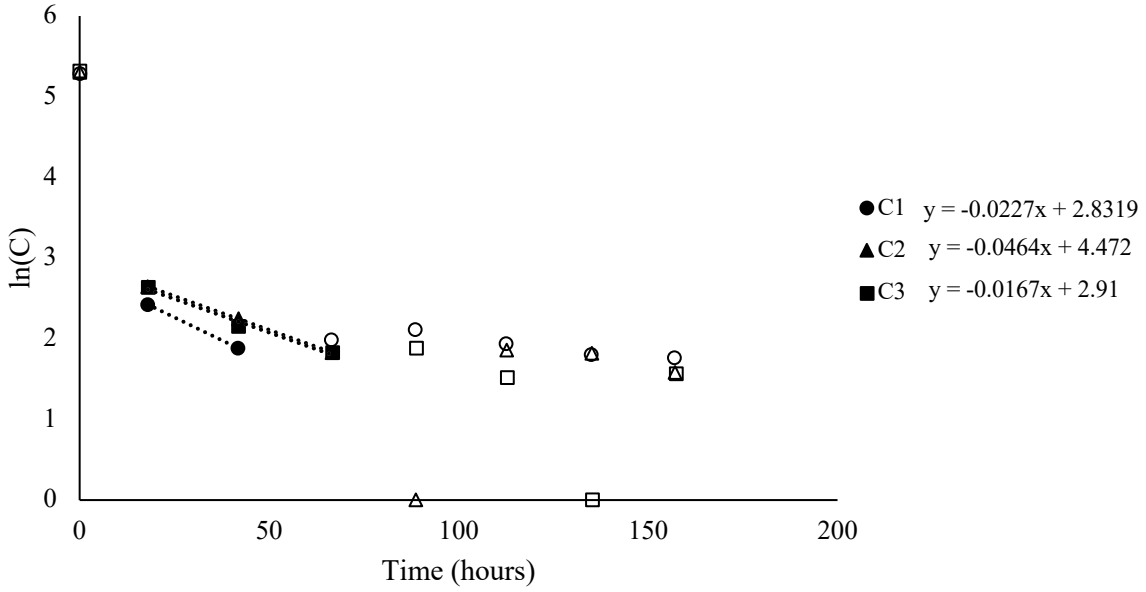


Figure A2: Linearized degradation of trace-level organic contaminants by Heyburn control (a-e) and treated (f-j) soils. Trendline equations are shown to the right of the legend.

a. BPA Control



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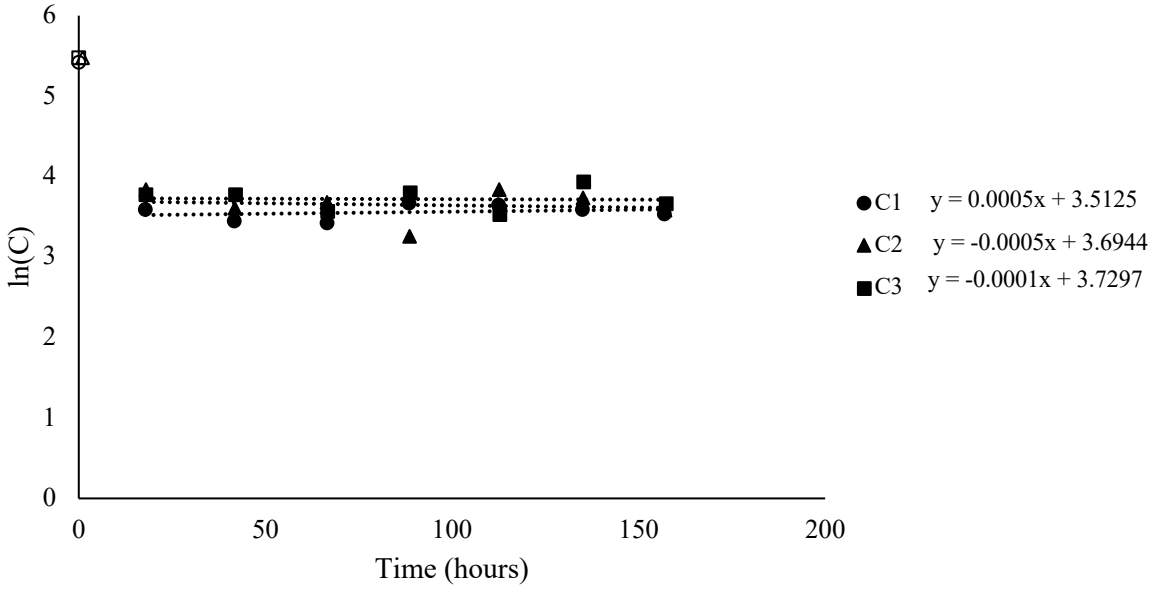
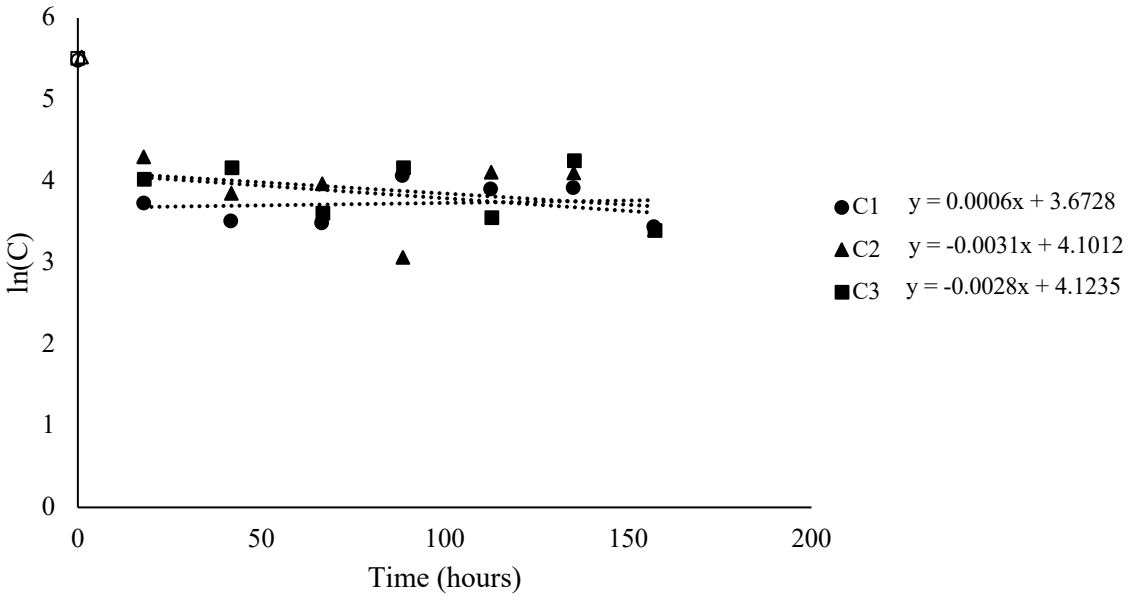
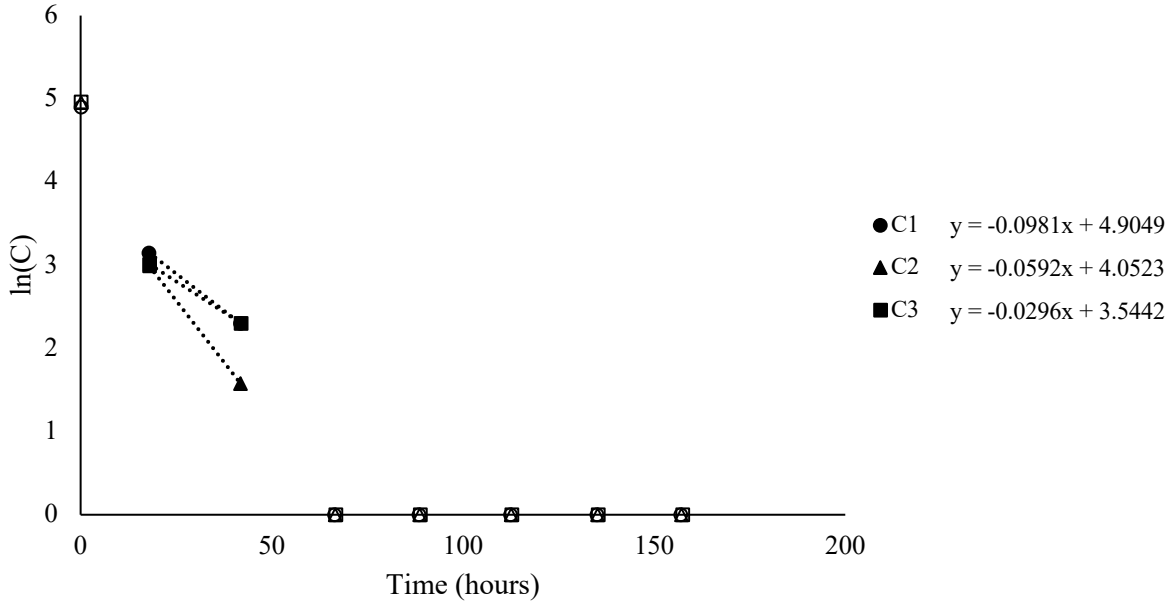


