Acetaminophen Concentrations Found in Recycled Wastewater alter Soil Microbial Community Structure and Functional Diversity

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Abstract

The practice of using recycled wastewater (RWW) (aka reclaimed water) has been adopted to address the growing demand for clean water and has been largely successful. However, chemicals of emerging concern (CECs) including pharmaceutical products such as acetaminophen (APAP) remain in the water even after additional cleaning. When recycled waste water is used to irrigate crops or landscapes these chemicals can then enter these and adjacent environments. APAP is one such contaminant and has the potential to disrupt the soil microbiome associated with agricultural crops. We evaluated this possibility using a greenhouse study to mimic agricultural field conditions, and applied treatments of APAP concentrations found within the ranges observed for RWW effluents. Using Illumina sequencing based approaches we observed that APAP was able to cause shifts in the microbial community and positively select for bacteria that are capable of metabolizing the breakdown products of APAP such as glycosides and carboxylic acids. Community-level physiological profiles of carbon metabolism were evaluated using Biolog EcoPlate as a proxy for community functions. The biolog plates indicated that the metabolism of amino acids, carbohydrates, carboxylic acids, phenolics, and polymers was significantly higher in the presence of APAP. Together our observations indicate that the soil microbial community and functions were altered by APAP and demonstrates the need to change current RWW policies to address the presence of these chemicals.

Introduction

Potable water supplies are becoming scarcer as population growth continues. Climate factors such as increased temperatures and altered precipitation patterns in many regions has limited the ability to regenerate these supplies, especially in arid regions[1–3]. Water conservation alone can only stretch existing water supplies so far and may not be enough to address the growing demands for clean water [2]. Alternative ways to generate usable or potable water, such as recycling waste water, are becoming essential to help meet the rising demand. The overall impacts of climate change make the practice of recycling wastewater more essential [2,4]. Globally, not all regions are impacted directly by drought conditions. The regions that are though, can import potable water when supplies run low. The practice of importing water means that even non-arid regions can be impacted by droughts in other distant regions. The practice of importing water can be more costly and have higher environmental impacts than recycling waste water depending on the location and quality of the water source and it is not an ideal long-term solution [5,6]. The environment can also suffer from the practice of withdrawing water from natural reservoirs, reducing habitat area and consumable water availability for the biota [7]. Therefore, alternative methods for generating potable water are becoming a necessity to meet rising water demands [3,8].

Fortunately, the practice of using recycled wastewater to supplement potable water supplies has been very successful [6,8,9]. Many arid regions, such as southern California, have used recycled waste water (RWW) (aka reclaimed water) to supplement the use of potable water. Currently, reclaimed water is

primarily used for agriculture and landscape irrigation [8–11]. The use of RWW has allowed farmers in arid regions of southern California to maintain high agricultural outputs without depleting the potable water supply or having to increase water withdrawal from natural aquifers [6,11]. Using RWW has been so successful that many water districts are planning on increasing their capacity for capturing and treating larger volumes of their waste water [12].

Despite the large conservation success of RWW use, this source of water may pose risks to natural and agricultural environments. Even though the treatment processes appears to be efficient at removing potentially disease causing biological contaminants, the technology is less effective at removing chemical contaminants that are generated from personal care or pharmaceutical products [13–16]. These contaminants include pharmaceuticals, chemicals from personal care products, detergents, and nanoparticles and have been collectively referred to as chemicals of emerging concern (CECs) [14,16,17]. Processing of wastewater is able to reduce the levels of these compounds, and it was originally believed that the concentrations of these chemicals found in RWW effluent (typically in the ug to mg / L range) were so low that they are biologically irrelevant [18–20]. However, more recent evidence suggests that the concentrations of CECs in RWW are able to impact microorganisms, insects, and plants [21–26]. These chemicals can end up in natural environments like streams and rivers [13]. Soils irrigated with RWW accumulate these contaminants [14], while plants have been observed to uptake some CECs and concentrate them in their tissues [18,19,27]. Therefore, CECs pose a risk in the agricultural setting where RWW is primarily used, and RWW should be evaluated extensively to manage or reduce any potential hazards.

The actual composition of CECs in RWW varies substantially. Nearly every RWW measured varies in the composition of the chemicals found and maybe related to the sources and human activities that generate the wastewater that is treated for reuse [28]. However, there are some chemicals, such as acetaminophen (APAP), that are consistently found in RWW effluent in many regions. APAP is typically found to be among the higher concentrated compounds compared to other CECs detected in a given source of RWW [13,16,28–30]. APAP is one of the most popular over-the- counter medications (it does have prescription strength variations as well) that is used as a pain reliever and fever reducer [31,32]. APAP is also a highly used veterinarian pharmaceutical compound that can be excreted by livestock [33]. APAP and other CECs are not fully broken down in animal metabolisms, thus the intact, active APAP compound is delivered into waste water and an appreciable amount remains behind even after the wastewater is treated for reuse [16].

APAP can act as an anthropogenic factor in agricultural soils and disrupt microbial functions that are essential to plant health such as nitrogen cycling [22]. Since CECs such as APAP can accumulate in soils irrigated with RWW, the soil microbiome that is typically associated with a given agricultural plant may become altered [22]. The addition of inorganic or organic matter into a given soil environment has the potential to select for a specific group of organisms, possibly ones that can benefit directly from the newly imputed factor. The rationale for our study is that, since APAP is broken down into a glucoside by

filamentous soil fungi or by plants [27,34,35] or into the carboxylic acid 2-hexenoic acid by soil microorganisms [30], the glucoside and the carboxylic acid can be utilized as a carbon source by some bacterial groups [36–38]. Bacterial groups that can utilize these carbon sources will most likely be selected for in soils contaminated by APAP, thus altering the soil microbiome. Soil microbiomes that are altered by anthropogenic factors can exhibit the loss or reduction of key functions, such as nutrient cycling [39–41]. Plant health is intimately related to its associated soil microbiome and its functions, thus any alterations to it could potentially have negative impacts [42]. Since it is likely that high APAP use will continue, and it has been found to impact soil microorganisms and their functions, we decided to evaluate its impacts directly on the soil microbial community of an important agricultural crop, *Solanum melongena* (eggplants) using a greenhouse study. We predict that levels of APAP found in RWW effluent will significantly alter the soil bacterial community structure associated with *S. melongena* and bacterial community functions.

