**The Identification of Carbamazepine Biodegrading Phylotypes and Phylotypes Sensitive to
Carbamazepine Exposure in Two Soil Microbial Communities**

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Abstract

Carbamazepine (CBZ), an antiepileptic drug, has been introduced into agricultural soils via irrigation with treated wastewater and biosolids application. Such contamination is problematic because CBZ is persistent and the risks to ecosystems or human health are unknown. The current study examined CBZ biodegradation in two agricultural soils (soil 1 and 2) and the effects on the soil microbial communities during CBZ exposure. The experimental design involved three CBZ concentrations (50, 500, 5000 ng/g), under aerobic as well as anaerobic conditions. CBZ concentrations were determined using solid phase extraction and LC MS/MS. The effect of CBZ on the soil microbial community was investigated using high throughput sequencing and a computational approach to predict functional composition of the metagenomes (phylogenetic investigation of communities by reconstruction of unobserved states, PICRUSt). The most significant CBZ biodegradation occurred in soil 1 under aerobic conditions. In contrast, CBZ biodegradation was limited under anaerobic conditions in soil 1 and under both conditions in soil 2. For soil 1, several phylotypes were enriched following CBZ degradation compared to the controls, including unclassified *Sphingomonadaceae*, *Xanthomonadaceae* and *Rhodobacteraceae*, as well as *Sphingomonas*, *Aquicella* and *Microvirga*. These phylotypes are considered putative CBZ degraders as they appear to be benefiting from CBZ biodegradation. PICRUSt revealed that soil 1 contained a greater abundance of xenobiotic degrading genes compared to soil 2, and thus, this analysis method offers a potential valuable approach for predicting CBZ attenuation in soils. PICRUSt analysis also implicated *Sphingomonadaceae* and *Xanthomonadaceae* in drug metabolism. Interestingly, numerous phylotypes decreased in abundance following CBZ exposure and these varied with soil type, concentration, duration of exposure, and the availability of oxygen. For three phylotypes (*Flavobacterium*, 3 *genus incertae*

sedis and *unclassified Bacteroidetes*), the relative abundance was reduced in both soils, indicating a notable sensitivity to CBZ for these microorganisms.

Keywords: Carbamazepine, biodegradation, *Sphingomonadaceae*, *Xanthomonadaceae*, *Rhodobacteraceae*, PICRUSt

1. Introduction (this has been reduced from 1382 words to 1043 words)

It is now widely recognized that pharmaceuticals and personal care products (PPCPs) are not fully eliminated during wastewater treatment and can be released into the environment via wastewater effluent and biosolids (Cha and Cupples, 2009; Chen et al., 2011; Clara et al., 2004; Gottschall et al., 2012; Kinney et al., 2006b; Lajeunesse et al., 2012; Miao et al., 2005; Miege et al., 2008; Ollers et al., 2001; Vieno and Sillanpaa, 2014; Williams and McLain, 2012; Wu et al., 2010). This is a cause for concern because these chemicals are being detected in surface water and groundwater (Gottschall et al., 2012; Kolpin et al., 2002; Lapworth et al., 2012; Nakada et al., 2008; Tixier et al., 2003; Tran et al., 2014). The reuse of wastewater for irrigation of agricultural areas can also increase the presence PPCPs in the environment (Dalkmann et al., 2014; Gibson et al., 2010; Grossberger et al., 2014; Kinney et al., 2006a). The long term ecotoxicity potential of individual and mixtures of PPCPs in the environment has not been determined (Rosi-Marshall and Royer, 2012). The continued acceptance of 1) biosolids for agricultural land application and 2) wastewater effluent reuse in arid and semi-arid areas will depend on the ecological and human risks associated with the fate and effect of PCPPs in the environment.

From the many PPCPs released into the environment, carbamazepine (*5H*-dibenzo [*b,f*]azepine-5-carboxamide, CBZ), an antiepileptic drug, is perhaps one of the most problematic. CBZ has been detected in many wastewater treatment plant (WWTP) samples (Metcalf et al., 2003; Miao and Metcalfe, 2003; Ternes, 1998; Tixier et al., 2003). It was found in all influent and effluent samples from 18 WWTPs across Canada (Metcalf et al., 2003). Another study detected CBZ in 30 WWTP effluents in Germany (Ternes, 1998). CBZ has also been detected in biosolids from WWTPs (Gottschall et al., 2012; Kinney et al., 2006b; Miao et al., 2005). CBZ was observed in nine different biosolids products in seven states with average concentrations ranging from 8 to 390 µg/Kg (Kinney et al., 2006b). A study involving the analysis of 110 biosolids samples suggested the projected land application rate of CBZ is 550-680 Kg/yr (McClellan and Halden, 2010). The frequency of detection measured for CBZ in this study was 100% and the mean concentration was 163 µg/kg. Thus, the frequent detection of CBZ in WWTP effluent and biosolids raises concerns about the fate and risks associated with applying treated wastewater for irrigation and land applying biosolids during crop production.

Several researchers have documented the recalcitrant nature of CBZ in soil following irrigation with wastewater effluent. For example, CBZ was one of the four most commonly detected pharmaceuticals in soil irrigated with reclaimed wastewater (Kinney et al., 2006a). In another study, CBZ exhibited half-life values ranging from 147 to >200 days following irrigation with treated wastewater (Grossberger et al., 2014). Further, soils exposed to CBZ through irrigation with untreated wastewater exhibited half-lives of 355-1,624 days (Dalkmann et al., 2014). CBZ has also been shown to be highly persistent in soils following the applications of biosolids. In outdoor mesocosms with municipal biosolids, CBZ exhibited an estimated half-life of 462-533

days (Walters et al., 2010). CBZ was found in dewatered municipal biosolids aggregates incorporated into soil, one year post application at a level of 30 ng/g (Gottschall et al., 2012). A recent manuscript reported that CBZ mineralization did not exceed 2% of the spiked rate through 120 days of aerobic incubation in different soils (Li et al., 2013). Others have also reported the recalcitrant nature of CBZ when present in soils (Maeng et al., 2011; Wu et al., 2010).

Surprisingly, given the common occurrence of CBZ, very little is known about the microorganisms able to degrade this compound. To our knowledge, only two reports have identified bacteria capable of CBZ degradation. *Rhodococcus rhodochrous* and *Aspergillus niger* were reported to remove 15% and 9% respectively when exposed to $\sim 10 \text{ mg L}^{-1}$ CBZ (Gauthier et al., 2010). Also, *Streptomyces* MIUG 4.89 degraded up to 14% CBZ with an initial concentration of 0.2 mg L^{-1} (Popa et al., 2014). Notably, no information exists as to which microorganisms might be responsible for CBZ degradation in soils. Further, no data is available linking specific bacteria to CBZ degradation at environmentally relevant concentrations.

The current research was designed to determine which phylotypes increased in abundance following CBZ biodegradation, and could therefore be putatively associated with CBZ degradation. Our hypothesis being that these microorganisms are obtaining a growth benefit from CBZ degradation, perhaps as a carbon, nitrogen or energy source. The phylotypes linked to CBZ biodegradation in two agricultural soils, at three CBZ concentrations (50 ng/g, 500 ng/g and 5000 ng/g), under aerobic and anaerobic conditions, were investigated. Further, PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) was used to identify important xenobiotic degradative genes under each treatment. PICRUSt is a

computational approach that uses evolutionary modeling to predict the functional composition of a metagenome using 16S rRNA gene data and a database of reference genomes (Langille et al., 2013). To our knowledge, this is the first attempt to identify CBZ degraders in soil communities.

The current research also included a secondary objective, which involved identifying the phylotypes detrimentally affected by CBZ. Although it is widely accepted that PPCPs are released into the environment, their unintended effects on receiving environments remain largely unknown. Exposure to PPCPs may alter the microbial community composition, which may in turn influence ecosystem function. For example, the expression of gene categories related to N, P and C cycling were strongly affected by the presence of pharmaceutical products (Yergeau et al., 2012). To date, research on the effects of PPCPs in soil microbial communities has primarily focused on the effects of antibiotics (Schmitt et al., 2004; Thiele-Bruhn, 2003; Thiele-Bruhn and Beck, 2005). Limited work has addressed on the impact of CBZ on microbial communities. The common occurrence of CBZ in wastewater effluent and biosolids and the recalcitrant nature of CBZ in soils suggest CBZ will continue to be a chemical of concern to many ecosystems. To address this knowledge gap, the current work examined the impact of CBZ on two soil microbial communities under both aerobic and anaerobic conditions. This is the first study on the impact of this chemical on specific soil microorganisms.

2. Methods

2.1. Chemicals and Materials

Carbamazepine (100%) and carbamazepine-d₁₀ (99.4%) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA) and C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada), respectively.

Acetonitrile (HPLC grade) and methanol (Optima™ LC/MS) were purchased from Fisher Scientific. QuECHERS (quick, easy, cheap, effective, rugged & safe) extract pouches (6.0 g magnesium sulfate and 1.5 g sodium acetate) were obtained from Agilent Technologies (Wilmington, DE, USA). Cleanert SAX cartridges (1000 mg, 6 mL) and Oasis HLB cartridges (200 mg, 6cc) were purchased from GS-Tek (Newark, DE, USA) and Waters (Milford, MA, USA), respectively. Stock CBZ solutions (100 mg L^{-1}) were prepared in DI water and stored at 4°C until use. Working CBZ solutions (0.5 mg L^{-1} , 5.0 mg L^{-1} , and 50 mg L^{-1}) were also prepared through dilution with an appropriate volume of the stock solution in DI water. The solution concentrations were chosen to ensure the same volume ($500 \mu\text{L}$) of CBZ solution was added to each microcosm. Agricultural soils were collected from close to the campus of Michigan State University ($42^\circ46'22''\text{N}$, $84^\circ21'25''\text{W}$) and were stored in the dark at 4°C until use. Select physical and chemical properties were determined by A&L Great Lakes Laboratories, Inc. (Fort Wayne, IN) (Supplementary Table 1).