c. GEM Control



d. IBU Control



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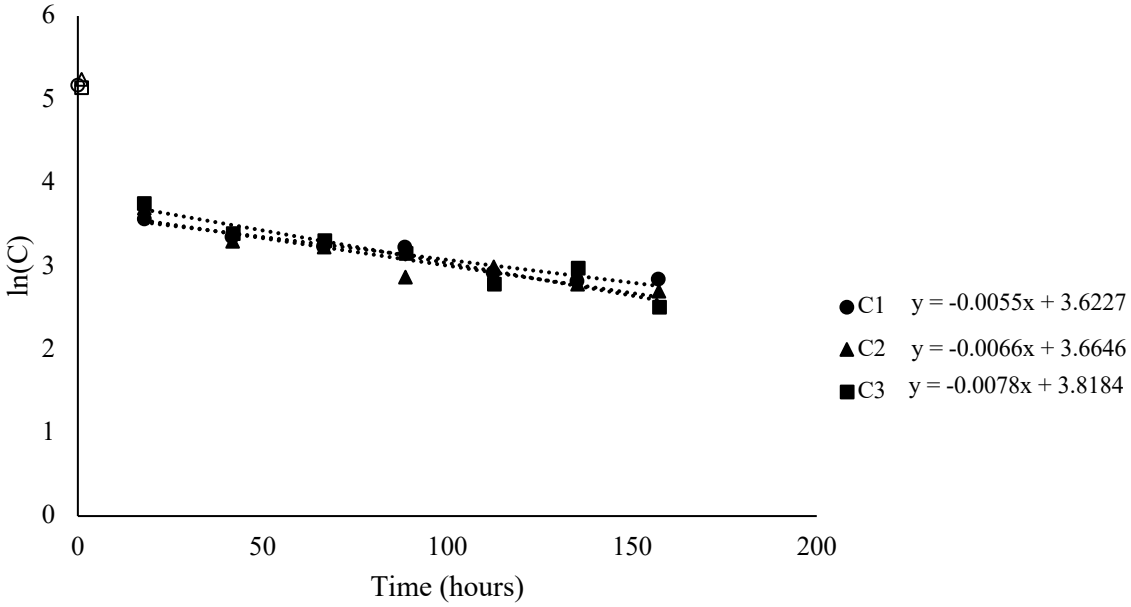
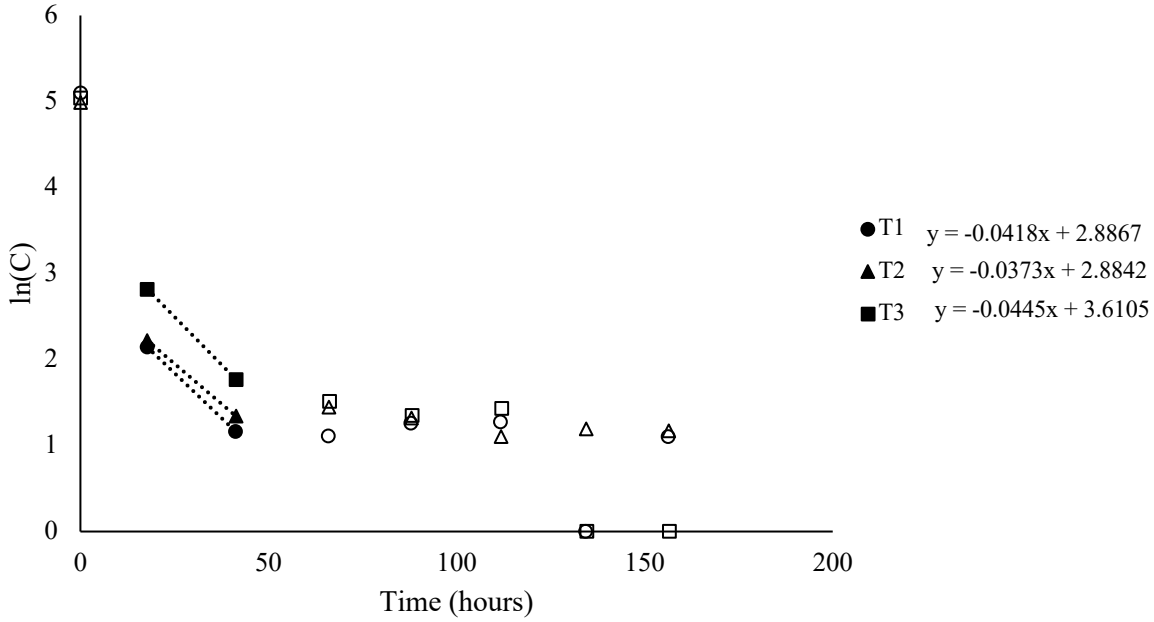


f. BPA Treated



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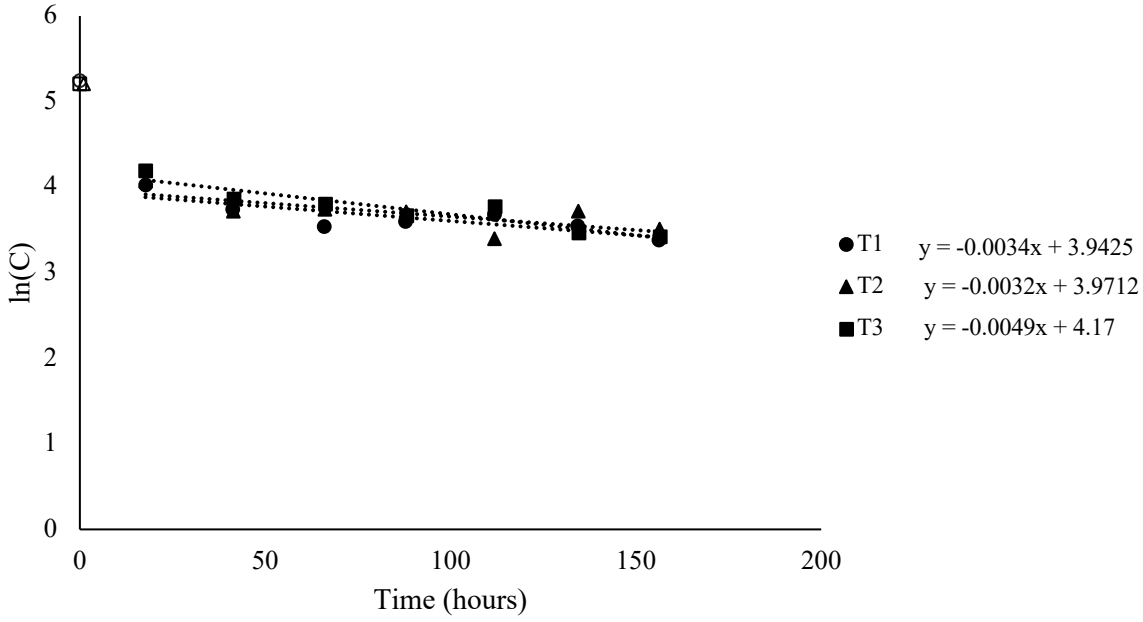