Materials And Methods

Eggplant Cultivation and Soil collection

The Patio Baby variety of *Solanum melongena* (eggplants) were cultivated as described in McLain and Gachomo, 2019. This study complies with relevant institutional, national, and international guidelines and legislation for the collection of plant material. Soil samples were collected at different times in the growing season of the crop. APAP treatments were not applied until one week after transplanting and the plants reached the 2 to 4 leaf stage. All plants were watered with one L of tap water 2 to 3 times a week. The water was added to the trays that all plants (n=4) from the same treatment were sitting in to ensure even distribution of water and APAP for treatments. Two different concentrations of APAP (10ug /L or 5 ug /L) were used and labelled for APAP-H and APAP-L respectively. APAP stock solution was diluted with tap water to obtain the desired final concentration while the no CEC treatment had no APAP dissolved in tap water used to irrigate the plants. For Illumina sequencing, 1 cm diameter by 2 cm deep soil samples were collected at three different time points. The initial soil collection occurred just before transplanting into the soil and before any treatments were applied (T0). The second (T1) and third (T2) soil collections occurred 3 and 7 weeks after treatments were initially applied.

DNA extractions and Illumina sequencing library prep

Total environmental DNA was extracted from 0.25 g of soil using the DNeasy Powersoil kit (Qiagen, Valencia, CA, USA), following manufacturer instructions, except only 50 uL of solution C6 was used in the final DNA elution step. DNA quality and purity was checked using an Implen NanoPhotometer (Implen, Westlake Village,CA, USA). Amplicon libraries of the bacterial 16s rRNA gene were generated from the extracted DNA to characterize the bacterial community. The 2 step PCR dual indexing inline barcoding procedure described by [43] was used to generate amplicons for Illumina sequencing [23]. The initial PCR

reaction was used to amplify the V5-V6 region of the bacterial 16s rRNA gene using the 799F forward and 1115R reverse primer pair, which have reduced specificity toward chloroplast DNA [23,43,44]. Both primers were modified on the 5' end to contain the Illumina adapter sequence followed by a unique 8 nucleotide barcode used to identify a given sample (Supplemental Table 1) [23]. Phusion High-Fidelity PCR master mix with HF buffer (Thermo Scientific) was used for the PCR reagents and 1 uL of extracted DNA was used for the template. PCR reactions were carried out on the BIO Rad T100 thermal cycler using the following protocol: initial denaturing at 98 °C for 30 seconds followed by 24 cycles of denature at 98 °C for 10 seconds, annealing at 56.5 °C for 30 seconds, elongation at 72 °C for 30 seconds; completed by one final elongation step for 5 minutes at 72 °C. All PCR reactions were screened using a 1% agarose gel for electrophoresis for quality and expected fragment size. Amplicons from successful PCR reactions were cleaned up following the Agencourt AMPure xp beads protocol (Beckman Coulter, Brea, CA, USA), with the exception that SPRI beads (Beckman Coulter, Brea, CA, USA) were used in place of AmPure beads and all ethanol washes were done using 80% ethanol. Cleaned DNA products were used as a template in a second PCR. The reaction reagents were the same except HPLC purified PCR2F and PCR2R primers were used instead of the 799F and 1115R primers to complete the 5' Illumina adaptor [23,43]. PCR's were carried out using the same thermal cycle protocol for PCR1 except only 7 cycles were used and the annealing temperature was increased to 65 °C. PCR reactions were screened as described for the initial PCR. DNA concentrations were measured using the nanodrop spectrophotometer, and amplicons were pooled in equal molar concentrations of 5 nM for sequencing. The samples were submitted to the UCR genomics core facility where the library quality was assessed using a 2100 Bioanalyzer (Agilent) and the libraries were sequenced using a MiSeq sequencer (Illumina) and Miseq Reagent kit version 3 (Illumina) with 2 x 150 cycles.

Data analysis - Processing and quality filtering

The forward and reverse Illumina sequencing reads were joined together using Qiime1 based on their overlapping regions. The joined sequences went through a preliminary quality filtering process based on their associated quality scores using default settings in QIIME [45]. The unique barcode pairs contained in the joined sequences (forward and reverse primers) were used to demultiplex and sort into their respective samples using QIIME1 and Fastx toolkit. Demultiplexed samples were uploaded into QIIME2 with their associated quality scores [46]. The sequences were then quality filtered further using the deblur method in QIIME2 [46–48]. Samples that contained less than 9,000 sequences were excluded from downstream analyses. Following the recommendations of the QIIME2 developers the number of sequences for all samples were rarefied down to the number of sequences contained by the sample with the lowest number (10,300), for downstream analyses [46]. Deblur classified these sequences into amplicon sequence variants (ASVs) that were taxonomically identified by matching to the Greengenes database (v 13.8) using QIIME2 default parameters [49]. The ASVs were identified down to the lowest possible taxonomic unit, the species level. The lowest identifiable taxonomic unit will be listed unless specified otherwise. Community alpha diversity was measured using the Shannon Wiener index calculated by QIIME2 and statistically compared

using generalized linear model (GLiM) with normal distribution and identity link function in SPSS (V. 27.0). Box plots of alpha diversity metrics were generated in QIIME2. Community differences among different samples (beta diversity) were evaluated using PERMANOVA [50,51] on Bray-Curtis distance matrices [52,53]. The beta diversity results were plotted as a boxplot in QIIME2. Community data produced in QIIME2 was used in the program PAST [54] to generate PCA graphs showing the vectors of the amplicon sequence variants (ASVs) that contributed to the most differences among samples. For clarity of visualization, only the major vectors are labeled with the most specific taxonomic classification determined by QIIME2. The group significance test in QIIME 1 was used to statistically compare the abundance of ASVs using pairwise t-tests [45]. Community data generated in QIIME2 was imported into Picurst [55] for metagenome predictions. The data was normalized by copy number and predictions were based on the KEGG orthologs. The level of KEGG categories was compared using ANOVA, and a post hoc Tukey test for pairwise comparisons. The 16s rRNA gene sequences were submitted to the SRA under NCBI and have been assigned biosample accession numbers ########.