2.2. Experimental Setup

Soil samples were stored at 4°C until use and were sieved (2-mm diameter) to remove the coarse particles. Background CBZ in the two soils (soils 1 and 2) were below the detection limit ($<0.39 \text{ ng/g}$). Sacrificial incubation experiments were performed to measure CBZ dissipation in the two soils and evaluate the effects of a range of initial CBZ concentrations (50 ng/g , 500 ng/g , 5000 ng/g) on the soil microbial community under both aerobic and anaerobic conditions. Although 50 ng/g is the most environmentally relevant concentration to consider, the higher level of 500 ng/g was investigated to be reflective of the higher concentrations found within biosolids (up to 390

ng/g (Kinney et al., 2006b)). The other higher level (5000 ng/g) was examined to provide a potential contrast in the resulting biodegradation rates.

The aerobic microcosms were established in amber glass serum bottles (30 mL) with 5.0 g (wet weight) of soil spiked with 500 μ L of each CBZ working stock solution to obtain 50 ng/g, 500 ng/g, or 5000 ng/g of CBZ/g soil. After CBZ addition, the contents were vortexed to mix the target PPCP throughout the sample. Then, the moisture content of each soil was adjusted to 60% of maximum water holding capacity (63 and 78 mL/g dry soil for soil 1 and 2). The aerobic microcosms were set up in triplicates and were opened periodically to enable oxygen replenishment. The anaerobic microcosms were established in similar manner with slight modifications. Briefly, 5.0 g of soil (wet weight) spiked with 500 μ L of each CBZ solution was vortexed before adding 10 mL DI water into each bottle. The water was added to imitate saturated conditions, typical after a heavy precipitation event. The headspace was purged with oxygen-free nitrogen for 10 minutes and the bottles were immediately sealed with septa and crimps. The anaerobic microcosms were set up in triplicates or duplicates and remained closed for the duration of the experiment. Live controls (no CBZ controls) for both aerobic and anaerobic microcosms were prepared in a similar manner with the exclusion of CBZ from the 500 μ L DI water spiking solution.

To assess the role of microorganisms in the degradation of CBZ, abiotic controls were also included (for soil 1 only under aerobic conditions, as soil 2 illustrated no significant degradation). For this, 5.0 g of soil (wet weight) were placed in serum bottles capped with aluminum foil; the bottles and their contents were autoclaved at 121°C for 45 minutes for three

consecutive days after cooling. CBZ solutions were filtered (22 micron nylon syringe filter) and the DI water used to adjust the soil maximum water holding capacity was also autoclaved at 121°C for 45 minutes.

All bottles were kept in the dark until they were sampled. CBZ was extracted (as described below) from the sacrificed microcosms and the abiotic controls on days 4 and 14. Before CBZ extraction, an aliquot of soil (0.35 g of wet soil) was taken from each sample and each live control for total DNA extraction (as described below).

Another set of experiments was performed to assess the long-term biodegradation potential of CBZ (at 50 ng/g) in soil 1. For this, CBZ concentrations were determined at days 3, 14, 33 and 50 for triplicates of live and abiotic controls under both aerobic and anaerobic conditions. The experimental setup was the same as that described above, except DNA was not extracted.

2.3. CBZ Extraction and Analytical Methods

Methods were developed to extract CBZ from soils using a modified QuEChERS approach based on previous work (Salvia et al., 2012). The CBZ internal standard (CBZ-d10) was spiked (200 ng/g) to the soil matrix before extraction. The extraction procedure consisted of adding 10 mL of DI water plus 15 mL of acetonitrile to the soil samples. The mixture was vortexed for 2 min; then slowly and continuously, the QuEChERS buffer (6 g of magnesium sulfate and 1.5 g of sodium acetate) was added while vortexing at a low speed (Bragança et al. 2012). The tubes were manually shaken for 30 sec and vortexed at high speed for 30 sec. The tubes were subsequently placed on a shaker (Lab-Line, Lab-Line Instruments, Inc. Melrose, IL) at 750 rpm

for 3 min before centrifugation at 3000 g (2 min). Then, 10 mL of the acetonitrile supernatant was placed in a 12-mL glass tube. Finally, the extract was evaporated to dryness through a gentle stream of nitrogen at 40 °C and reconstituted with 60 mL of methanol-water (3:97, v/v). The pH of the extract was adjusted to an average of 7.3. The above technique was used to extract CBZ from all the aerobic as well as the anaerobic microcosms. However, only acetonitrile was added to the anaerobic microcosms since water was already present (each sample already contained 10 mL DI water). To determine CBZ extraction efficiency, 5.0 g of soil (wet weight) was placed in 50 mL polypropylene tubes and samples were spiked with 500 µL of CBZ in methanol (5 mg/L) to obtain 500 ng/g of CBZ/g soil. Then, the above CBZ extraction method was followed.

2.4. Processing of Soil Extracts

The reconstituted soil extracts underwent a clean-up step prior to their analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS). The solid phase extraction (SPE) technique presented in this work is a modification of the SPE reported by Salvia et al. (2012) and Vazquez-Roig et al. (2010) to purify soil extracts containing pharmaceuticals. First, a strong anion exchange cartridge (SAX) was placed in tandem with a polymeric cartridge (Oasis HLB). The SAX cartridge was used to minimize matrix interferences due to the presence of humic and fulvic acids in the soil extract (Vazquez-Roig et al., 2010). Next, both cartridges were preconditioned successively with methanol (5 mL) and deionized water (5 mL). Then the reconstituted soil extracts (60 mL) were loaded into the cartridges. The bottles containing the soil extracts were rinsed with 10 mL of DI water, which was then loaded into the cartridges. The cartridges were subsequently washed with 5 mL of DI water before being dried under vacuum for 30 min. The SAX cartridges were removed prior to eluting CBZ from the HLB cartridges.

The elution solvent consisted of 2 x 5 mL of methanol. The flow rate for both the loading and elution steps was < 1 mL/min. The collected eluate was evaporated to dryness under a stream of nitrogen at 40°C and reconstituted with 1 mL of methanol before transferring to 1.5 mL HPLC vials and storing at -15°C until LC-MS/MS analysis.

2.5. LC-MS/MS Analysis

Mass spectrometry was performed using an LC-MS/MS system comprising a Shimadzu high performance liquid chromatograph (HPLC, Columbia, MD, USA) coupled with an API SCIEX 3200 triple quadrupole mass spectrometer (Foster City, CA, USA) equipped with TurboIonSpray ion source. CBZ and CBZ-d10 were ionized in positive mode with ion spray voltage and temperature set to 5000 V and 750 °C, respectively. The curtain gas pressure was set to 10 psi, whereas the collision gas pressure and the ion source gas pressure were set to 9 psi and 30 psi, respectively. The MS/MS parameters were optimized for both CBZ and CBZ-d10 (Supplementary Table 2). The HPLC system utilized comprised of two LC-20AD pumps, an SIL-20A autosampler and a DGU-20A degasser with a CBM-20A controller. Gemini C18 column (50 x 2.00 mm, 5µm, Phenomenex) was used for the LC separation. The mobile phase consisted of 0.3% formic acid in MilliQ water (A) and acetonitrile (B) with a flow rate of 0.35 mL/min. A gradient involved 10% of eluent B for 0.5 min; then the eluent B underwent an increase to 100% between 0.5-2 min and was held constant between 2-3 min. Finally, a decrease from 100% to 10% was effected between 3-3.5 min. The injection volume per sample was 10 µL and the equilibration time between run was set to 2 min. The average recovery for CBZ in soil was $103.78 \pm 1.42\%$ for the tested concentration (500 ng/g).

2.6. DNA Extraction, MiSeq Illumina Sequencing and PICRUSt

DNA was extracted using the Power Soil DNA extraction kit (MO BIO Laboratories, Inc. Carlsbad, CA) following the manufacturer's instructions. DNA was extracted from replicate microcosms on day 4 and day 14 for all experimental conditions (three CBZ concentrations, two soils, aerobic and saturated conditions). Total genomic DNA extracts were submitted for high throughput amplicon sequencing following the protocol described elsewhere (Caporaso et al., 2012; Caporaso et al., 2011) at the Research Technology Support Facility (RTSF) at Michigan State University. Illumina specific fusion primers were used to amplify the V4 region of the 16S rRNA gene and to add unique barcodes to samples in each well to enable pooling and sequencing. After the amplicons were checked on 1% agarose gel, equimolar amounts of the sample were pooled to normalize results, purified and then sequenced on the Illumina MiSeq™ Personal Sequencing System. The amplicon sequencing data in the fastq file format was analyzed on Mothur version 1.33.0 from Patrick D. Schloss Laboratory (Schloss, 2009) using the MiSeq standard operating procedure (Schloss, 2013). Barcode information was removed from the sequence data and contiguous sequences were created using the forward and reverse reads, were analyzed for errors and then classified. Samples were checked for the proper read length (<275 bp), ambiguous bases and homopolymer length greater than 8 to eliminate such sequences. These sequences were then aligned with the SILVA bacteria database (Pruesse et al., 2007) for the V4 region. Chimeras, mitochondrial and chloroplast lineage sequences were removed and then the sequences were classified into OTU's. Cho1 and Shannon values were calculated and principle component analyses were performed using Mothur. Illumina sequencing data was deposited in the NCBI Sequence Read Archive under Bioproject: PRJNA311080 and Biosample: SAMN04461763.