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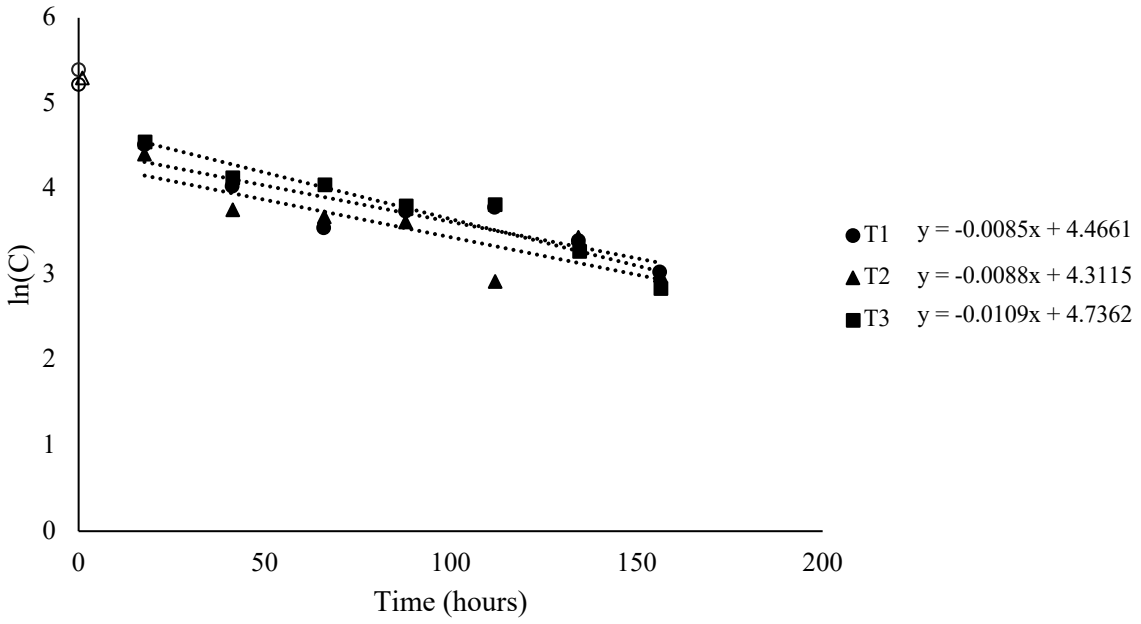
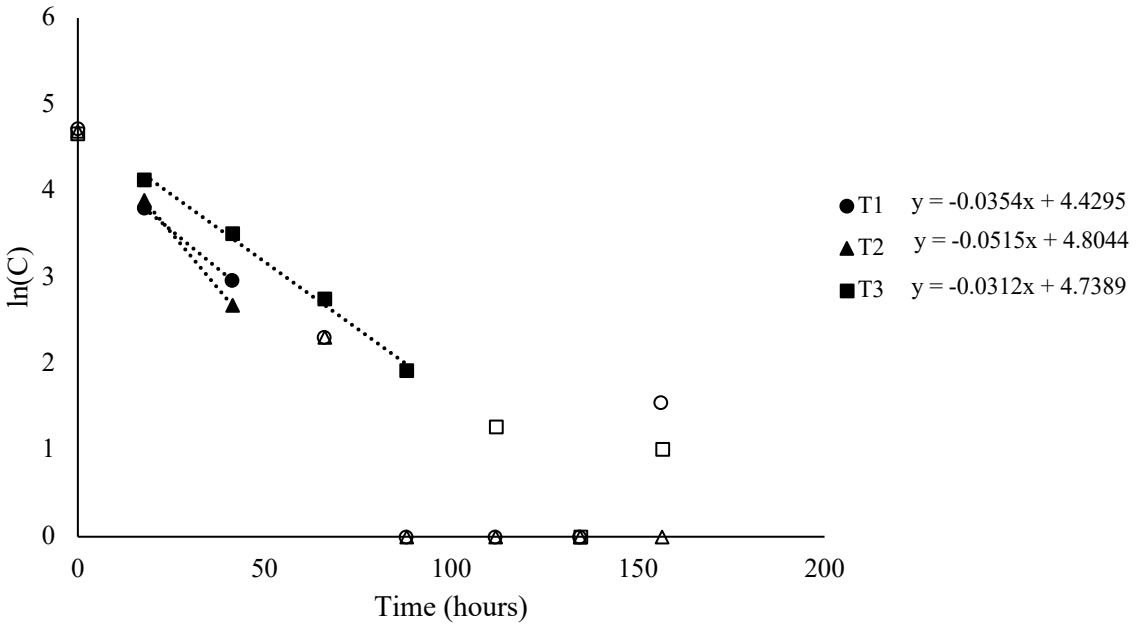


i. IBU Treated



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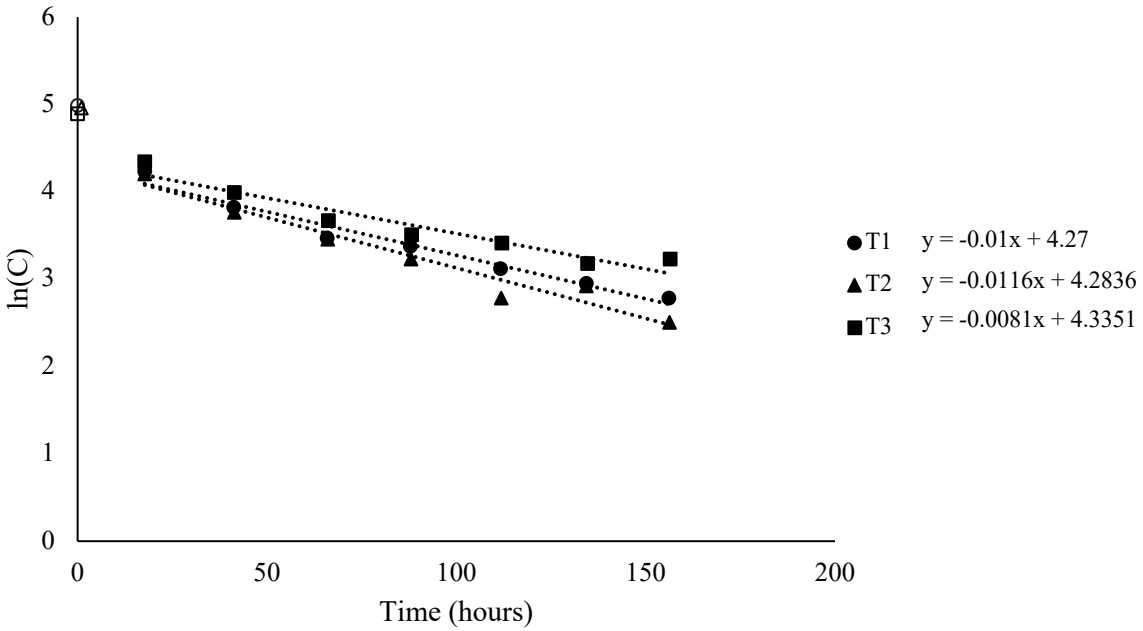
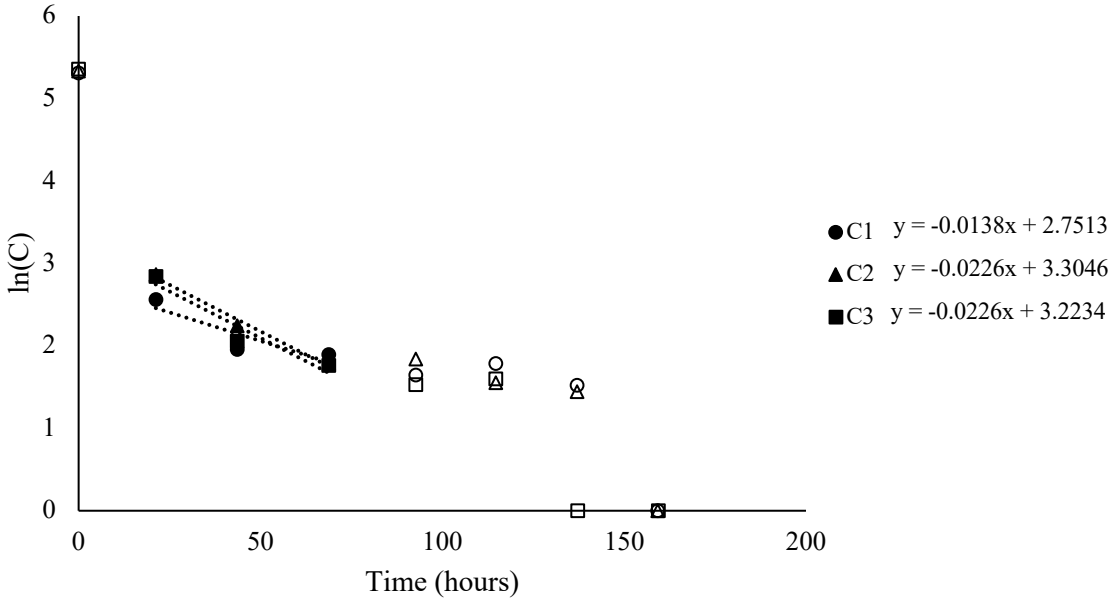
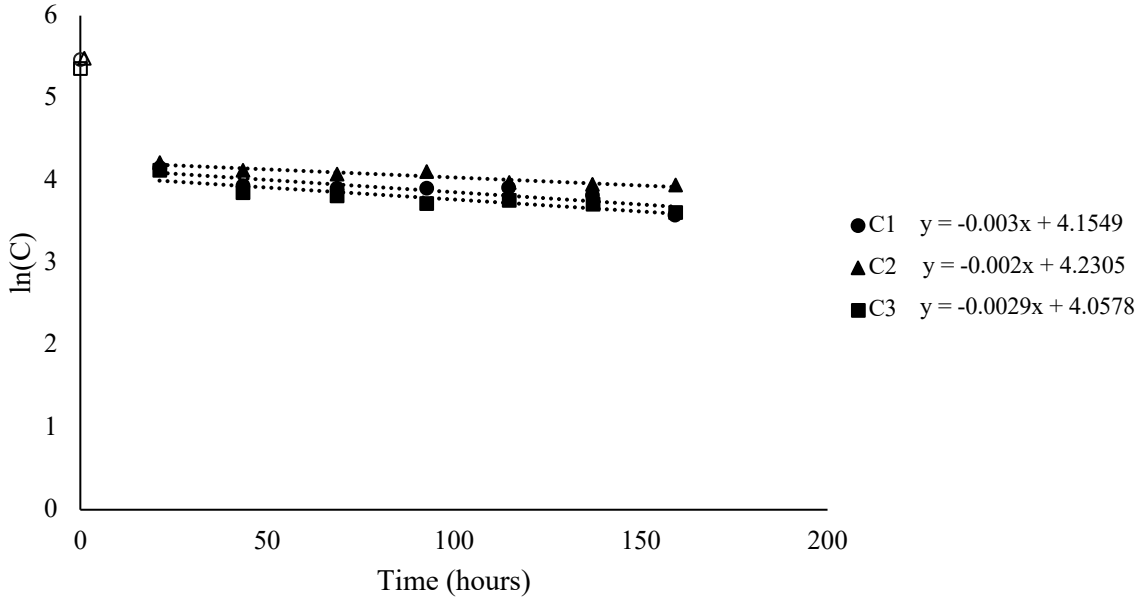