Evaluating changes in microbial functional diversity:

In order to determine changes in functional diversity of soil microbial communities after exposure to APAP, samples from APAP-H (10ug /L) treatment and no CECs treatment (control) were used to evaluate utilization of different carbon sources. This was done using the Biolog EcoPlate which contains 31 ecologically relevant carbon sources and water (control) in triplicates within a 96 well plate (Supplementary Table 2). Soil samples collected at 7 weeks after treatment with APAP (T2) were added to the wells and analysis of the pattern of utilization of the carbon substrates in the Biolog EcoPlate (Matrix Technologies Corporation, USA) used to determine the community-level physiological profiles (CLPP) as described below.

One gram of soil was diluted with dH2O to make 10 ml and shaken for 30 min at 210 rpm at room temperature, then allowed to settle for 30 min. A 10^{-2} dilution was prepared for each sample and 150µL was added to each well [56]. EcoPlates were incubated in a dark incubator at 25°C for 6 days. A Promega GloMax-Multi Detection System was used to measure the absorbance at 590nm of EcoPlates at 12, 24, 48, 72, 96, 120, and 144 hours post inoculation (hpi). The absorbance of each well was standardized by subtracting the absorbance for the control substrate (water). Average well color development (AWCD) was used as a measure utilization of the carbon source in each well by the microbial community. The formula used to calculate AWCD was as follows:

$$AWCD = \sum \frac{(Optical\ Density\ in\ Carbon\ Source\ Well - Optical\ Density\ of\ Control)}{31}$$

Carbon sources were grouped according to major functional groups [57] and the substrate utilization patterns for each group analyzed. Independent t-tests were conducted between the control and soil

irrigated with acetaminophen (10µg/L APAP) for each timepoint and functional group using IBM SPSS Statistics 26. Overall ACWD data is presented for each timepoint, and carbon utilization patterns for the 48 hpi is shown as a representative of the overall trend.

Data from the biolog plates and community data generated in QIIME2 were combined and imported into the PAST software. PAST was used to conduct canonical correspondence analysis (CCAs) to determine which compounds were having the most impact on community composition. The points that represented individual sample communities were plotted as a PCA graph, but vectors representing the effects of the chemicals were plotted over the PCA as determined by the CCA analyses. The longer the vectors the more of an impact the respective chemicals had on overall beta-diversity differences.

Results

The bacterial community data indicates that the level of diversity (measured as the Shannon-Wiener index) among different treatments at all time points remained relatively steady. This may be an indication that these soil communities exhibited a high degree of resiliency. However, community structure (evaluated as β-diversity discussed below) did change among APAP treatment and untreated soils. Together this may suggest these soil communities may have a high amount of community redundancy. Bacterial groups are either being replaced, or becoming less abundant, allowing low abundant organisms to increase. The possibility of resiliency and redundancy was reinforced by the observations that no significant changes in the predicted meta-genome occurred among APAP treated and untreated samples. On the other hand, metabolic rates were found to be higher in all treated samples according to the biolog assay. Specific metabolic processes were also found to be higher in APAP treated soil and appear to be related to some of the breakdown products of APAP. Therefore, our data suggest that even though the soil bacteria community exhibited signs of resiliency and redundancy, the treated communities appear to be shifting toward a community that can tolerate, or benefit from the presence of APAP and possibly other sources of contamination.

The Illumina sequencing data indicated that the presence of APAP did not have a strong effect on community □-diversity, as measured by the Shannon-Wiener index. APAP-H at T2 had the highest Shannon-Wiener index value of 10.18, while the no CEC treatment at T2 had the lowest measured diversity at 9.63 (Fig.1). Interestingly the initial diversity present in the soil community at T0 was 10.04, indicating that diversity did slightly increase by T2 with the APAP present, but decreased slightly in the untreated soil community. The changes in diversity were only minor, however, as there were no significant interactions among treatment and time point, nor were any significant differences found among treatments or time points (2-way GLiM: χ^2_2= 0.774, P = 0.679; χ^2_2= 1.874, P = 0.392; χ^2_2= 0.078, P = 0.780; respectively)

Despite only minor changes in □-diversity being observed, community structure was impacted by the addition of APAP as measured by Bray-Curtis distance matrices. The soil community treated with higher concentrations of APAP (APAP-H-T1) for 3 weeks was found to be significantly different from the initial

soil community at T0 (PERMANOVA pseudo-F = 1.743, pseudo-P = 0.047) (Fig. 2). The lower concentration of APAP (APAP-L) did not have a significant impact on the community structure after 3 weeks of application. However, by seven weeks of treatment (T2) using the low or high concentration of APAP, soil communities were significantly different than the initial soil community (PERMANOVA pseudo-F = 2.100, pseudo-P = 0.026; pseudo-F = 1.749, pseudo-P = 0.016; respectively). The untreated soil bacteria communities were not significantly different from the initial T0 community at 3 and 7 weeks (all pseudo-P > 0.05), indicating that treatment with APAP was the reason for the change in the soil community structure.

Looking at specific taxonomic groups, shifts in relative abundance did occur among different treatments. The increase of APAP in the soil led to shifts in the relative abundance of different bacterial groups by promoting or inhibiting their growth. In all treatments, Proteobacteria were the most abundant in the soil with a relative abundance between 40-60%. Application of APAP at either of the concentrations (high or low) more than doubled the relative abundance of Chloroflexi class of bacteria between weeks 3 and 7 of application. During treatment with APAP-H and APAP-L, the relative abundance of the Actinobacteria class was decreased to 6.3% to 8.6% of the total population in week 3 and 9.8% to 11.0% by week 7 respectively. At both time points this was lower than the untreated samples that had relative abundance of 9.4% at week 3 and 17.8% by week 7 (Fig. 3). Bacteroidetes phylum abundance was lower in APAP treated soil than untreated samples by week 7, wtih APAP treated samples containing only 11.9% to 13.2% compared to 15.2% for the untreated soil community. The Gemmatimonadetes class had higher relative abundance in the APAP-L and -H treated soil (6.5% and 8.3%, respectively) compared to the untreated soil

(4.4%) by week 7 (Fig.3). The relative abundance of Firmicutes did not change significantly with APAP treatment or with time, and remained between 2.3% and 3.6%. However, the relative abundance of Acidobacteria decreased in all treatments compared to the original soil and the most significant decrease was observed in the untreated soil (3.3% to 1.3%; T0 to no CEC T2 respectively) (Fig 4)