To obtain the inferred metagenome from the 16S rRNA gene data, all sequences were first reclassified to the Greengenes gene database (DeSantis et al., 2006) and an OTU biom table was created using Mothur. Following this, normalized OTU tables were generated using PICRUSt (Langille et al., 2013), and these were used to create a functional metagenome prediction file for each sample. The functional metagenome predictions files were analyzed using the “categorize by function” command (level 3) using the KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathways. Only the KEGG pathways concerning the degradation and metabolism of xenobiotic compounds were considered in the data analysis. Following this, the OTUs responsible for contributing to the drug metabolism pathways (cytochrome P450 and other enzymes) were determined using the “metagenome contributions” command in PICRUSt. The drug metabolism pathways were selected for analysis because of the differences between the two soils for these pathways (see results section). The three OTUs with the highest gene count contribution values for these pathways were determined for soils 1 and 2 under aerobic conditions.

2.7. Data Analysis

Statistical analyses included two-tailed t-tests assuming unequal variances. This approach was used to determine if CBZ removal was significant compared to the abiotic controls (soil 1). Further, t-tests were applied to investigate which phylotypes illustrated a difference in relative abundance between the CBZ-amended samples and the live controls (no CBZ added). Finally, the PICRUSt generated data were analyzed to investigate the differences in the abundance of xenobiotic degrading genes between the two soils, at both time points, for the three concentrations under aerobic and anaerobic conditions.

3. Results and Discussion

3.1. Carbamazepine Biodegradation

The differences in CBZ concentrations between day 4 and 14 were only statistically significant for soil 1. Specifically, under aerobic conditions and under all three concentrations tested, the percent CBZ remaining significantly decreased between days 4 and 14 (Supplementary Table 3). The percent decrease under aerobic conditions between these two sampling events was similar at all three concentrations and ranged between 12.8% and 14.5% (Supplementary Figure 1A). In contrast, for soil 1, under anaerobic conditions, a statistically significant decrease was observed for only the lowest concentration ($p=0.0089$). Notably, only duplicates were included for day 14 under anaerobic conditions and this may have influenced the statistical tests. When all samples were considered collectively for all three concentrations under anaerobic conditions, the difference between the two time points was significant ($p=0.0261$) (Supplementary Table 3) and ranged from 6.2% to 14.9%. A follow up experiment with abiotic controls was completed to determine if the removal in soil 1 was biological. For all treatments tested (aerobic and anaerobic at three CBZ concentrations), there was no statistical difference in percent CBZ remaining between days 4 and 14 in the abiotic controls, indicating the removal in soil 1 was indeed biological (Supplementary Table 3).

The results from soil 2 differed considerably from those for soil 1. Although the average CBZ remaining in all treatments was lower at day 14 compared to day 4 (Supplementary Figure 1B), the differences were not significant in the majority of the treatments (Supplementary Table 3). There was only a statistically significant difference between the two time points for the highest CBZ concentration (5000 ng/g) (4.8% decrease) under aerobic conditions ($p=0.0314$). As

limited removal was observed in soil 2, the follow up experiment with abiotic controls was not performed.

The contrasting results from soil 1 and soil 2 may have important implications for the application of biosolids or wastewater effluent to agricultural areas. Soil 2 has a high organic matter content (8%) and this could impact the bioavailability of CBZ to the soil microbial community for microbial degradation. On the other hand, the organic matter content of soil 1 was low (1.5%), which may have resulted in CBZ being more readily available for biodegradation. Previous research has indicated a correlation between CBZ sorption and organic matter content (Arye et al., 2011). Therefore, soils with higher organic matter may slow CBZ degradation by limiting bioavailability to soil microorganisms. An alternative hypothesis is that the observed differences in biodegradation results from differences in the functional abilities of the two microbial communities (see PICRUS data later).

In the current study, for soil 1, the concentration of CBZ did not appear to impact removal trends. In contrast, others have reported no degradation at low CBZ concentrations (50 ng/g), but notable degradation at higher concentrations (up to ~25% at 5000 ng/g) (Grossberger et al., 2014). Conversely, higher initial CBZ concentrations have produced longer half-life values (990 days compared to 138 days for lower CBZ concentrations) in other soil biodegradation experiments (Duran-Alvarez et al., 2015). High CBZ half-life values have also been reported in studies using samples containing environmentally relevant CBZ concentrations. For example, long half-lives were reported in soils amended with untreated wastewater (between 355 and 1624

days) (Dalkmann et al., 2014) and in soils amended with biosolids in an outdoor mesocosm study (between 462 and 533 days) (Walters et al., 2010).

Limited data exist on the susceptibility of CBZ to degradation under anaerobic conditions. Here, the percent CBZ remaining decreased in soil 1 at all three concentrations under anaerobic conditions, but this decrease was only significant at the lowest concentration. Further, no CBZ removal was observed in soil 2 under anaerobic conditions. At least one other study has compared CBZ removal under aerobic and anaerobic conditions. In that research, sediment from three wetlands was examined to evaluate CBZ degradation (Conkle et al., 2012). CBZ half-life values between 207 and 546 days were reported under anaerobic conditions compared to between 165 and 264 days under aerobic conditions. The research presented here, along with the above results, indicates CBZ is more recalcitrant under anaerobic conditions and may therefore persist longer in saturated soils.

An additional set of experiments was performed to determine the long term biodegradation potential of CBZ in soil 1 at the lowest concentration (50 ng/g) under both aerobic and anaerobic conditions (Supplementary Figure 2). CBZ biodegradation under aerobic conditions was only observed at the last sampling point (day 50, $p=0.005$ for a t-test between control and samples). Further, no removal was observed under anaerobic conditions. The increased time required for CBZ degradation under aerobic conditions and the lack of degradation under anaerobic conditions can likely be attributed to soil storage time, as the experiment was performed one year after the short term experiment.

3.2. Impact of CBZ on Microbial Community

A summary of the analyzed sequence data is shown (Supplementary Table 4). The total number of OTUs or species richness was estimated using the Chao1 estimator (Supplementary Table 5). Soil 2 appeared to have greater species richness compared to soil 1. Also, species richness was lower under anaerobic conditions compared to aerobic conditions for both soils. There was only a statistically significant difference in species richness for the samples compared to the live controls (no CBZ added) in the those amended with 500 ng/g CBZ in soil 1 at day 14 and soil 2 at day 4 (both aerobic conditions).

The calculated Shannon diversity values were also generally higher for soil 2 compared to soil 1 (Supplementary Table 6). There was a statistically significant decrease in the diversity values between the CBZ-amended samples for soil 1 (at all three concentrations) and the controls at day 14. In contrast, under anaerobic conditions, there was a statistically significant increase in the diversity values between the CBZ-amended samples for soil 1 (higher two concentrations) and the controls at day 14.

The sequencing data were classified into phyla to determine the overall microbial diversity in the samples and CBZ-free controls for both soils. In soil 1, under aerobic and anaerobic conditions, the most dominant phyla were *Proteobacteria*, followed by *Bacteroidetes*, *Acidobacteria*, *Verrucomicrobia* and *Actinobacteria* (Supplementary Figure 3). In this soil, the abundance of several phyla were impacted by the presence of CBZ. In three of the four treatments (two time points, aerobic and anaerobic conditions) studied, *Actinobacteria* and *Bacteroidetes* were significantly impacted. In two of the four treatments, the phyla *WS3* and *Gemmatimonadetes*

were affected. In one of the four treatments, other impacted phyla included *BRC1*, *Spirochaetes*, *Chloroflexi*, *Nitrospira* and *Proteobacteria*.

Similar phyla were present in soil 2 compared to soil 1 (Supplementary Figure 4). *Proteobacteria* was also the most abundant, but in this case, *Acidobacteria* was the second most abundant. The third most abundant was *Bacteroidetes*, followed by *Verrucomicrobia* and then *Actinobacteria*. Similar to soil 1, the abundance of *Bacteroidetes* was significantly different in the CBZ-amended samples compared to the controls in two of the four treatments (aerobic day 4 and 14). Other phyla were only impacted in one of the four treatments and these included *Actinobacteria*, *Acidobacteria*, *WS3*, *Chloroflexi*, *Gemmatimonadetes*, *Chloroflexi* and *Proteobacteria*. Principal component analysis was performed on the sequencing data. Only one dataset exhibited significant clustering for the CBZ-amended samples compared to the controls and the initial samples (soil 1, aerobic conditions at day 14) (Supplementary Figure 5).

3.3. Identifying Putative Carbamazepine Degrading Phylotypes

The microorganisms putatively linked to CBZ degradation were only investigated for soil 1, as there was limited removal in soil 2. The aim was to determine which phylotypes increased in abundance following CBZ biodegradation, and could therefore be putatively associated with CBZ degradation. These microorganisms could be using CBZ as a carbon, nitrogen or an energy source. For this, percent relative abundance values were compared between CBZ-amended microcosms and live control microcosms (CBZ-free controls) to determine which phylotypes increased in abundance. Specifically, t-tests (two-tailed, unequal variance) were performed to

determine which phylotypes were more abundant in the CBZ-amended samples compared to the live controls.