Figure A3: Linearized degradation of trace-level organic contaminants by Ellisport control (a-e) and treated (f-j) soils. Trendline equations are shown to the right of the legend.

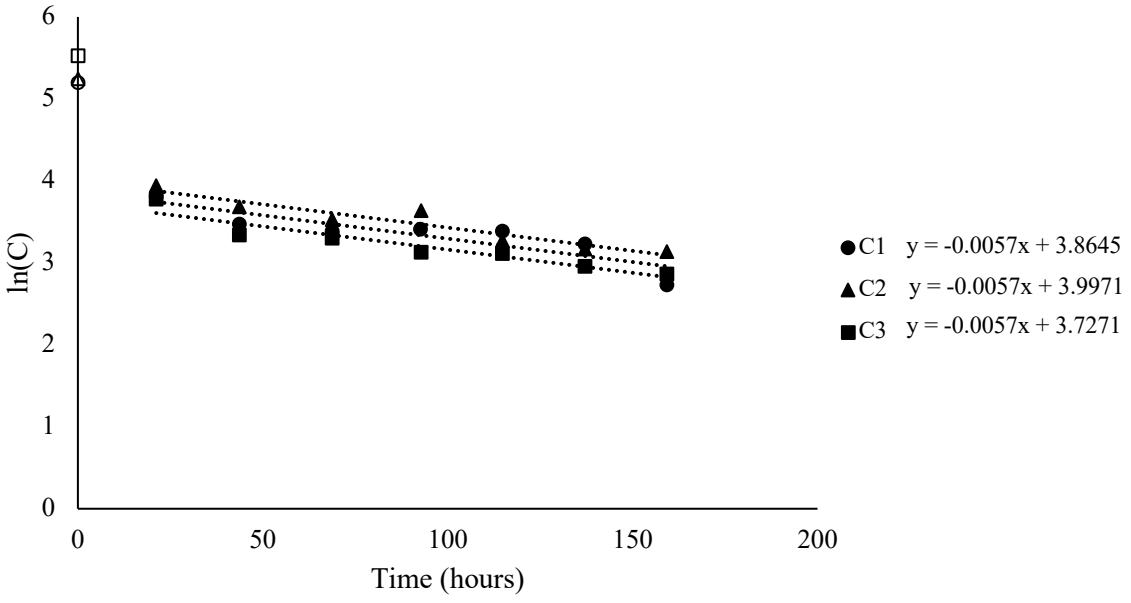
a. BPA Control



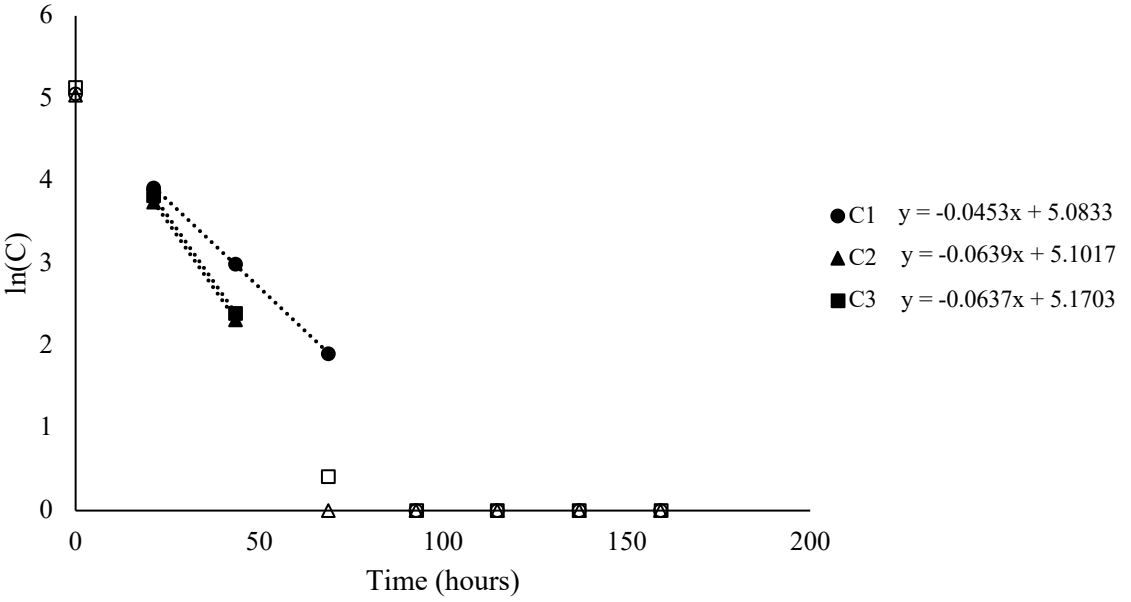
b. CAR Control



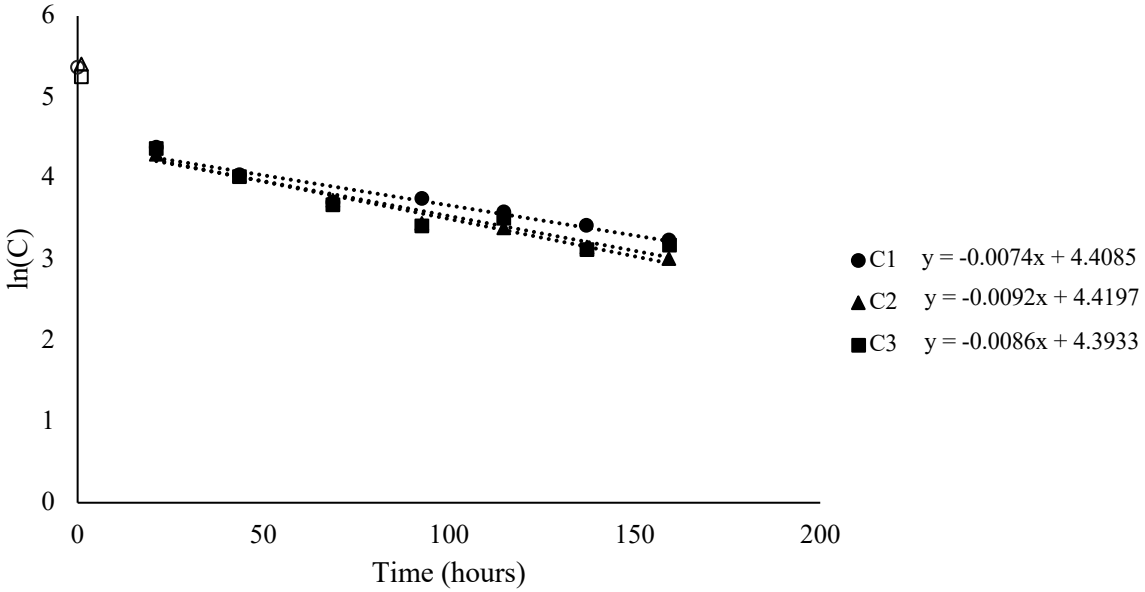
c. GEM Control



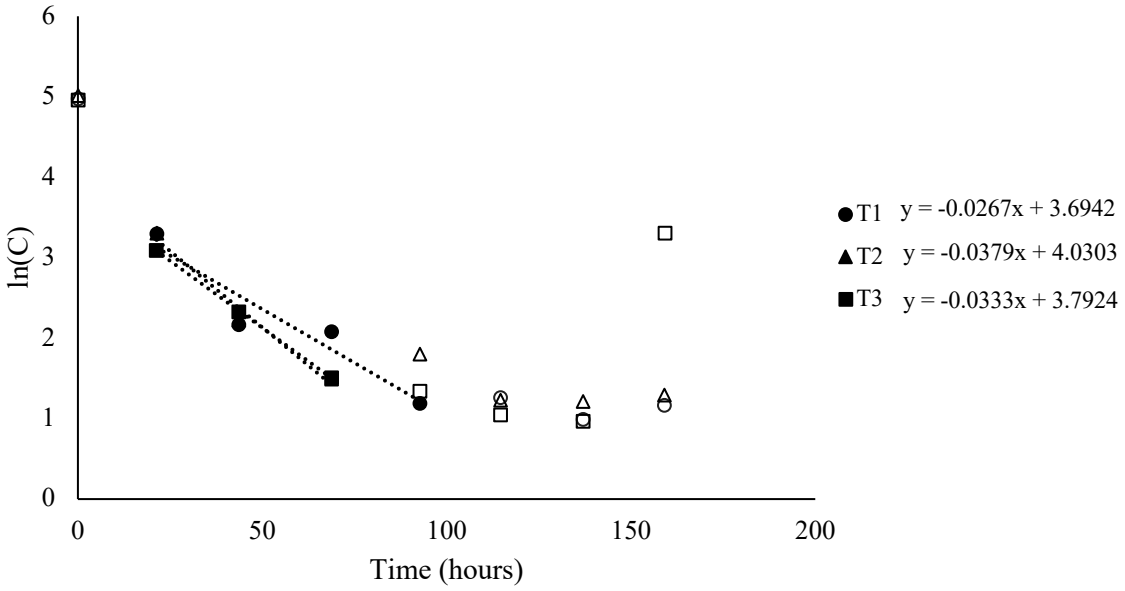
d. IBU Control



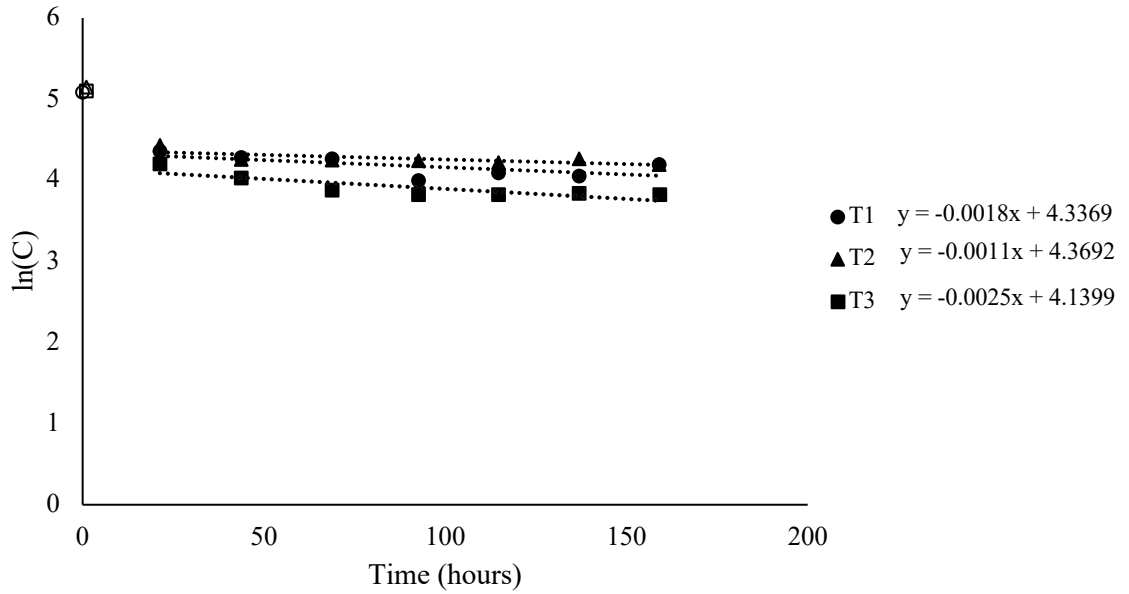
e. NAP Control



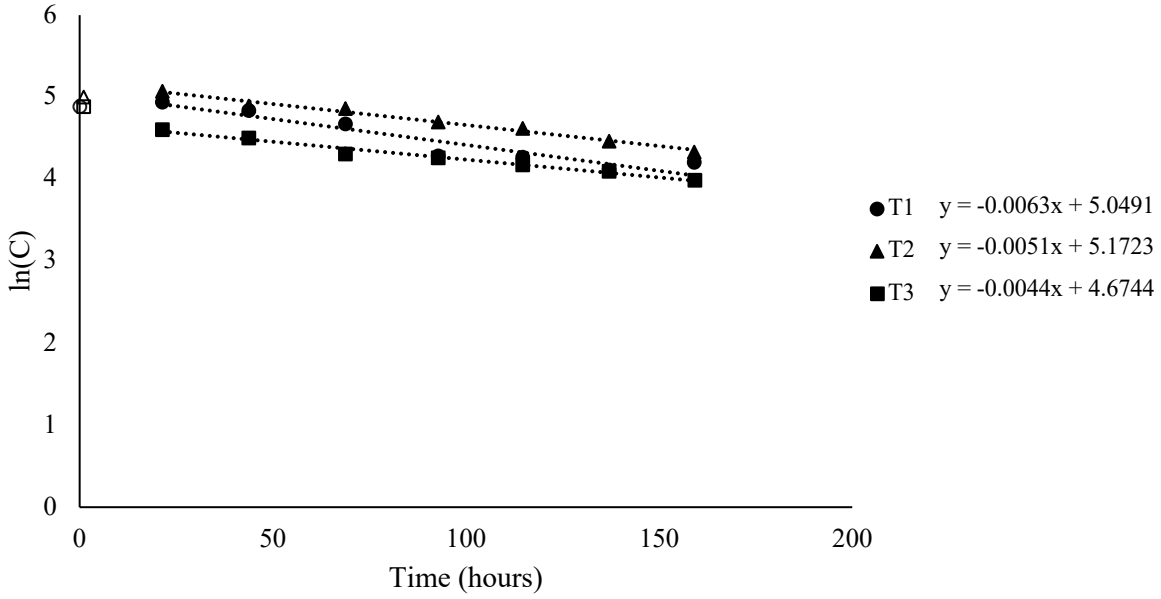
f. BPA Treated



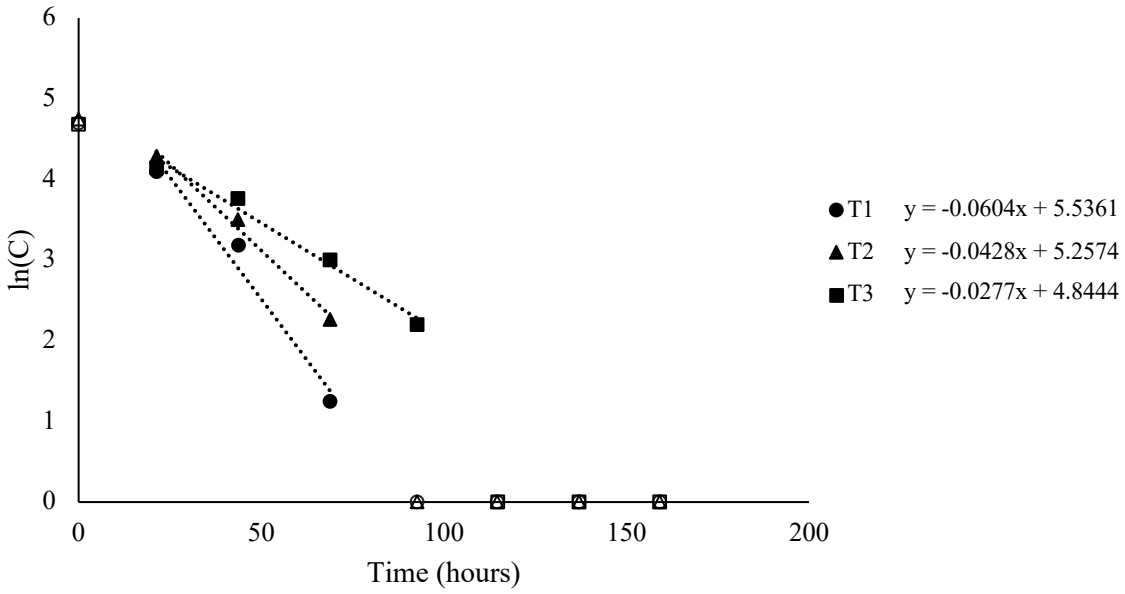
g. CAR Treated



h. GEM Treated



i. IBU Treated



j. NAP Treated

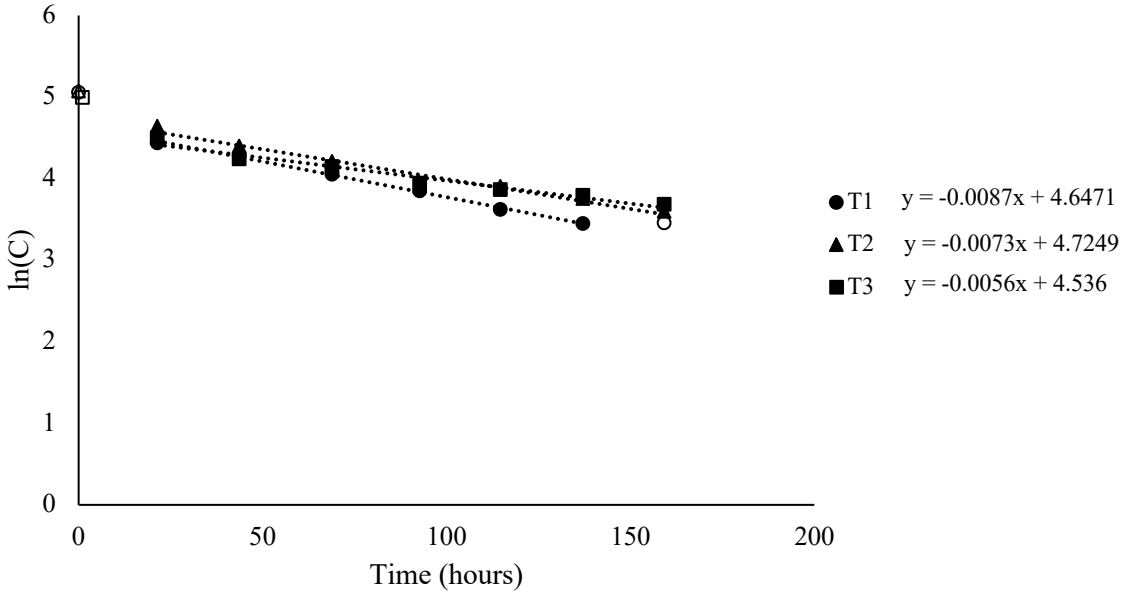


Figure A4: Linearized degradation of trace-level organic contaminants by Garfield Bay control (a-e) and treated (f-j) soils. Trendline equations are shown to the right of the legend.

Table A1: Droplet digital PCR methods development and optimization.

Date	DNA	Target	Primers	Annealing temp	Results	Notes
1/21/20	BiD32, soil	16S	8F/987R	55	Righthand peak observed, lots of rain	