While identifying artificial sequencing variants (ASVs) down to the lowest taxonomic level, the species, a total of 748 uniquely identified ASVs were found. Out of all the identified ASVs, 247 were determined to have significantly different abundance between T0 and the T2 APAP-H treatment (QIIME 1 group significance t-test; all P's < 0.05). The PCA plot of the sequencing data revealed 9 taxonomic groups that had a strong impact on determining community differences among the samples (Fig. 5). A group of organisms within the Gemmatimonadetes phylum, the Chloroflexi phylum, the Caulobacteraceae family, the *Cellvibrio* genus, the *Pseudomonas* genus, *Enhydrobacteria aerosaccus,* and *Amycolatopsis thermoflava* were found to increase in abundance between the T0 sample and the T2 APAP-H sample, all significantly (QIIME 1 group significance t-test; all P's < 0.05) except for the bacterium in the Cellvibrio genus. The increases of these microbial groups match well with the increases of metabolic rates observed in the Biolog plates described below. However, a different group within the class Gemmatimonadetes and a group in the family Xanthomonadaceae decreased significantly in abundance between the T0 and T2 APAP-H treatment (QIIME 1 group significance t-test; all P's < 0.05).

Metagenome prediction

Interestingly, even though there were significant differences in community structure, no significant differences in the Picurst gene predictions were detected through analyses with Stamp (ANOVA all P's > 0.05) except for the subcategory metabolic diseases under the human disease category. However, a few metabolic pathways predicted to be present in the soil contributed a substantial portion of the overall predicted metagenome. Genes for the metabolic pathways were detected for amino acid metabolism (11.3 to 11.1%), carbohydrate metabolism (10.4 to 10.0%) energy metabolism (5.81 to 5.55%), metabolism of cofactors and vitamins (4.28 to 4.18%), and Xenobiotics biodegradation and metabolism (3.70 to 4.33%). While two non-metabolic gene expression pathways, Membrane transport (10.2 to 10.0%) and replication and repair genes (7.32 to 6.98%) also contributed a relatively large portion of the overall predicted metagenome in the samples.

Evaluating changes in microbial functional diversity

Overall, Average Well Color Development (AWCD) values for APAP treated soils were higher than control soils. At 12 hours post inoculation (hpi), there was little measurable microbial activity in the Biolog EcoPlate and no significant difference in AWCD was observed. However, after 24 hpi, the AWCD of APAP treated soils was significantly higher than that of control soils. We observed significant differences in the utilization of different carbon groups between APAP treated soil and the control at different time points (ANOVA post hoc tukey test, all P's < 0.05) The AWCD of treated soils increased by 69%, 251%, 98%, 70%, 52%, and 42% at 24, 48, 72, 96, 120, and 144 hpi, respectively (Fig. 6A). The AWCD reached a peak at 48 hpi and then started to decline (Fig. 6A). At 48 hpi APAP significantly increased the AWCD through utilization of amino acids, carbohydrates, carboxylic acids, phenolics and polymers by 828%, 108%, 388%, 690%, and 276% respectively (Fig 6B). At the 96 hpi the AWCD for amino acids, carbohydrates carboxylic acids, and polymers had increased by 110%, 611%, 906%, and 101%, respectively compared to the control. This suggests that either the microbial density was higher in APAP treated soils or that APAP treatment selected for bacteria species that were better adapted to utilizing more varied carbon sources. At 96 hpi there was no significant change in the utilization of amines and phenolics between APAP treated soils and the control. However, there were no significant differences in the utilization of amines during the time of the experiment. Metabolism was higher in the APAP-H treated samples than untreated samples at all time points tested in the biolog assay (ANOVA post hoc Tukey test, all P's < 0.05) except for at 12H's. The CCA analyses of the 96H biolog results further indicated that amino acid, carbohydrate, carboxylic acids, and polymer metabolism contributed to community structural differences. These had the largest vectors on the CCA plot, suggesting that differences in these metabolic pathways between treated and untreated soil communities had a large effect on influencing community structure (Fig.7 and see Supplementary Fig. S1)

Discussion

The combined observations of the 16s rRNA data, predicted metagenome, and Biolog EcoPlate assays suggest that the soil bacterial community in our samples was in fact sensitive to the levels of APAP used in this study. Significant community differences were observed by 3 weeks of APAP treatment and significant differences in carbon metabolism were observed between treated and untreated samples collected at 7 weeks. Taken together, our results show that APAP was able to alter the soil bacterial communities and impact their functions.

APAP addition did not change individual community α-diversity levels as measured by the Shannon-Wiener index. The Shannon-Wiener diversity index remained relatively constant among treatments for the duration of the study. Although microbial communities can be sensitive to perturbations, plants can act to stabilize their associated soil microbial communities [58,59]. Previous observations suggest that all microbes are not impacted equally by a given disturbance and some are more resistant to perturbations than others [39,40]. Our results suggest that APAP was not able to completely displace many taxonomic groups of bacteria that were present in the soil. Instead, APAP caused certain bacterial groups to decrease in relative abundance and other groups to increase, thus allowing the community to maintain a similar level of α-diversity. Measured Shannon-wiener diversity would be less sensitive to community shifts in which the number of bacterial groups present remained the same. However, β-diversity, measured as Bray-Curtis distance matrices, would be more sensitive since it is affected by changes of relative abundance within and among different groups.

The observed significant differences of β-diversity between APAP treated and the initial sampling point suggests that the overall community structure (β-diversity) was sensitive to the addition of APAP at concentrations found in RWW, especially after 7 weeks of exposure. Our observations are consistent with previous ones that indicate pharmaceutical products, including APAP, can impact microbial communities, and even hinder or disrupt key microbial functions [20–22,60]. The APAP concentrations used in our study may appear low, but they represent levels typically found in RWW effluent [15,29,61]. Our results suggest that the concentrations of APAP found in RWW can impact soil microbial communities, especially if they are repeatedly exposed to the CECs. These results are consistent with previous observations of microbes in agricultural soils that were observed to be sensitive to other pharmaceutical products present in treated wastewater [56,62]. However, in these studies the resolution for detecting specific microbial community members was limited because they relied on non-sequencing-based approaches to characterize changes in the soil microbial community. In contrast, our study was able to detect specific shifts in the microbial community and identify specific bacterial groups that were impacted by APAP treatment by using Illumina sequencing based approaches.