In soil 1, at day 4, under aerobic conditions, no phylotypes statistically significantly increased in abundance in the CBZ-amended samples compared to the live controls. However, by day 14, many phylotypes were more abundant in the CBZ-amended samples compared to the controls. In these experiments, 44, 17 and 22 phylotypes were enriched in the samples amended with 50, 500 and 5000 ng/g CBZ, respectively (data not shown). From these, 16 phylotypes were enriched in two or more of the concentrations tested (Figure 1). The highest levels of enrichment were noted for unclassified *Sphingomonadaceae*, unclassified *Xanthomonadaceae* and *Sphingomonas*. Only three phylotypes were enriched at all three concentrations, including *Aquicella*, *Microvirga* and unclassified *Rhodobacteraceae*. These enrichment patterns indicate these specific phylotypes are benefiting from CBZ degradation and are likely CBZ degraders.

It is difficult to compare the above findings concerning unclassified *Sphingomonadaceae* and unclassified *Xanthomonadaceae* to previous research, as in the current work, the specific genera responsible were not determined. However, many studies have illustrated the ability of *Sphingomonas* spp. to degrade organic environmental contaminants. For example, just within the past couple of years, microorganisms in the genus *Sphingomonas* spp. have been linked to the degradation of dibenzofuran (Coronado et al., 2015), phenanthrene (Pan et al., 2016), azo dyes (Nadh et al., 2015), the fungicide ortho-phenylphenol (Perruchon et al., 2016), the chloroacetamide herbicides, butachlor, acetochlor and alachlor (Chen et al., 2015) and bisphenol-A (Matsumura et al., 2015). The data suggest that *Sphingomonas* are important for the

biodegradation of many organic chemicals and, as shown here, are likely also involved in CBZ biodegradation.

Limited information exists on the genus *Aquicella* (*Gammaproteobacteria*, *Legionellales*, *Coxiellaceae*). Two novel isolates were obtained from well and spa water in central Portugal (Santos et al., 2003) and sequences classifying as *Aquicella* were reported in rhizospheres of field-grown potato plants (Kobayashi et al., 2015). However, previous research has documented that *Aquicella* illustrated a positive response in soils amended with phenanthrene (Ding et al., 2012). Microorganisms in the genus *Microvirga* (*Alphaproteobacteria*, *Rhizobiales*, *Methylobacteriaceae*) are symbiotic nitrogen-fixing bacteria (Ardley et al., 2012; Radl et al., 2014; Reeve et al., 2014) and have also been isolated from free-flowing geothermal waters (Kanso and Patel, 2003). Notably, microorganisms classifying as *Microvirga* were found in a biofilm exposed to crude oil, n-hexadecane and phenanthrene contaminated sewage effluent (Al-Mailem et al., 2014). Microorganisms in the family *Rhodobacteraceae* have been found in coastline water samples exposed to crude oil (Acosta-Gonzalez et al., 2015) and have been associated with the degradation of different polyaromatic hydrocarbon (PAH) compounds (Gutierrez et al., 2011; Harwati et al., 2009; Pinyakong et al., 2012; Syakti et al., 2013). The results presented here, along the studies discussed above, suggest similarities in the phylotypes able to degrade PAH and CBZ. This pattern is perhaps not surprising, as CBZ contains two aromatic ring moieties.

In soil 1, under anaerobic conditions, there was only a statistically significant difference between CBZ remaining at the two time points at the lowest CBZ concentration (Supplementary Table 3).

However, when the data were combined from all three concentrations, a significant decrease between the two time points was observed ($p=0.0261$). Therefore, the phylotypes that increased in abundance in CBZ treated samples were compared to the live CBZ-free controls for the anaerobic experiments. At the early time point (day 4), 5, 14 and 5 phylotypes were enriched in the CBZ-amended microcosms compared to the controls at 50, 500 and 5000 ng/g, respectively (Supplementary Figure 6). Only three phylotypes were enriched in more than one of the concentrations tested, included unclassified *Desulfuromonadaceae*, *Pusillimonas* (both with relative abundances of $< 0.009\%$) and *Armatimonadetes gp5* (relative abundance of $> 0.5\%$). Very little is known about the genus *Armatimonadetes gp5* (phylum *Armatimonadetes*) and, according to the Ribosomal Database Project, the genus contains only 14 uncultured microorganisms. The data generated in the current study indicate a potentially significant role of this phylotype in CBZ degradation. The low relative abundance values of the other two phylotypes suggests their roles are likely minor.

A more limited effect on the microbial community was noted for soil 1, under anaerobic conditions, at the later time point (day 14). For this, only one phylotype was enriched at 50 and 5000 ng/g and nine phylotypes were enriched at 500 ng/g (Supplementary Figure 7). No phylotypes were enriched in more than one concentration and only two phylotypes (*Cryobacterium* and *Solirubrobacter*) were enriched at more than 0.3% relative abundance (Supplementary Figure 7B, C). These weak trends make it difficult to link specific phylotypes with CBZ removal under anaerobic conditions.

As stated above, only *Rhodococcus rhodochrous*, *Aspergillus niger* and *Streptomyces* MIUG 4.89 have been previously linked to CBZ degradation (Gauthier et al., 2010; Popa et al., 2014). Interestingly, no enrichment of these genera was observed here following CBZ degradation, suggesting that pure culture research may inaccurately portray the microorganisms responsible for CBZ in soil communities when exposed to environmentally relevant CBZ concentrations.

3.4. PICRUST Analysis

PICRUST was used to predict the functional composition of the metagenomes from soils 1 and 2 under the different treatments and time points. The analysis focused on the genes associated with xenobiotic degradation pathways. The percent predicted abundance of these genes was compared between the soils. Under aerobic conditions, there was a statistically significant difference between the two soils for several pathways, including DDT degradation, atrazine degradation, chlorocyclohexane and chlorobenzene degradation, drug metabolism-other enzymes, drug metabolism- cytochrome P450, fluorobenzoate degradation and nitrotoluene degradation (Table 1). For the majority of these pathways (except nitrotoluene degradation), the genes were more abundant in soil 1 compared to soil 2 in most of the DNA extracts examined (in bold in Table 1). The trends were less pronounced under anaerobic conditions, which involved a statistically significant difference for three pathways (chlorocyclohexane and chlorobenzene degradation, drug metabolism-other enzymes, and nitrotoluene degradation) (Table 2). Two of the three pathways (except nitrotoluene) were more important in soil 1. The drug metabolism pathways were selected for further analysis using PICRUST because of the consistent difference between the two soils for these pathways. Specifically, the analysis involved the identification of the phylotypes in soils 1 and 2 (aerobic conditions) linked to the KEGG entries in the drug

metabolism pathways (other enzymes and cytochrome P450) (Supplementary Tables 7 and 8). The phylotypes contributing to the high gene counts for these KEGG entries were summarized for soil 1 (Table 3). The most important microorganisms in soil 1 contributing to the drug metabolism KEGG entries include unclassified *Chitinophagaceae*, unclassified *Saprospiraceae*, Ellin6075 (phylum *Acidobacteria*), *Kaistobacter* (family *Sphingomonadaceae*) and *Arenimonas oryzae* (family *Xanthomonadaceae*). It is interesting to note that both *Sphingomonadaceae* and *Xanthomonadaceae* were again identified as being implicated in drug metabolism.

Overall, the PICRUSt data support the experimental data described above, that is 1) biodegradation potential was more important in soil 1 compared to soil 2 and 2) biodegradation was more likely to occur under aerobic conditions. It would be interesting to apply this analysis method to other samples, to determine if this approach can predict CBZ biodegradation in other agricultural soils or in WWTP samples.

3.5. Phylotypes Negatively Impacted by Carbamazepine Exposure

A secondary objective of this study was to determine if exposure to CBZ negatively affected specific phylotypes in the soil microbial community. For this, the relative abundance of phylotypes in the CBZ-amended samples was compared to the live CBZ-free controls to determine if any significantly ($p < 0.05$) decreased in abundance because of the addition of CBZ. This analysis was performed in soils 1 and 2, under both aerobic and anaerobic conditions.

In soil 1, under aerobic conditions, at day 4, four and two phylotypes decreased in abundance at 50 ng/g and 5000 ng/g, respectively, and these were different for each treatment (Figure 2A and

2B). The difference in relative abundance, compared to the controls, was greatest at 5000 ng/g (~0.3% for *Geobacter* and *Flavobacterium*). At the 500 ng/g treatment level, no effect was noted for any phylotype. These data indicate a limited effect of CBZ on the soil microbial community for this short time period. In contrast, in soil 1, by day 14 (also under aerobic conditions) a much greater effect was observed following exposure to CBZ. The greatest decrease, compared to the controls, was noted for unclassified *Bacteria* (1.3%), unclassified *Bacteroidetes* (1.5%) and 3 *genus incertae sedis* (2.2%). Many common phylotypes decreased in abundance compared to the controls at all three concentrations (Figure 2C). These included unclassified *Bacteroidetes*, unclassified *Gammaproteobacteria*, 3 *genus incertae sedis*, *Ohtaekwangia*, *Nitrospira*, *Steroidobacter*, *Gp1*, unclassified *Opitutaceae*, *Cellvibrio*, *Anaeromyxobacter*, unclassified *Ruminococcaceae*, *Aureispira* and *Emticicia*. In addition, a further ten phylotypes decrease in abundance in two of the three concentrations. These data imply that a longer time period is needed for CBZ to significantly affect the microbial community.

In soil 1, under anaerobic conditions at day 4 and 14, a number of phylotypes were also negatively affected by exposure to CBZ (Figure 3). At day 4, under 12 phylotypes decreased in abundance at each treatment level (Figure 3A, B, C). From these, three microorganisms (unclassified *Polyangiaceae*, *Rhizobium* and *Armatimonadetes gp4*) decreased in abundance in at least two of the treatments. The difference in relative abundance, compared to the controls, was greatest for *Methylophilus* (~3%) (Figure 3A). By day 14, a smaller number of phylotypes were affected (6 or less) and two different microorganisms (*Iamia* and *Luteibacter*) decreased in abundance in at least two of the treatment concentrations (Figure 3D, E, F).