2/25/20	BiD32, soil	16S	8F/987R	63	Spotty, arching droplets, righthand peak, no clear negative/positive drops	Move reactions to column 3 to avoid edge effects
3/5/20	BiD32 (new)	16S	1114F/1275R	63	Manual calls	Vortex thoroughly to avoid arching dots Blank had positive droplets- contamination?
7/21/20	Nepal	16S	111F/1275R 1079F/1468R 2363F/3362R	63	No visible upper threshold, consistent rain	
8/10/20	BiD32, IBU4	BPA	490F/895R	63	1079/1468 and 490/895 worked, 2363F/3362R did not	
8/26/20	Heyburn	BPA	1079F/1468R		No righthand peaks	
9/9/20	BiD32, Hey	Arc	109F/348R		787 did not work as well	A109 is the best primer
10/20/20	BiD32, Ell, Bay	Bac/Arc	109F/348R 1114F/1275R	63	Bad run	Try adjusting annealing temp
10/21/20	Hey, Ell, Bay	16S	1114/1275	55	Overwhelmed reactions, no righthand peak	Dilute DNA
11/2/20	Hey, Ell, Bay	16S	1114/1275	53	Righthand peak for all, good numerical answers	
11/13/20	BiD32	16S	1114F/1275R	55	Low amplification	Microfuge arriving soon- re-extract DNA Purchase new MM? Used C1000 thermocycler, changed up protocol (extend/anneal together)