The changes in the microbial community were most likely driven by changes in soil conditions caused by the addition of APAP. Many soil microbial communities are sensitive to soil conditions (such as pH, organic matter content, and mineral composition), and those caused by anthropogenic inputs such as pharmaceutical products [22,62]. When the microbial community changes, the overall functions may

change as well. Introduction of antibiotics like sulfamethoxazole into soil can cause decreases in the metabolism of a number of carbon substrates [56] or disrupt nitrogen cycling in bacterial communities [39,63]. The Addition of APAP to soils has also been found to disrupt key aspects of nitrogen cycling although the concentrations of APAP (50 to 1,000 mg/L) used were greater than those found in RWW effluent [22,60]. Our results from the Biolog assay showed altered microbial functions while using lower concentrations of APAP (5 ug/ L and 10 ug/L) that are in the range of those found in RWW effluent. Besides lower APAP concentration, our study distinguishes itself from previous ones in a few other ways. Unlike previous studies that focused on nitrogen cycling [39,63], our study examined a wide range of carbon metabolism pathways by using 31 ecologically relevant carbon sources (Supplementary Table 2). This is a very robust approach that can encompass nearly every member of the microbial community. The biolog approach allowed us to widen our scope beyond a specific set of community members such as anammox bacteria [60], or bacteria that contain amoA, napaA, or nifH genes for nitrification, denitrification, or nitrogen fixation respectively [39]. Nitrogen cycling is critical for the soil microbiome and its associated environment, but some organisms that are not efficient nitrogen cyclers may be overlooked. Thus, by looking for variations in metabolic rates of various carbon sources we could screen for a wide variety of bacterial groups that were impacted by the addition of APAP to the soil. The study Liu et al., 2012[56]did examine microbial community functions using biolog plates. However, their study did not simultaneously employ a method that would allow for the identification of specific microbial organisms that are shifting in abundance or that may be responsible for the changes in carbon metabolism they observed.

In this study, APAP treated samples had significantly higher rates of carbon metabolism in nearly every category measured (carbohydrate, amino acid, carboxylic acid, and polymer metabolism) compared to the control. We think this occurred because APAP is a carbon source for some organisms, therefore, its addition to the soil selects for microbes that use different sources of carbon more efficiently. Li et al., 2013[56] demonstrated that APAP is broken down in non-sterilized soil but not in sterilized soil. Indicating that soil microbes are able to break down APAP and use it as a carbon source. Metabolomics analyses revealed that when APAP is added to soil, the microbes were able to break it down to 8 identifiable intermediates, namely: 3-hydroxyacetaminophen, hydroquinone, 1, 4-benzoquinone, N-acetyl-p-benzoquinone imine, p-acetanisidide, 4-methoxyphenol, 2-hexenoic acid, and 1, 4-dimethoxybenzene [56]. The intermediate 2-hexenoic acid is a carboxylic acid and it was found to be the most abundant metabolite in the soil after APAP treatment by Li and his colleagues (2013). This led us to conclude that treatment of soil with APAP increases carboxylic acid content in the soil, which in turn increases microbes that can use it as a carbon source. We were able to confirm this using our Biolog assay, which showed that soil treated with APAP had a significantly higher carboxylic acid metabolism compared to the control (ANOVA post hoc Tukey test, $P< 0.01$). Other carbon sources whose metabolism was found to be significantly higher in APAP treated soils were amino acids, carbohydrates, polymers and phenolics (Fig 6).

Furthermore, the Picrust analyses indicated that amino acid and carbohydrate metabolisms constituted the most prominently expressed genes within the soil communities from APAP treated soils, which was

consistent with the Biolog assay results. However, the Picrust data did not detect differences in gene expression among APAP treatments that were used in the Biolog assay. This is not too surprising because Picrust predicted metagenomes rely on detecting the presences of different 16s rRNA genes from bacterial groups and it is based on previously established databases [55,64]. Also many soil microbial communities can exhibit a high degree of genomic, and therefore metabolic, redundancy [65,66]. It is possible that different soil microbes contained a high degree of overlapping genes. Therefore, Picrust predictions would be insensitive to changes in gene expression, if the total metagenome pool remains stable. Picrust may be only able to detect changes in genome expression among drastically different microbial communities with major differences in the constituting microbial groups. The Biolog plates, on the other hand, can detect changes in the community-level physiological profiles even when the microbial shifts are only in relative abundance changes of constituent organisms. Thus, the Biolog assay may be a better indicator of gene expression levels, especially for genes related to metabolic pathways.

The specific shifts in relative abundance of the soil microbial community members were very consistent with the observed changes in the microbial community function determined in the carbon substrate metabolism (i.e. BIOLOG assay). *A. thermoflava* and *Cellvibrio* sp. were two microbial groups that increased in relative abundance after APAP application. These groups were major contributors to community differences among the different soil communities and are capable of metabolizing a diverse set of carbon substrates, including glycosides [36–38]. Glycosides are major breakdown products of APAP in the soil as a result of fungal [34,35] and plant detoxification activities [27]. Another major breakdown product of APAP due to microbial activity is a carboxylic acid [30], which can be utilized by these two bacteria as well as some of the others that were observed to increase in abundance. In addition, *A. thermoflava* is also capable of degrading xanthine, a compound that caffeine is derived from and salicin which is a compound similar to aspirin [37]. Both caffeine and salicin are also considered to be CECs, thus this particular group of soil bacteria may be positively selected for in soils contaminated with a variety of different CECs.

Cellvibrio genus was also a major driver in community structure differences as determined by the canonical correlation analysis (CCA). *Cellvibrio* is a genus of cellulolytic bacteria, capable of degrading plant cell walls. Members of *Cellvibrio* genus increased in abundance in the APAP treatment. Some Cellvibrios have been found to be able to utilize many different carbohydrates and can even utilize α- and β- glycosides [36,38]. When APAP contaminated water is used for irrigation, plants translocate APAP and detoxify it into a glutathionyl and a glycoside conjugate, which accumulate in the roots [27] and can get into the soil. Soil fungi have also been reported to break down APAP to a glycoside conjugate [34]. The presence of glycosides in plant roots or in the soil probably led to the increase of glycoside metabolising organisms like Cellvibrio bacteria after APAP treatment in our study. This is a particularly interesting group because cellulolytic organisms can have major impacts to the soil community in general by degrading refractory cellulose, making the substrates available to other community members [67,68].