In soil 2, under aerobic conditions, the effect of CBZ on the soil microbial community was more pronounced at day 4. In this soil, an effect was noted at all three concentrations at day 4 and up to eight phylotypes were impacted in any one treatment (Figure 4A, B C). Three phylotypes were commonly impacted in at least two of the concentrations tested, including *unclassified Chitinophagaceae*, *Aureispira* (also affected in soil 1) and *Paenibacillus*. Similar to soil 1 under aerobic conditions, *Flavobacterium* illustrated a decrease in relative abundance compared to the controls (Figure 4A). By day 14, only two phylotypes were affected by the presence of CBZ. However, one phylotype, unclassified *Bacteroidetes*, illustrated a large decrease in abundance at all three concentrations (~1%) (Figure 4D). This phylotype was also impacted in soil 1 (under aerobic conditions). Similar to the data from day 4, unclassified *Chitinophagaceae* also exhibited a decrease in abundance at day 14 (for 50 ng/g only) (Figure 4D).

The difference between the effect of CBZ under aerobic conditions between soil 1 and soil 2 may be explained by a decreased bioavailability in soil 2 due to increased sorption to soil organic matter (higher % organic matter). In soil 1, many phylotypes declined in abundance by day 14, however, in soil 2, only two decreased in abundance by this time.

In soil 2, under anaerobic conditions, different microorganisms were impacted by CBZ. Between three and ten microorganisms decreased in abundance compared to the controls at the three concentrations investigated (Figure 5). At day 4, only two, *Pasteuria* and unclassified *Bacilli*, were impacted in more than one treatment (Figure 5A, B, C). Similar to soil 1 (aerobic conditions) and soil 2 (aerobic conditions), *Flavobacterium* again exhibited a decrease in abundance compared to the controls (Figure 5A). By day 14, fewer phylotypes (three or less for

each treatment) were influenced by the presence of CBZ. Only one, *Lewinella*, decreased in abundance compared to the controls in more than one of the treatments (Figure 5D, E). Interestingly, similar to soil 1, the phylotype *3 genus incertae sedis* illustrated a large decrease (~0.8%) compared to the control at day 14 (Figure 5F).

The implications of these findings on ecosystem function are uncertain, but it is clear that the presence of CBZ does impact the microbial community. In many cases, the differences in relative abundances between the CBZ-free controls and the CBZ-amended samples were small. However, for other phylotypes, including *Methylophilus*, *Lacibacter*, *Geobacter*, *Flavobacterium*, *3 genus incertae sedis*, *Gp4*, unclassified *Chitinophagaceae* and unclassified *Bacteroidetes*, the percent decrease was considerably higher. Further, for several phylotypes the relative abundance was reduced in both soils (*Flavobacterium*, *3 genus incertae sedis* and unclassified *Bacteroidetes*), indicating a notable sensitivity to CBZ for these microorganisms.

As two of the phylotypes listed above could not be classified to the genus level (unclassified *Chitinophagaceae* and unclassified *Bacteroidetes*) it is difficult to predict the effect of their reduced population on the function of the soil community. The family *Chitinophagaceae* (phylum *Bacteroidetes*) contains several genera, including *Lacibacter* (also impacted), *Balneola*, *Filimonas*, *Flavisolibacter*, *Gracilimonas*, *Niastella*, *Terrimonas* and the type genus *Chitinophaga* (Rosenberg, 2014). Some species can degrade chitin and the hydrolysis of cellulose is also associated with some species (Rosenberg, 2014). Therefore, it is possible that a reduction in the population of this family could impact soil carbon cycling. It is impossible to predict the effect of a reduction in unclassified *Bacteroidetes*, as this phylum contains many

genera. The decreased relative abundance of *Methylophilus* may also have implications for carbon cycling, as this genus has been associated with methanol utilization (Xia et al., 2015; Yomantas et al., 2010). *Flavobacterium* (phylum *Bacteroidetes*) are chemoorganotrophic and so their decreased abundance could also influence the carbon cycle. At least one isolate from this genus has been associated with pesticide degradation (Nayariseri et al., 2015). The impact on the *Geobacter* population may influence iron cycling in soils, as a key characteristic of *Geobacter* is iron reduction (Zacharoff et al., 2016).

The phylotype 3 *genus incertae sedis* belongs to the class *Subdivision 3*, within the phylum *Verrucomicrobia*. Bacteria belonging to this phylum are commonly present in surface soils, however they are typically difficult to cultivate (Bergmann et al., 2011; Janssen, 2006; Janssen et al., 1997; Joseph et al., 2003). The ecology of this phylum remains poorly understood (Bergmann et al., 2011) and hence it is difficult to predict the effect of a reduced population on soil function. CBZ may also impact other functions associated with microorganisms for which little information exists, e.g. *Gp4*.

4. Conclusions (this section has been reduced from 578 words to 424 words).

CBZ biodegradation was observed in soil 1, but not in soil 2, within the time period investigated. In soil 1, under aerobic conditions, the percent CBZ decrease between days 4 and 14 was similar at all three concentrations. In contrast, for soil 1, CBZ biodegradation was less consistent under anaerobic conditions, indicating CBZ will likely be more recalcitrant under water saturated and oxygen depleted conditions. In soil 1, many phylotypes were more abundant in the CBZ-amended samples compared to CBZ-free controls. Higher levels of enrichment were noted for

unclassified *Sphingomonadaceae*, unclassified *Xanthomonadaceae* and *Sphingomonas*. Only three phylotypes were enriched at all three concentrations, including *Aquicella*, *Microvirga* and unclassified *Rhodobacteraceae*. The enrichment patterns indicate these specific phylotypes are benefiting from CBZ degradation. The results presented here, along with previous research on these microorganisms, suggest similarities in the phylotypes able to degrade PAHs and CBZ. The current research did not observe an enrichment of any of the three isolates previously linked to CBZ degradation. These trends suggest that pure culture research may inaccurately portray the microorganisms responsible for CBZ degradation in soil communities. Using PICRUSt, the genes associated with drug metabolism were more important in soil 1 compared to soil 2 and these were also linked to unclassified *Sphingomonadaceae*, unclassified *Xanthomonadaceae*. These results lend support to the hypothesis that the difference in CBZ biodegradation capacity between the two soils is, in part, a result of differences in the microbial communities. This work offers a potentially useful platform to study CBZ biodegradation potential in other soils.

A secondary objective was to determine if exposure to CBZ negatively affected specific phylotypes in the soil microbial community. For several phylotypes, including *Methylophilus*, *Lacibacter*, *Geobacter*, *Flavobacterium*, 3 *genus incertae sedis*, *Gp4*, unclassified *Chitinophagaceae* and unclassified *Bacteroidetes*, a particularly notable decrease in relative abundance was observed in the CBZ-amended samples compared to the controls. For three of these phylotypes (*Flavobacterium*, 3 *genus incertae sedis* and unclassified *Bacteroidetes*), the relative abundance was reduced in both soils, indicating a notable sensitivity to CBZ for these microorganisms. The observed impact of CBZ on some phylotypes may have implications for carbon cycling, pesticide degradation or iron reduction in soils.

In summary, this is the first study to identify putative CBZ degraders in soil communities. The results are particularly important because environmental relevant CBZ concentrations were examined. In addition, this is the first report on the effect of CBZ on specific soil phylotypes. Further, the work suggests that insights into CBZ biodegradation potential can be obtained from examining the abundance of xenobiotic degrading genes using PICRUSt.