1/27/21	BiD32	16S	1114F/1275R 515F/806R		Both primer sets, moving forward with 1114/1275	
2/3/21	BiD32	16S	1114F/1275R		Too much DNA, overwhelmed reaction	Next time, try 2x primer concentration
2/10/21	BiD32	16S	1114F/1275R	57	Barely any amplification despite higher primer concentrations	Add signal stabilization step as described in BioRad protocol
2/10/21	BiD32	16S	1114F/1275R	57	Righthand peak clearly visible	
2/16/21	Hey, Ell, Bay	16S	1114F/1275R	57	Clear negative and righthand peaks	Run again in triplicate for final results
2/18/21	Hey, Ell, Bay	BPA	1079F/1468R		No righthand peaks	Run 1:10 dilution instead
3/4/21	Hey, Ell, Bay	BPA	1079F/1468R		Temperature around 51ºC looked best in the gradient	Set ramp rate to 2C/s
3/10/21	Hey, Ell, Bay	BPA	1079F/1468R	51	Results were consistently lower than previous BPA run	
3/11/21	Hey, Ell, Bay	16S	1114F/1275R	58	Final bacterial 16S results	

3/16/21	Hey	Arc	109F/348R		59ºC was best temp
3/16/21	Hey, Ell, Bay	Arc	109F/348R	59	Final archaeal 16S results
3/23/21	Hey, Ell, Bay	BPA	1079F/1468R	51	Final BPA gene results