Enhydrobacter aerosaccus is another group of bacteria that increased in relative abundance in the APAP treated soils. This organism also seems to be positively selected for by the conditions generated by the addition of APAP as this organism is known to ferment many different carbohydrates, and can also utilize many amino acids for a carbon source [69]. Metabolism of carbohydrates and amino acids was found to be significantly higher in APAP treated soils compared to the non-treated controls and both functional groups were found to be major drivers of the community differences as determined by the CCA.

The observed increases in metabolic rates of many carbon sources also suggest that there will be community members that do not tolerate the soil conditions caused by APAP addition, and will decline in relative abundance. Our results showed a decrease in the relative abundance of actinobacteria in APAP treated soil compared to the untreated control. [34] found that many strains of actinomyces (which is a genus of class Actinobacteria) were not able to metabolize APAP. This could explain the reduction in the relative abundance of Actinobacteria observed in our study. A large group of bacteria identified to the Xanthomonadaceae family also decreased in relative abundance in the presence of APAP. This particular bacteria family is very diverse and contains numerous plant beneficial or deleterious organisms [70]. At this point it is unclear if this particular decrease in Xanthomonadaceae family was a result of its direct interactions with APAP, or if this was the result of indirect interactions, such as APAP benefiting a competitor. This warrants further investigation. One group of bacteria identified to the Gemmatiomadetes phylum decreased in abundance in APAP treated samples. Gemmatimonadetes bacteria are normally found in high abundance in a variety of different soil environments but, not much is known about the group as there are virtually no culturable representatives that can be studied directly at this point [71].

Conclusions

The overall results of this study are evidence that CECs such as APAP at the levels found in treated RWW have the ability to alter the soil microbial communities and functions in agricultural soil. Although, not all changes in the soil microbiome will be deleterious towards plant health or agriculture production, plant health is directly related to its associated soil microbiome, and shifts in this community may be beneficial or deleterious to a given plant. Therefore, the relationship, and specific plant-microbe interactions require further investigation inorder to better predict how APAP-induced microbial communities shifts impact specific plants or plant communities.

Our findings suggest that APAP can select for a set of bacteria within a given soil community. Groups of bacteria that are capable of utilizing many different carbon substrates were positively selected for by APAP. One group in particular, *A. thermoflava* appears to thrive in the APAP treated soils, and possess the ability to metabolize a wide range of carbon sources, including some metabolites of CECs. While other groups, such as Xanthomonadaceae appear to be selected against by the addition of APAP. At this point however, it is unclear if this selection effect is acting specifically on the organisms themselves or acting indirectly by interacting with competing or complementary organisms. Additional, culture-based studies

may be employed to further elucidate the specific effects or mechanisms of action APAP has on a particular bacterium.

RWW use has been a tremendous success for conserving clean water supplies and has allowed many water districts to stretch the supplies of clean water further to meet the growing demands associated with population increases. Since RWW is so successful, it's more than likely RWW use will increase in the future. Therefore, more studies on the impacts of CECs in RWW need to be conducted to ensure that this source of water does not have negative impacts on food and feed production. Our previous study [26] showed that CECs impact plant disease development, which may consequently affect food production.

We hope that our study and others that suggest CECs within RWW can disrupt microbial community composition and function will demonstrate the need to update the technology used for cleaning treated wastewater for reuse. Traditionally, legislation and policies addressing RWW use focused on the removal of potential human pathogens and soil physicochemical factors (e.g. pH, salinity, organic load, heavy metal) [72] to minimize risks to human health and changes in soil properties [3]. The current study and many others [21–26] indicate that policies need to address the level of chemical contaminants, such as CECs, that may pose risks to natural and agricultural environments, thus indirectly affecting human health as well. Hopefully these policies will lead to the development of technology that can more effectively clean wastewater for reuse to such a degree that there will be no risk in using the invaluable source of water.

Declarations

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Authors contributions statement

E.W.G. conceived the study; EWG & NKM designed of the work; NKM & MYG the acquired and analyzed the data; EWG, NKM & MYG interpretation of data and wrote the manuscript.