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References

- Acosta-Gonzalez, A., Martirani-von Abercron, S.M., Rossello-Mora, R., Wittich, R.M., Marques, S. 2015. The effect of oil spills on the bacterial diversity and catabolic function in coastal sediments: a case study on the Prestige oil spill. *Environ. Sci. Pollut. R.* 22, 15200-15214.
- Al-Mailem, D.M., Kansour, M.K., Radwan, S.S. 2014. Bioremediation of hydrocarbons contaminating sewage effluent using man-made biofilms: effects of some variables. *Appl. Biochem. Biotech.* 174, 1736-1751.
- Ardley, J.K., Parker, M.A., De Meyer, S.E., Trengove, R.D., O'Hara, G.W., Reeve, W.G., et al. 2012. *Microvirga lupini* sp nov., *Microvirga lotononidis* sp nov and *Microvirga zambiensis* sp nov are alphaproteobacterial root-nodule bacteria that specifically nodulate and fix nitrogen with geographically and taxonomically separate legume hosts. *Int. J. Syst. Evol. Micro.* 62, 2579-2588.
- Arye, G., Dror, I., Berkowitz, B. 2011. Fate and transport of carbamazepine in soil aquifer treatment (SAT) infiltration basin soils. *Chemosphere* 82, 244-252.
- Bergmann, G.T., Bates, S.T., Eilers, K.G., Lauber, C.L., Caporaso, J.G., Walters, W.A., et al. 2011. The under-recognized dominance of *Verrucomicrobia* in soil bacterial communities. *Soil Biol. Biochem.* 43, 1450-1455.
- Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621-1624.
- Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., et al. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *P. Natl. Acad. Sci. USA* 108, 4516-4522.
- Cha, J.M., Cupples, A.M. 2009. Detection of the antimicrobials triclocarban and triclosan in agricultural soils following land application of municipal biosolids. *Water Res.* 43, 2522-2530.
- Chen, F., Ying, G.G., Kong, L.X., Wang, L., Zhao, J.L., Zhou, L.J., et al. 2011. Distribution and accumulation of endocrine-disrupting chemicals and pharmaceuticals in wastewater irrigated soils in Hebei, China. *Environ. Pollut.* 159, 1490-1498.
- Chen, K., Chen, Q., Wang, G.X., Ni, H.Y., He, J., Yan, X., et al. 2015. *Sphingomonas chloroacetimidivorans* sp. nov., a chloroacetamide herbicide-degrading bacterium isolated from activated sludge. *Antonie van Leeuwenhoek* 108, 703-10.
- Clara, M., Strenn, B., Kreuzinger, N. 2004. Carbamazepine as a possible anthropogenic marker in the aquatic environment: investigations on the behaviour of Carbamazepine in wastewater treatment and during groundwater infiltration. *Water Res.* 38, 947-954.
- Conkle, J.L., Gan, J., Anderson, M.A. 2012. Degradation and sorption of commonly detected PPCPs in wetland sediments under aerobic and anaerobic conditions. *J. Soil Sediment* 12, 1164-1173.
- Coronado, E., Valtat, A., van der Meer, J.R. 2015. *Sphingomonas wittichii* RW1 gene reporters interrogating the dibenzofuran metabolic network highlight conditions for early successful development in contaminated microcosms. *Env. Micro. Rep.* 7, 480-8.
- Dalkmann, P., Siebe, C., Amelung, W., Schlöter, M., Siemens, J. 2014. Does long-term irrigation with untreated wastewater accelerate the dissipation of pharmaceuticals in soil? *Environ. Sci. Technol.* 48, 4963-4970.
- DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., et al. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microb.* 72, 5069-5072.
- Ding, G.C., Heuer, H., Smalla, K. 2012. Dynamics of bacterial communities in two unpolluted soils after spiking with phenanthrene: soil type specific and common responders. *Front. in Micro.* 3, 290.

- Duran-Alvarez, J.C., Prado, B., Gonzalez, D., Sanchez, Y., Jimenez-Cisneros, B. 2015. Environmental fate of naproxen, carbamazepine and triclosan in wastewater, surface water and wastewater irrigated soil - Results of laboratory scale experiments. *Sci. Total Environ.* 538, 350-362.
- Gauthier, H., Yargeau, V., Cooper, D.G. 2010. Biodegradation of pharmaceuticals by *Rhodococcus rhodochrous* and *Aspergillus niger* by co-metabolism. *Sci. Total Environ.* 408, 1701-1706.
- Gibson, R., Duran-Alvarez, J.C., Estrada, K.L., Chavez, A., Cisneros, B.J. 2010. Accumulation and leaching potential of some pharmaceuticals and potential endocrine disruptors in soils irrigated with wastewater in the Tula Valley, Mexico. *Chemosphere* 81, 1437-1445.
- Gottschall, N., Topp, E., Metcalfe, C., Edwards, M., Payne, M., Kleywegt, S., et al. 2012. Pharmaceutical and personal care products in groundwater, subsurface drainage, soil, and wheat grain, following a high single application of municipal biosolids to a field. *Chemosphere* 87, 194-203.
- Grossberger, A., Hadar, Y., Borch, T., Chefetz, B. 2014. Biodegradability of pharmaceutical compounds in agricultural soils irrigated with treated wastewater. *Environ. Pollut.* 185, 168-177.
- Gutierrez, T., Singleton, D.R., Aitken, M.D., Semple, K.T. 2011. Stable isotope probing of an algal bloom to identify uncultivated members of the *Rhodobacteraceae* associated with low-molecular-weight polycyclic aromatic hydrocarbon degradation. *Appl. Environ. Micro.* 77, 7856-60.
- Harwati, T.U., Kasai, Y., Kodama, Y., Susilaningih, D., Watanabe, K. 2009. *Tropicibacter naphthalenivorans* gen. nov., sp nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from Semarang Port in Indonesia. *Int. J. Syst. Evol. Micro.* 59, 392-396.
- Janssen, P.H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microb.* 72, 1719-1728.
- Janssen, P.H., Schuhmann, A., Morschel, E., Rainey, F.A. 1997. Novel anaerobic ultramicrobacteria belonging to the *Verrucomicrobiales* lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. *Appl. Environ. Microb.* 63, 1382-1388.
- Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.A., Janssen, P.H. 2003. Laboratory cultivation of widespread and previously uncultured soil bacteria. *Appl. Environ. Microb.* 69, 7210-7215.
- Kanso, S., Patel, B.K.C. 2003. *Microvirga subterranea* gen. nov., sp nov., a moderate thermophile from a deep subsurface Australian thermal aquifer. *Int. J. Syst. Evol. Micro.* 53, 401-406.
- Kinney, C.A., Furlong, E.T., Werner, S.L., Cahill, J.D. 2006a. Presence and distribution of wastewater-derived pharmaceuticals in soil irrigated with reclaimed water. *Environ. Toxicol. Chem.* 25, 317-326.
- Kinney, C.A., Furlong, E.T., Zaugg, S.D., Burkhardt, M.R., Werner, S.L., Cahill, J.D., et al. 2006b. Survey of organic wastewater contaminants in biosolids destined for land application. *Environ. Sci. Technol.* 40, 7207-7215.
- Kobayashi, A., Kobayashi, Y.O., Someya, N., Ikeda, S. 2015. Community analysis of root- and tuber-associated bacteria in field-grown potato plants harboring different resistance levels against common scab. *Microbes Environ.* 30, 301-309.
- Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., et al. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environ. Sci. Technol.* 36, 1202-1211.
- Lajeunesse, A., Smyth, S.A., Barclay, K., Sauve, S., Gagnon, C. 2012. Distribution of antidepressant residues in wastewater and biosolids following different treatment processes by municipal wastewater treatment plants in Canada. *Water Res.* 46, 5600-5612.
- Langille, M.G.I., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., et al. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814-+.
- Lapworth, D.J., Baran, N., Stuart, M.E., Ward, R.S. 2012. Emerging organic contaminants in groundwater: A review of sources, fate and occurrence. *Environ. Pollut.* 163, 287-303.

- Li, J.Y., Dodgen, L., Ye, Q.F., Gan, J. 2013. Degradation kinetics and metabolites of carbamazepine in soil. *Environ. Sci. Technol.* 47, 3678-3684.
- Maeng, S.K., Sharma, S.K., Abel, C.D.T., Magic-Knezev, A., Amy, G.L. 2011. Role of biodegradation in the removal of pharmaceutically active compounds with different bulk organic matter characteristics through managed aquifer recharge: Batch and column studies. *Water Res.* 45, 4722-4736.
- Matsumura, Y., Akahira-Moriya, A., Sasaki-Mori, M. 2015. Bioremediation of bisphenol-A polluted soil by *Sphingomonas bisphenolicum* AO1 and the microbial community existing in the soil. *Biocontrol Sci.* 20, 35-42.
- McClellan, K., Halden, R.U. 2010. Pharmaceuticals and personal care products in archived US biosolids from the 2001 EPA national sewage sludge survey. *Water Res.* 44, 658-668.
- Metcalfe, C.D., Koenig, B.G., Bennie, D.T., Servos, M., Ternes, T.A., Hirsch, R. 2003. Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. *Environ. Toxicol. Chem.* 22, 2872-2880.
- Miao, X.S., Metcalfe, C.D. 2003. Determination of carbamazepine and its metabolites in aqueous samples using liquid chromatography-electrospray tandem mass spectrometry. *Anal. Chem.* 75, 3731-3738.
- Miao, X.S., Yang, J.J., Metcalfe, C.D. 2005. Carbamazepine and its metabolites in wastewater and in biosolids in a municipal wastewater treatment plant. *Environ. Sci. Technol.* 39, 7469-7475.
- Miege, C., Choubert, J.M., Ribeiro, L., Eusebe, M., Coquery, M. 2008. Removal efficiency of pharmaceuticals and personal care products with varying wastewater treatment processes and operating conditions - conception of a database and first results. *Water Sci. Technol.* 57, 49-56.
- Nadh, A.G., Akare, U.R., Sharma, P., Yadav, P., Bandaru, S., Nayariseri, A., et al. 2015. Identification of azo dye degrading *Sphingomonas* strain EMBS022 and EMBS023 using 16S rRNA gene sequencing. *Curr. Bioinform.* 10, 599-605.
- Nakada, N., Kiri, K., Shinohara, H., Harada, A., Kuroda, K., Takizawa, S., et al. 2008. Evaluation of pharmaceuticals and personal care products as water-soluble molecular markers of sewage. *Environ. Sci. Technol.* 42, 6347-6353.
- Nayariseri, A., Suppahia, A., Nadh, A.G., Nair, A.S. 2015. Identification and characterization of a pesticide degrading *Flavobacterium* species EMBS0145 by 16S rRNA gene sequencing. *Interdiscip. Sci.* 7, 93-99.
- Ollers, S., Singer, H.P., Fassler, P., Muller, S.R. 2001. Simultaneous quantification of neutral and acidic pharmaceuticals and pesticides at the low-ng/l level in surface and waste water. *J. Chromatogr. A* 911, 225-234.
- Pan, T., Deng, T., Zeng, X.Y., Dong, W., Yu, S.J. 2016. Extractive biodegradation and bioavailability assessment of phenanthrene in the cloud point system by *Sphingomonas polyaromaticivorans*. *Appl. Microbiol. Biot.* 100, 431-437.
- Perruchon, C., Patsioura, V., Vasileiadis, S., Karpouzias, D.G. 2016. Isolation and characterisation of a *Sphingomonas* strain able to degrade the fungicide ortho-phenylphenol. *Pest. Manag. Sci.* 72, 113-124.
- Pinyakong, O., Tiangda, K., Iwata, K., Omori, T. 2012. Isolation of novel phenanthrene-degrading bacteria from seawater and the influence of its physical factors on the degradation of phenanthrene. *Scienceasia.* 38, 36-43.
- Popa, C., Favier, L., Dinica, R., Semrany, S., Djelal, H., Amrane, A., et al. 2014. Potential of newly isolated wild *Streptomyces* strains as agents for the biodegradation of a recalcitrant pharmaceutical, carbamazepine. *Environ. Technol.* 35, 3082-3091.

- Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., et al. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nuc. Acids Res.* 35, 7188-7196.
- Radl, V., Simoes-Araujo, J.L., Leite, J., Passos, S.R., Martins, L.M.V., Xavier, G.R., et al. 2014. *Microvirga vignae* sp nov., a root nodule symbiotic bacterium isolated from cowpea grown in semi-arid Brazil. *Int. J. Syst. Evol. Micr.* 64, 725-730.
- Reeve, W., Ardley, J., Tian, R., De Meyer, S., Terpolilli, J., Melino, V., et al. 2014. Genome sequence of the *Listia angolensis* microsymbiont *Microvirga lotononidis* strain WSM3557(T). *Stand. Genomic Sci.* 9, 540-550.
- Rosenberg, E. The Family *Chitinophagaceae*. In: E. R, F. DE, S. L, E. S, F. T, editors. *The Prokaryotes - Other Major Lineages of Bacteria and Archaea*. Springer-Verlag, 2014, pp. pp.493-495.
- Rosi-Marshall, E.J., Royer, T.V. 2012. Pharmaceutical compounds and ecosystem function: an emerging research challenge for aquatic ecologists. *Ecosystems*. 15, 867-880.
- Salvia, M.V., Vulliet, E., Wiest, L., Baudot, R., Cren-Olive, C. 2012. Development of a multi-residue method using acetonitrile-based extraction followed by liquid chromatography-tandem mass spectrometry for the analysis of steroids and veterinary and human drugs at trace levels in soil. *J. Chromatogr. A* 1245, 122-133.
- Santos, P., Pinhal, I., Rainey, F.A., Empadinhas, N., Costa, J., Fields, B., et al. 2003. Gamma-proteobacteria *Aquicella lusitana* gen. nov., sp nov., and *Aquicella siphonis* sp nov infect protozoa and require activated charcoal for growth in laboratory media. *Appl. Environ. Microb.* 69, 6533-6540.
- Schloss, P.D. 2009. A High-Throughput DNA Sequence Aligner for Microbial Ecology Studies. *PLOS One* 4.
- Schloss, P.D. MiSeq SOP. In: Schloss PD, editor. *Mothur wiki*. 2013, 2013, pp. This is a page describing the standard operating procedure for analysing MiSeq data using Mothur.
- Schmitt, H., Van Beelen, P., Tolls, J., Van Leeuwen, C.L. 2004. Pollution-induced community tolerance of soil microbial communities caused by the antibiotic sulfachloropyridazine. *Environ. Sci. Technol.* 38, 1148-1153.
- Syakti, A.D., Yani, M., Hidayati, N.V., Siregar, A.S., Doumenq, P., Sudiana, I.M.M. 2013. The bioremediation potential of hydrocarbonoclastic bacteria isolated From a mangrove contaminated by petroleum hydrocarbons on the Cilacap Coast, Indonesia. *Bioremediat. J.* 17, 11-20.
- Ternes, T.A. 1998. Occurrence of drugs in German sewage treatment plants and rivers. *Water Res.* 32, 3245-3260.
- Thiele-Bruhn, S. 2003. Pharmaceutical antibiotic compounds in soils - a review. *J. Plant Nutr. Soil Sc.* 166, 145-167.
- Thiele-Bruhn, S., Beck, I.C. 2005. Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass. *Chemosphere*. 59, 457-465.
- Tixier, C., Singer, H.P., Oellers, S., Muller, S.R. 2003. Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. *Environ. Sci. Technol.* 37, 1061-1068.
- Tran, N.H., Li, J.H., Hu, J.Y., Ong, S.L. 2014. Occurrence and suitability of pharmaceuticals and personal care products as molecular markers for raw wastewater contamination in surface water and groundwater. *Environ. Sci. Pollut. R.* 21, 4727-4740.
- Vazquez-Roig, P., Segarra, R., Blasco, C., Andreu, V., Pico, Y. 2010. Determination of pharmaceuticals in soils and sediments by pressurized liquid extraction and liquid chromatography tandem mass spectrometry. *J. Chromatogr. A* 1217, 2471-2483.
- Vieno, N., Sillanpaa, M. 2014. Fate of diclofenac in municipal wastewater treatment plant - A review. *Environ. Int.* 69, 28-39.

- Walters, E., McClellan, K., Halden, R.U. 2010. Occurrence and loss over three years of 72 pharmaceuticals and personal care products from biosolids-soil mixtures in outdoor mesocosms. *Water Res.* 44, 6011-6020.
- Williams, C.F., McLain, J.E.T. 2012. Soil persistence and fate of carbamazepine, lincomycin, caffeine, and ibuprofen from wastewater reuse. *J. Environ. Qual.* 41, 1473-1480.
- Wu, C.X., Spongberg, A.L., Witter, J.D., Fang, M., Czajkowski, K.P., Ames, A. 2010. Dissipation and leaching potential of selected pharmaceutically active compounds in soils amended with biosolids. *Arch. Environ. Con. Tox.* 59, 343-351.
- Xia, F., Zou, B., Shen, C., Zhu, T., Gao, X.H., Quan, Z.X. 2015. Complete genome sequence of *Methylophilus* sp TWE2 isolated from methane oxidation enrichment culture of tap-water. *J. Biotechnol.* 211, 121-122.
- Yergeau, E., Sanschagrin, S., Waiser, M.J., Lawrence, J.R., Greer, C.W. 2012. Sub-inhibitory concentrations of different pharmaceutical products affect the meta-transcriptome of river biofilm communities cultivated in rotating annular reactors. *Env. Microbiol. Rep.* 4, 350-359.
- Yomantas, Y.A.V., Tokmakova, I.L., Gorshkova, N.V., Abalakina, E.G., Kazakova, S.M., Gak, E.R., et al. 2010. Aromatic amino acid auxotrophs constructed by recombinant marker exchange in *Methylophilus methylotrophus* AS1 cells expressing the *aroP*-encoded transporter of *Escherichia coli*. *Appl. Environ. Microb.* 76, 75-83.
- Zacharoff, L., Chan, C.H., Bond, D.R. 2016. Reduction of low potential electron acceptors requires the CbcL inner membrane cytochrome of *Geobacter sulfurreducens*. *Bioelectrochemistry.* 107, 7-13.

Table 1. T-test (two tailed, unequal variance) comparison of xenobiotic degradation pathways (generated from PiCRUST) between soil 1 and 2 under aerobic conditions. Those significantly different in 4 or more from the 6 comparisons are shown in bold. Note: NS - not significant.

	50 - Day 4		50 - Day 14		500 - Day 4		500 - Day 14		5000 - Day 4		5000 - Day 14	
	p value	trend	p value	trend	p value	trend	p value	trend	p value	trend	p value	trend
Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT)	0.006	1 > 2	0.010	1 > 2	0.001	1 > 2	0.008	1 > 2	0.012	1 > 2	0.043	1 > 2
Aminobenzoate	> 0.05		0.015	1 > 2	NS		NS		NS		0.026	1 > 2
Atrazine	NS		0.002	2 > 1	0.040	2 > 1	0.039	2 > 1	NS		0.002	2 > 1
Benzoate	NS		0.005	1 > 2	NS		NS		NS		0.015	1 > 2
Bisphenol	NS		0.038	1 > 2	NS		NS		NS		NS	
Caprolactam	NS		0.029	1 > 2	NS		NS		NS		NS	
Chloroalkane & chloroalkene	NS		0.001	1 > 2	0.013	1 > 2	0.044	1 > 2	NS		0.002	1 > 2
Chlorocyclohexane & chlorobenzene	NS		0.000	1 > 2	0.005	1 > 2	0.004	1 > 2	0.015	1 > 2	0.000	1 > 2
Dioxin	NS		0.003	1 > 2	NS		NS		NS		0.034	1 > 2
Drug metabolism - cytochrome P450	NS		0.001	1 > 2	0.001	1 > 2	0.048	1 > 2	NS		0.012	1 > 2
Drug metabolism - other enzymes	0.003	1 > 2	0.001	1 > 2	0.000	1 > 2	0.000	1 > 2	0.001	1 > 2	0.002	1 > 2
Ethylbenzene	NS		0.003	1 > 2	0.034	1 > 2	NS		NS		0.004	1 > 2
Fluorobenzoate	NS		0.000	1 > 2	0.034	1 > 2	0.007	1 > 2	0.035	1 > 2	0.000	1 > 2
Metabolism of xenobiotics by cytochrome P450	NS		0.002	1 > 2	0.002	1 > 2	NS		NS		0.020	1 > 2
Naphthalene	NS		0.012	1 > 2	NS		NS		NS		0.020	1 > 2
Nitrotoluene	0.021	2 > 1	0.000	2 > 1	0.001	2 > 1	0.002	2 > 1	0.018	2 > 1	0.008	2 > 1
Polycyclic aromatic hydrocarbon	NS		NS		NS		NS		NS		NS	
Styrene	NS		0.002	1 > 2	0.017	1 > 2	NS		NS		0.001	1 > 2
Toluene	NS		0.002	1 > 2	NS		0.006	1 > 2	NS		0.000	1 > 2
Xylene	NS		0.004	1 > 2	NS		NS		NS		0.003	1 > 2

Table 2. T-test (two tailed, unequal variance) comparison of xenobiotic degradation pathways (generated from PiCRUST) between soil 1 and 2 under anaerobic conditions. Those significantly different in 4 or more from the 6 comparisons are shown in bold. Note: NS - not significant.