R code for statistical analysis

```
#PCA / microbial community analysis
library(dplyr)
library(tibble)
library(vegan)
library(tidyr)
library(ggplot2)

orig <- data.frame(read.csv(("otu_taxa_tableCOPY.csv"),
                            header=T,
                            stringsAsFactors = F, dec="."))
orig <- orig[-1,]
orig <- data.frame(orig, row.names = orig$OTU_ID)
orig <- data.frame(orig, header=T, stringsAsFactors = F,
                   row.names = orig$OTU_ID, dec=".")


otus <- data.frame(read.csv("otu_tableCOPY.csv"),
                   stringsAsFactors = F)
otus <- data.frame(read.csv("otu_tableCOPY.csv"),
                   stringsAsFactors = F,
                          row.names=otus$OTU_ID)
otus <- data.frame(otus[,-1])
otus <- as.data.frame(otus)
class(otus)

pca1 <- prcomp(t(otus), scale.=T)
summary(pca1) # all summary
names(summary(pca1)) # names of summary list elements
summary(pca1)$importance # importance only
summary(pca1)$importance[,1:6] # importance only of PC1-6

# Find the statistical results of the PCA
str(pca1)

#write csv
write.csv ((pca1)$rotation[,1:6],
           "~PCA.rotations.csv" )
write.csv (summary(pca1)$importance[,1:6],
           "~PCA.importance.csv" )
write.csv((pca1$x), "PCA.xy.csv")

#scree plot
pca.var <- pca1$sdev^2
pca.var.per <- round(pca.var/sum(pca.var)*100, 1)
pca.var.per
barplot(pca.var.per, main="Scree Plot",
        xlab="Principal Component",
        ylab="Percent Variation")
```

```
# Plot the PCA
library(ggplot2)
pca.data <- data.frame(Sample=rownames(pca1$x),
                       X=pca1$x[,1],
                       Y=pca1$x[,2])

l.x <- pca1$rotation[,1]
l.y <- pca1$rotation[,2]

ggbiplot(pca1, choices=1:2, scale=1)

?ggbiplot
library(devtools)
install_github("vqv/ggbiplot")
library(ggbiplot)

#loadings
load.scores <- pca1$rotation[,1]
gene.scores <- abs(load.scores)
gene.ranked <- sort(gene.scores, decreasing=T)
top.genes <- names(gene.ranked[1:10])
top.genes
load.scores
gene.scores
gene.ranked

#Diversity stats
shannon <- read.csv("idaho_microorganism_stats.csv")
aov.shan <- aov(Shannon~Treatment+Site, data=shannon)
summary(aov.shan)
aov.shan.interact <- aov(Shannon~Treatment+Site+
                           Treatment*Site, data=shannon)
summary(aov.shan.interact)

#Which model to use
library(AICcmodavg)
model.set <- list(aov.shan, aov.shan.interact)
model.names <- c("aov.shan", "aov.shan.interact")
aictab(model.set, modnames = model.names)
plot(aov.shan.interact)
tuk <- TukeyHSD(aov.shan.interact)
tuk

#Don't scale in pca
pca2 <- prcomp(t(otus), scale.=F)
summary(pca2)
pca.data2 <- data.frame(Sample=rownames(pca2$x),
                       X=pca2$x[,1],
                       Y=pca2$x[,2])

l.x <- pca2$rotation[,1]
l.y <- pca2$rotation[,2]
```

```
#write csv
write.csv ((pca2)$rotation[,1:6],
           "~PCA2.rotations.csv" )
write.csv (summary(pca2)$importance[,1:6],
           "~PCA2.importance.csv" )
write.csv((pca2$x), "PCA2.xy.csv")

#Phyla abundance
#Solirubrobacter
Soli.B.T <- c(1.61,1.69)
Soli.B.C <- c(4.09,3.81)
t.test(Soli.B.T, Soli.B.C, alternative="two.sided")

Soli.H.T <- c(3.58,4.09)
Soli.H.C <- c(6.58,6.11)
t.test(Soli.H.T, Soli.H.C, alternative="two.sided")

Soli.E.T <- c(2.21,2.32)
Soli.E.C <- c(5.06,5.04)
t.test(Soli.E.T, Soli.E.C, alternative="two.sided")

#Gaiellales
Ga.B.T <- c(1.45,1.47)
Ga.B.C <- c(3.03,3.13)
t.test(Ga.B.T, Ga.B.C, alternative="two.sided")

Ga.H.T <- c(3.44,3.44)
Ga.H.C <- c(3.94,4.24)
t.test(Ga.H.T, Ga.H.C, alternative="two.sided")

Ga.E.T <- c(1.69,1.79)
Ga.E.C <- c(2.37,2.47)
t.test(Ga.E.T, Ga.E.C, alternative="two.sided")

#Bacilli
Baci.B.T <- c(0.14,0.13)
Baci.B.C <- c(0.18,0.15)
t.test(Baci.B.T, Baci.B.C, alternative="two.sided")

Baci.H.T <- c(2.3,2.2)
Baci.H.C <- c(0.17,0.19)
t.test(Baci.H.T, Baci.H.C, alternative="two.sided")

Baci.E.T <- c(0.18,0.18)
Baci.E.C <- c(0.19,0.16)
t.test(Baci.E.T, Baci.E.C, alternative="two.sided")

#Flavobacteria
Flav.B.T <- c(2.26,2.12)
Flav.B.C <- c(0.36,0.42)
t.test(Flav.B.T, Flav.B.C, alternative="two.sided")
```

```
Flav.H.T <- c(0.5,0.53)
Flav.H.C <- c(0.3,0.31)
t.test(Flav.H.T, Flav.H.C, alternative="two.sided")

Flav.E.T <- c(0.5,0.58)
Flav.E.C <- c(0.31,0.29)
t.test(Flav.E.T, Flav.E.C, alternative="two.sided")

#Actinobacteria
Act.B.T <- c(7.72,7.76)
Act.B.C <- c(16.52,16.54)
t.test(Act.B.T, Act.B.C, alternative="two.sided")

Act.H.T <- c(16.26,16.94)
Act.H.C <- c(21.36,20.73)
t.test(Act.H.T, Act.H.C, alternative="two.sided")

Act.E.T <- c(9.25,9.76)
Act.E.C <- c(20.14,20.07)
t.test(Act.E.T, Act.E.C, alternative="two.sided")

#Gemmatimonadetes
Gemm.B.T <- c(4.23,4.47)
Gemm.B.C <- c(4.16,4.19)
t.test(Gemm.B.T, Gemm.B.C, alternative="two.sided")

Gemm.H.T <- c(4.11,4.34)
Gemm.H.C <- c(3.34,3.36)
t.test(Gemm.H.T, Gemm.H.C, alternative="two.sided")

Gemm.E.T <- c(6.51,6.85)
Gemm.E.C <- c(3.35,3.34)
t.test(Gemm.E.T, Gemm.E.C, alternative="two.sided")

#Nitrospirae
Nitr.B.T <- c(3.35,2.92)
Nitr.B.C <- c(2.92,3.38)
t.test(Nitr.B.T, Nitr.B.C, alternative="two.sided")

Nitr.H.T <- c(2.66,2.15)
Nitr.H.C <- c(1.77,2.15)
t.test(Nitr.H.T, Nitr.H.C, alternative="two.sided")

Nitr.E.T <- c(6.05,5.25)
Nitr.E.C <- c(2.04,1.85)
t.test(Nitr.E.T, Nitr.E.C, alternative="two.sided")

#Firmicutes
Firm.B.T <- c(0.15,0.16)
Firm.B.C <- c(0.19,0.17)
t.test(Firm.B.T, Firm.B.C, alternative="two.sided")
```

```
Firm.H.T <- c(2.33,2.22)
Firm.H.C <- c(0.18,0.21)
t.test(Firm.H.T, Firm.H.C, alternative="two.sided")

Firm.E.T <- c(0.21,0.21)
Firm.E.C <- c(0.22,0.18)
t.test(Firm.E.T, Firm.E.C, alternative="two.sided")

#Bacteroidetes
Bact.B.T <- c(9.59,9.19)
Bact.B.C <- c(4.86,4.77)
t.test(Bact.B.T, Bact.B.C, alternative="two.sided")

Bact.H.T <- c(6.65,6.61)
Bact.H.C <- c(5.7,5.95)
t.test(Bact.H.T, Bact.H.C, alternative="two.sided")

Bact.E.T <- c(4.43,4.38)
Bact.E.C <- c(4.21,4.37)
t.test(Bact.E.T, Bact.E.C, alternative="two.sided")

ggbiplot(pca2, choices=1:2, scale=1)

#####

#ddPCR analysis
setwd("~/Desktop/Grad School Things/")
library(plyr)

ddpcr <- read.csv("ddPCR_forR.csv")
ddpcr.ratios <- ddpcr
Bac.Arc <- ddpcr$Bacteria/ddpcr$Archaea
Bac.BPA <- ddpcr$Bacteria/ddpcr$BPA
Arc.BPA <- ddpcr$Archaea/ddpcr$BPA
ddpcr$Bac.Arc <- cbind(Bac.Arc)
ddpcr$Bac.BPA <- cbind(Bac.BPA)
ddpcr$Arc.BPA <- cbind(Arc.BPA)

#subset dataset into bact/arc/bpa gene
bact <- data.frame(ddpcr[,1:4])
arc <- data.frame(ddpcr[,1:3],ddpcr[,5])
bpa <- data.frame(ddpcr[,1:3],ddpcr[,6])

#subset ratios
bacarc <- data.frame(ddpcr[,1:3], ddpcr[,7])
bacbpa <- data.frame(ddpcr[,1:3], ddpcr[,8])
arcbpa <- data.frame(ddpcr[,1:3], ddpcr[,9])

#create lists of replicates - bacteria
HeyT.B <- c(bact[1:3,4])
HeyC.B <- c(bact[4:6,4])
```

```
EllT.B <- c(bact[7:9,4])