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Figures

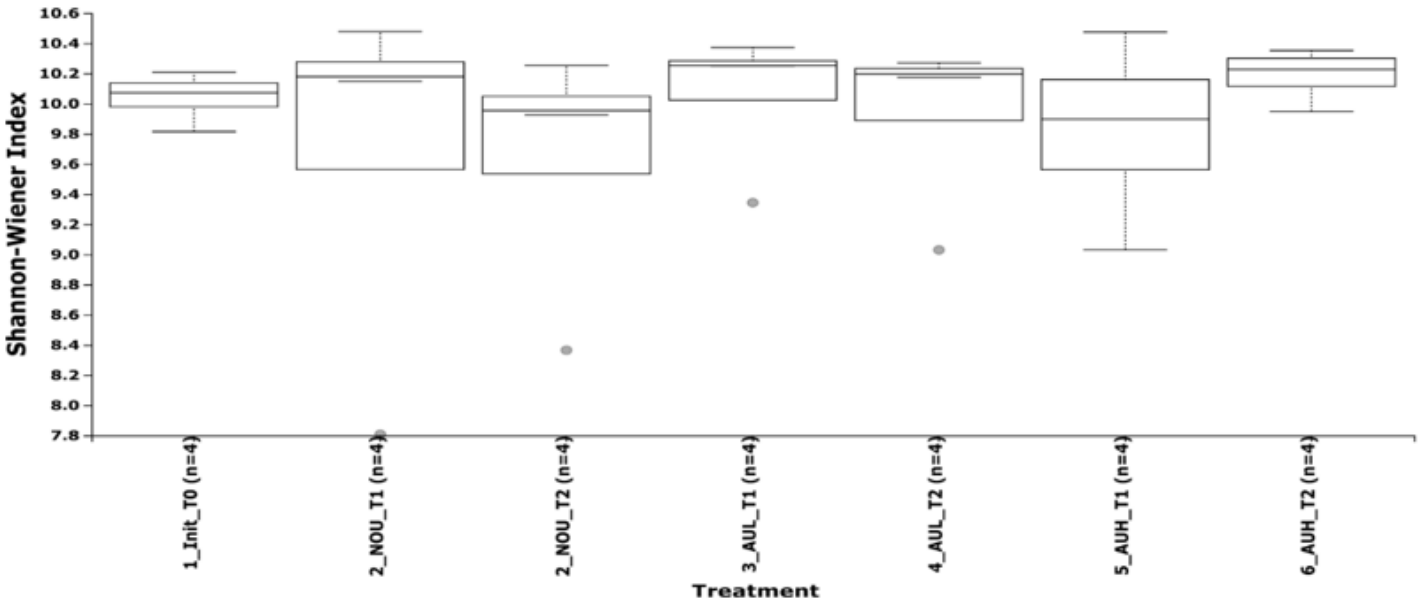

Figure 1

Box plots comparing the Shannon Weaver index among all

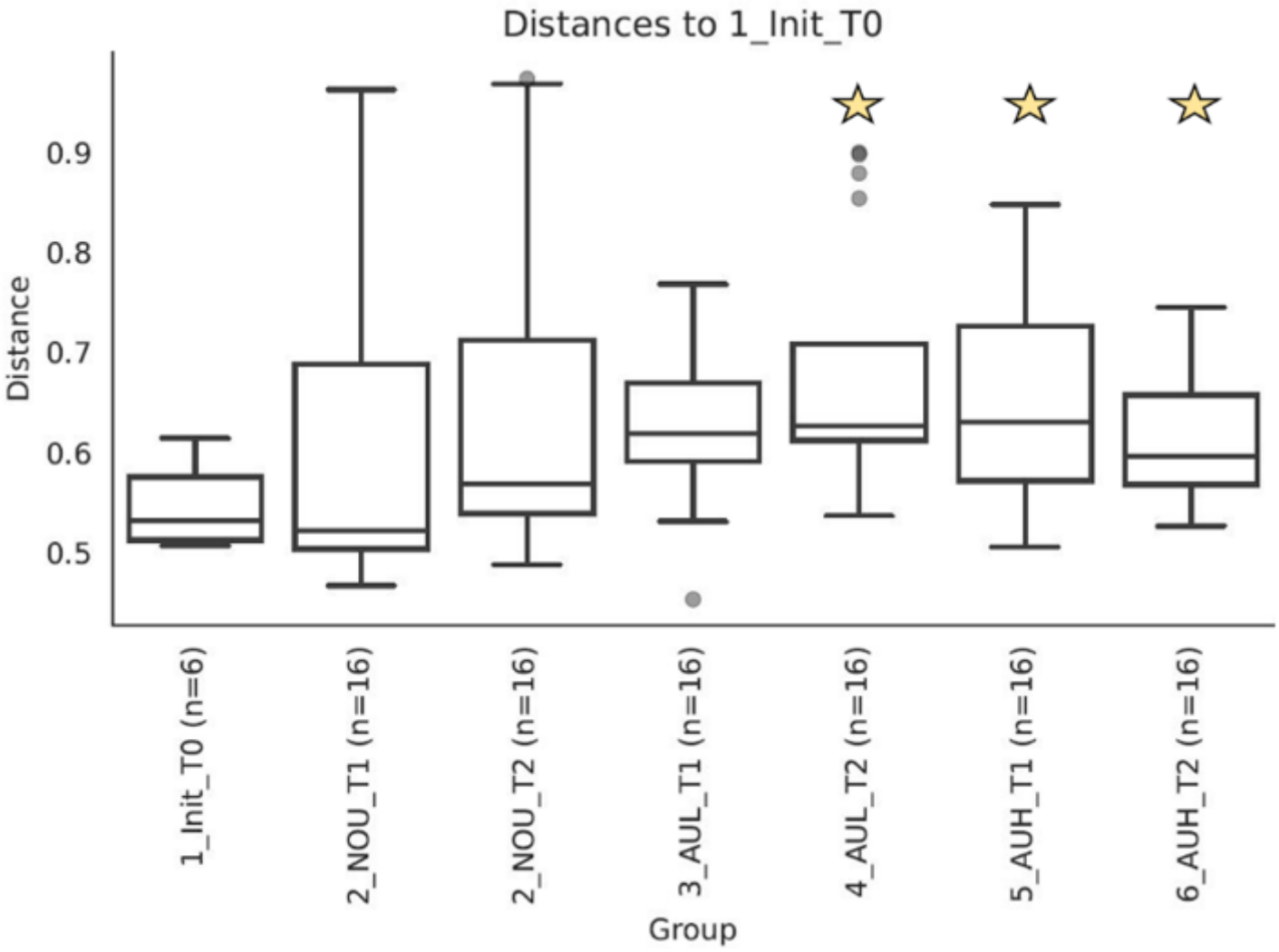

Figure 2

Box plot of Bray Curtis distances among the different samples. Star denotes samples that were significantly different **β**-diversity values compared to the initial, T0, samples. Pairwise Permanova all P's <0.05.