	50 - Day 4		50 - Day 14		500 - Day 4		500 - Day 14		5000 - Day 4		5000 - Day 14	
	p value	trend	p value	trend	p value	trend	p value	trend	p value	trend	p value	trend
Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT)	0.026	1 > 2	NS		0.017	1 > 2	NS		NS		NS	
Aminobenzoate	NS		NS		NS		NS		NS		NS	
Atrazine	NS		NS		0.023	2 > 1	NS		NS		NS	
Benzoate	0.021	1 > 2	NS		NS		NS		NS		NS	
Bisphenol	NS		NS		NS		NS		NS		NS	
Caprolactam	NS		0.043	2 > 1	NS		NS		NS		NS	
Chloroalkane & chloroalkene	0.001	1 > 2	0.039	1 > 2	0.008	1 > 2	0.038	1 > 2	NS		NS	
Chlorocyclohexane & chlorobenzene	0.001	1 > 2	NS		0.006	1 > 2	0.014	1 > 2	NS		NS	
Dioxin	NS		0.035	1 > 2	NS		NS		NS		NS	
Drug metabolism - cytochrome P450	0.008	1 > 2	NS		0.002	1 > 2	0.001	1 > 2	NS		NS	
Drug metabolism - other enzymes	0.001	1 > 2	0.041	1 > 2	0.008	1 > 2	0.024	1 > 2	NS		NS	
Ethylbenzene	0.001	1 > 2	0.008	1 > 2	0.009	1 > 2	NS		NS		NS	
Fluorobenzoate	NS		NS		NS		0.048	1 > 2	NS		NS	
Metabolism of xenobiotics by cytochrome P450	0.016	1 > 2	NS		0.007	1 > 2	0.002	1 > 2	NS		NS	
Naphthalene	0.008	1 > 2	0.040	1 > 2	0.028	1 > 2	NS		NS		NS	
Nitrotoluene	0.000	2 > 1	0.001	2 > 1	0.022	2 > 1	0.043	2 > 1	NS		0.009	2 > 1
Polycyclic aromatic hydrocarbon	NS		NS		NS		0.017	2 > 1	NS		NS	
Styrene	0.006	1 > 2	NS		0.021	1 > 2	NS		NS		NS	
Toluene	NS		0.042	1 > 2	NS		NS		NS		NS	
Xylene	0.007	1 > 2	NS		0.038	1 > 2	NS		NS		NS	

Table 3. The OTUs in soil 1 primarily responsible for the KEGG entries for the drug metabolism pathways. Values are gene counts (average and standard deviations for triplicate samples) contributed by each OTU. See Supplementary Tables 7 and 8 for the complete lists.

KEGG Entry (Drug metabolism - other enzymes)	Day 4		Day 14		Operational Taxonomic Unit
	Average	Stdev	Average	Stdev	
Uridine phosphorylase (K00757)	1232	170	1329	202	<i>Bacteroidetes, Saprospirae, Saprospirales, Chitinophagaceae, unclassified</i>
	1262	147	1101	205	<i>Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae, unclassified</i>
Uridine kinase (K00876)	2062	225	2327	472	<i>Acidobacteria, Chloracidobacteria, RB41, Ellin6075, unclassified</i>
	1262	147	1101	205	<i>Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae, unclassified</i>
Thymidine kinase (K00857)	2062	225	2327	472	<i>Acidobacteria, Chloracidobacteria, RB41, Ellin6075, unclassified</i>
	1232	170	1329	202	<i>Bacteroidetes, Saprospirae, Saprospirales, Chitinophagaceae, unclassified</i>
Hypoxanthine phosphoribosyltransferase (K00760)	1232	147	1101	205	<i>Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae, unclassified</i>
	2062	225	2327	472	<i>Acidobacteria, Chloracidobacteria, RB41, Ellin6075, unclassified</i>
Cytidine deaminase (K01489)	1213	244	1728	157	<i>Acidobacteria, Solibacteres, Solibacterales, Solibacteraceae, Candidatus Solibacter, unclassified</i>
	1232	170	1329	202	<i>Bacteroidetes, Saprospirae, Saprospirales, Chitinophagaceae, unclassified</i>
Beta-glucuronidase (K01195)	2062	225	2327	472	<i>Acidobacteria, Chloracidobacteria, RB41, Ellin6075, unclassified</i>
	1235	170	1329	202	<i>Bacteroidetes, Saprospirae, Saprospirales, Chitinophagaceae, unclassified</i>
GMP synthase (glutamine-hydrolysing) (K01951)	1259	147	1101	205	<i>Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae, unclassified</i>
	2062	225	2327	472	<i>Acidobacteria, Chloracidobacteria, RB41, Ellin6075, unclassified</i>
IMP dehydrogenase (K00088)	1232	170	1329	202	<i>Bacteroidetes, Saprospirae, Saprospirales, Chitinophagaceae, unclassified</i>
	2062	225	2327	472	<i>Acidobacteria, Chloracidobacteria, RB41, Ellin6075, unclassified</i>
Uridine monophosphate synthetase (K13421)	1232	170	1329	202	<i>Bacteroidetes, Saprospirae, Saprospirales, Chitinophagaceae, unclassified</i>
	1262	147	1101	205	<i>Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae, unclassified</i>
Thiopurine S-methyltransferase (K00569)	406	114	509	147	<i>Chloroflexi, Anaerolineae, SBR1031, A4b, unclassified</i>
	1262	147	826	205	<i>Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae, unclassified</i>
	689	110	640	112	<i>Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae, Arenimonas, oryzae</i>
KEGG Entry (drug metabolism - cytochrome P450)					
Monoamine oxidase (K00274)	2464	340	2658	403	<i>Bacteroidetes, Saprospirae, Saprospirales, Chitinophagaceae, unclassified</i>
	2062	225	2327	472	<i>Acidobacteria, Chloracidobacteria, RB41, Ellin6075, unclassified</i>
Glutathione S-transferase (K00799)	1262	147	1101	205	<i>Bacteroidetes, Saprospirae, Saprospirales, Saprospiraceae, unclassified</i>
	5457	891	6958	1727	<i>Proteobacteria, Alphaproteobacteria, Sphingomonadales, Sphingomonadaceae, Kaistobacter,</i>
	5457	901	6242	198	<i>Proteobacteria, Alphaproteobacteria, Rhizobiales, Hyphomicrobiaceae, Rhodoplanes, unclassified</i>

Figure 1. Common enriched phylotypes ($p < 0.05$) in at least two CBZ-amended treatments (50 ng/g, 500 ng/g and 5000 ng/g CBZ) compared to the controls in the aerobic treatments at day 14 (soil 1). Three asterisks indicate the phylotypes enriched ($p < 0.05$) at all three concentrations. The bars represent standard deviations from triplicate microcosms. No phylotypes were enriched at day 4.

Figure 2. Phylotypes that decreased in abundance in the CBZ-amended samples compared to the controls ($p < 0.05$) in soil 1 at 50 ng/g (A) and 5000 ng/g CBZ (B) in the aerobic treatments at day 4 (no differences were noted for 500 ng/g). The phylotypes that significantly decreased at the three concentrations at day 14 are also shown (C). Two and three asterisks indicate the phylotypes decreased in abundance at two and three concentrations, respectively. The bars represent standard deviations from replicates or triplicates.

Figure 3. Phylotypes that decreased in abundance in the CBZ-amended samples compared to the controls ($p < 0.05$) in soil 1 at 50 ng/g (A, D), 500 ng/g (B, E) and 5000 ng/g CBZ (C, F) in the anaerobic treatments at day 4 (A, B, C) and 14 (D, E, F). Two asterisks indicate the phylotypes decreased in abundance in at least two concentrations. The bars represent standard deviations from replicates or triplicates.

Figure 4. Phylotypes that decreased in abundance in the CBZ-amended samples compared to the controls ($p < 0.05$) in soil 2 at 50 ng/g (A), 500 ng/g (B) and 5000 ng/g CBZ (C) in the aerobic treatments at day 4 (A, B, C) and 14 (D). Two asterisks indicate the phylotypes decreased in abundance in at least two concentrations. The bars represent standard deviations from replicates or triplicate microcosms.

Figure 5. Phylotypes that decreased in abundance in the CBZ-amended samples compared to the controls ($p < 0.05$) in soil 2 at 50 ng/g (A, D), 500 ng/g (B, E) and 5000 ng/g CBZ (C, F) in the anaerobic treatments at days 4 (A, B, C) and 14 (D, E, F). Two asterisks indicate the phylotypes decreased in abundance in at least two concentrations. The bars represent standard deviations from replicates or triplicates.

*Graphical Abstract

Controls (no Carbamazepine, CBZ)

Samples (w/ CBZ 50, 500, 5000 ng/g)

Nucleic acid
extraction

Illumina
sequencing

Mothur analysis

PICRUSt analysis

QuECHERS
extraction

Solid phase
extraction

LC-MS/MS

Enriched Phylotypes Following Carbamazepine Biodegradation