EllC.B <- c(bact[10:12,4])
BayT.B <- c(bact[13:15,4])
BayC.B <- c(bact[16:18,4])

#t tests - bacteria
t.test(HeyT.B, HeyC.B)
t.test(EllT.B, EllC.B)
t.test(BayT.B, BayC.B)

#create lists of replicates - archaea
HeyT.A <- c(arc[1:3,4])
HeyC.A <- c(arc[4:6,4])
EllT.A <- c(arc[7:9,4])
EllC.A <- c(arc[10:12,4])
BayT.A <- c(arc[13:15,4])
BayC.A <- c(arc[16:18,4])

#t tests - archaea
t.test(HeyT.A, HeyC.A)
t.test(EllT.A, EllC.A)
t.test(BayT.A, BayC.A)

#create lists of replicates - bpa gene
HeyT.BP <- c(bpa[1:3,4])
HeyC.BP <- c(bpa[4:6,4])
EllT.BP <- c(bpa[7:9,4])
EllC.BP <- c(bpa[10:12,4])
BayT.BP <- c(bpa[13:15,4])
BayC.BP <- c(bpa[16:18,4])

#t tests - bpa gene
t.test(HeyT.BP, HeyC.BP)
t.test(EllT.BP, EllC.BP)
t.test(BayT.BP, BayC.BP)

#Interactions
interaction.plot(ddpcr$Treatment, ddpcr$Site, response =
ddpcr$Bacteria)
interaction.plot(ddpcr$Treatment, ddpcr$Site, response =
ddpcr$Archaea)
interaction.plot(ddpcr$Treatment, ddpcr$Site, response = ddpcr$BPA)

#anova
aovB <- aov(Bacteria~Site*Treatment, data=ddpcr)
summary(aovB)

aovA <- aov(Archaea~Site*Treatment, data=ddpcr)
summary(aovA)

aovBPA <- aov(BPA~Site*Treatment, data=ddpcr)
summary(aovBPA)
```

```
#TukeyHSD
TukeyHSD(aovB)
TukeyHSD(aovA)
TukeyHSD(aovBPA)
shapiro.test(BayC.A)
qqplot(BayC.A)
library(ggpubr)
ggdensity(EllT.BP)

#Wilcox??
wilcox.test(HeyT.BP, HeyC.BP, alternative="two.sided")
wilcox.test(HeyT.A, HeyC.A, alternative="two.sided")

#Ratio stat tests
#Create individual variables bac/arc
HeyT.ba <- c(bacarc[1:3,4])
HeyC.ba <- c(bacarc[4:6,4])
EllT.ba <- c(bacarc[7:9,4])
EllC.ba <- c(bacarc[10:12,4])
BayT.ba <- c(bacarc[13:15,4])
BayC.ba <- c(bacarc[16:18,4])

#T tests bac/arc
t.test(HeyT.ba, HeyC.ba)
t.test(EllT.ba, EllC.ba)
t.test(BayT.ba, BayC.ba)

#Bac/BPA
HeyT.bbpa <- c(bacbpa[1:3,4])
HeyC.bbpa <- c(bacbpa[4:6,4])
EllT.bbpa <- c(bacbpa[7:9,4])
EllC.bbpa <- c(bacbpa[10:12,4])
BayT.bbpa <- c(bacbpa[13:15,4])
BayC.bbpa <- c(bacbpa[16:18,4])

#T tests bac/bpa
t.test(HeyT.bbpa, HeyC.bbpa)
t.test(EllT.bbpa, EllC.bbpa)
t.test(BayT.bbpa, BayC.bbpa)

#Arc/BPA
HeyT.abpa <- c(arcbpa[1:3,4])
HeyC.abpa <- c(arcbpa[4:6,4])
EllT.abpa <- c(arcbpa[7:9,4])
EllC.abpa <- c(arcbpa[10:12,4])
BayT.abpa <- c(arcbpa[13:15,4])
BayC.abpa <- c(arcbpa[16:18,4])

#T tests arc/bpa
t.test(HeyT.abpa, HeyC.abpa)
t.test(EllT.abpa, EllC.abpa)
```

```
t.test(BayT.abpa, BayC.abpa)
#####

#Degradation rate analysis
library(plyr)
library(tibble)
library(tidyr)
kvals <- read.csv("kvalues.csv")
kvals <- column_to_rownames(kvals, var="Sample")

#ANOVA simple
BPA.anova <- aov(BPA~Soil+Treatment, data=kvals)
summary(BPA.anova)

CAR.anova <- aov(CAR~Soil+Treatment, data=kvals)
summary(CAR.anova)

GEM.anova <- aov(GEM~Soil+Treatment, data=kvals)
summary(GEM.anova)

IBU.anova <- aov(IBU~Soil+Treatment, data=kvals)
summary(IBU.anova)

NAP.anova <- aov(NAP~Soil+Treatment, data=kvals)
summary(NAP.anova)

#ANOVA with interaction effects
BPA.anova2 <- aov(BPA~Soil*Treatment, data=kvals)
summary(BPA.anova2)

CAR.anova2 <- aov(CAR~Soil*Treatment, data=kvals)
summary(CAR.anova2)

GEM.anova2 <- aov(GEM~Soil*Treatment, data=kvals)
summary(GEM.anova2)

IBU.anova2 <- aov(IBU~Soil*Treatment, data=kvals)
summary(IBU.anova2)

NAP.anova2 <- aov(NAP~Soil*Treatment, data=kvals)
summary(NAP.anova2)

#T tests for interesting k values
#Heyburn BPA
BPA.C.H <- c(-0.019183549,
             -0.023428303,
             -0.022094312)
BPA.T.H <- c(-0.027901412,
             -0.029822616)
t.test(x=BPA.C.H, y=BPA.T.H, alternative="two.sided")

#Heyburn CAR
```

```
CAR.C.H <- c(-0.009381198,
             -0.007611461,
             -0.006324807)
CAR.T.H <- c(-0.003359201,
             -0.006939864,
             -0.0048288)
t.test(x=CAR.C.H, CAR.T.H, alternative="two.sided")

#Heyburn GEM
GEM.C.H <- c(-0.008510888,
             -0.006771801,
             -0.006404375)
GEM.T.H <- c(-0.010266036,
             -0.007556521,
             -0.00848671)
t.test(x=GEM.C.H, y=GEM.T.H, alternative="two.sided")

#Heyburn IBU
IBU.C.H <- c(-0.031126345,
             -0.035684359,
             -0.024886343)
IBU.T.H <- c(-0.047146784,
             -0.040569594,
             -0.06582774)
t.test(x=IBU.C.H, y=IBU.T.H, alternative="two.sided")

#Heyburn NAP
NAP.C.H <- c(-0.008098037,
             -0.014470454,
             -0.00663227)
NAP.T.H <- c(-0.009037212,
             -0.006840868,
             -0.007647794)
t.test(x=NAP.C.H, y=NAP.T.H, alternative="two.sided")

#Ellisport BPA
BPA.C.E <- c(-0.022685718,
             -0.016794504,
             -0.016654917)
BPA.T.E <- c(-0.041770993,
             -0.03731735,
             -0.044543554)
t.test(x=BPA.C.E, y=BPA.T.E, alternative="two.sided")

#Ellisport IBU
IBU.C.E <- c(-0.035609238,
             -0.059163922,
             -0.029610011)
IBU.T.E <- c(-0.035417512,
             -0.051483189,
             -0.031162717)
t.test(x=IBU.C.E, y=IBU.T.E, alternative="two.sided")
```

```
#Ellisport GEM
GEM.C.E <- c(-0.01488951,
             -0.002753331)
GEM.T.E <- c(-0.008508994,
             -0.008757968,
             -0.010884302)
t.test(x=GEM.C.E, y=GEM.T.E, alternative="two.sided")

#Ellisport CAR
CAR.C.E <- c(-0.000525391,
             -0.000111154)
CAR.T.E <- c(-0.0033773,
             -0.003152953,
             -0.004876231)
t.test(x=CAR.C.E, y=CAR.T.E, alternative="two.sided")

#Ellisport NAP
NAP.C.E <- c(-0.005483509,
             -0.006595776,
             -0.007820321)
NAP.T.E <- c(-0.009963082,
             -0.011550347,
             -0.00812419)
t.test(x=NAP.C.E, y=NAP.T.E, alternative="two.sided")

#Garfield BPA
BPA.C.B <- c(-0.013831898,
             -0.02258339,
             -0.022550335)
BPA.T.B <- c(-0.02667477,
             -0.037919965,
             -0.033268854)
t.test(x=BPA.C.B, y=BPA.T.B, alternative="two.sided")

#Garfield CAR
CAR.C.B <- c(-0.002986121,
             -0.001953976,
             -0.002904651)
CAR.T.B <- c(-0.001770149,
             -0.001131471,
             -0.002465772)
t.test(x=CAR.C.B, y=CAR.T.B, alternative="two.sided")

#Garfield GEM
GEM.C.B <- c(-0.005694645,
             -0.005674593,
             -0.005659743)
GEM.T.B <- c(-0.006314019,
             -0.005121436,
             -0.004359948)
t.test(x=GEM.C.B, y=GEM.T.B, alternative="two.sided")
```

```
#Garfield IBU
IBU.C.B<- c(-0.042322875,
            -0.063899344,
            -0.063731234)
IBU.T.B <- c(-0.060383938,
             -0.042768458,
             -0.027688317)
t.test(x=IBU.C.B, y=IBU.T.B, alternative="two.sided")

#Garfield NAP
NAP.C.B <- c(-0.007427155,
             -0.00921431,
             -0.008586389)
NAP.T.B <- c(-0.008748497,
             -0.007310544,
             -0.005615383)
t.test(x=NAP.C.B, y=NAP.T.B, alternative="two.sided")
```