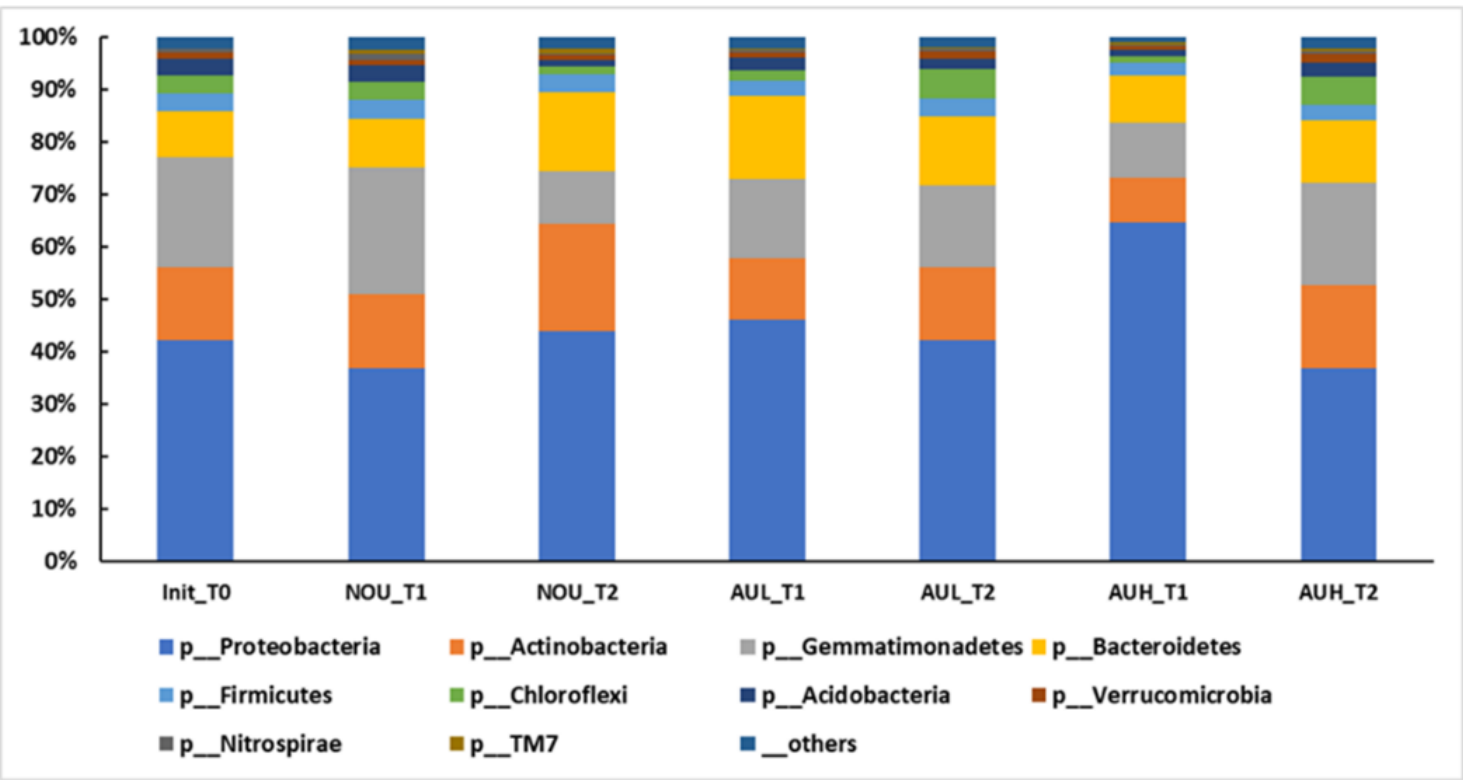

Figure 3

Showing the relative abundance of bacteria at the phylum level only. p = phylum.

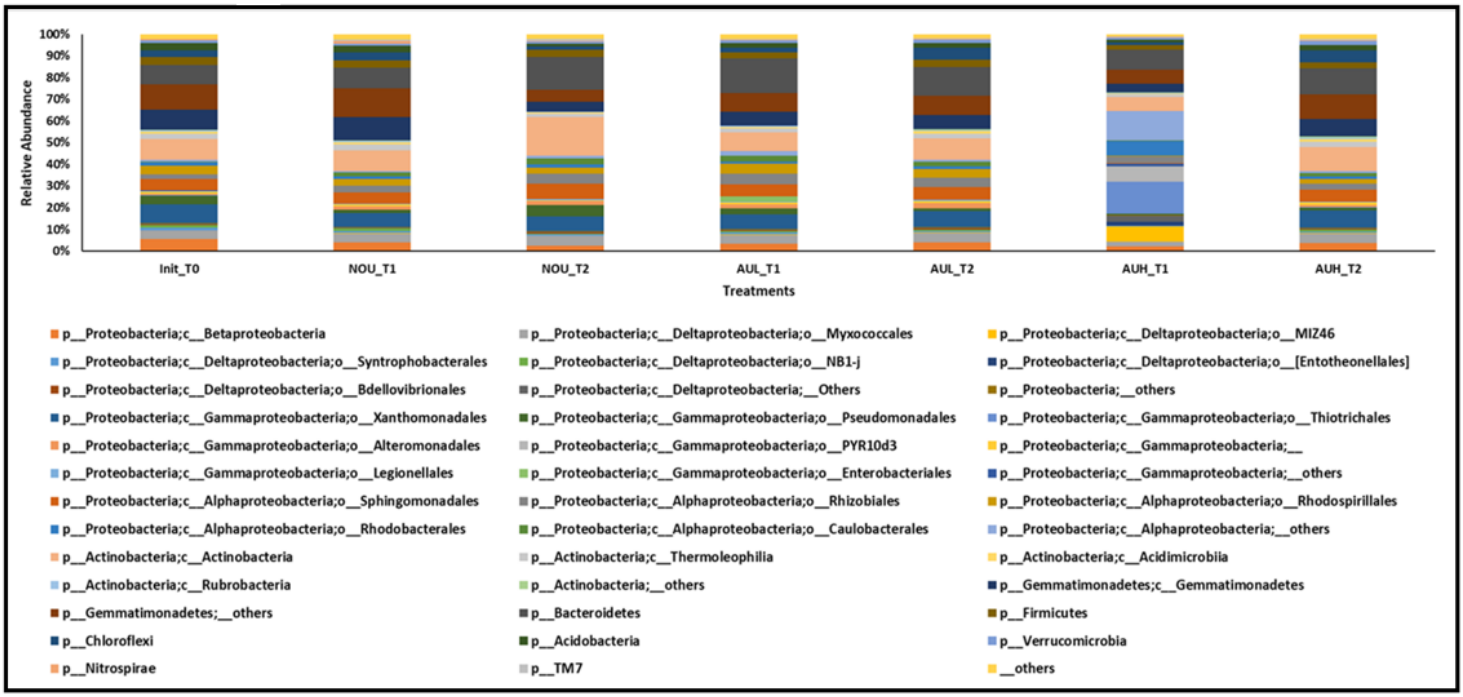

Figure 4

Relative abundance of the microorganisms detected in each sample. Some groups were collapsed to less specific taxonomic groups for clarity. p = phylum, c = class, o = order.

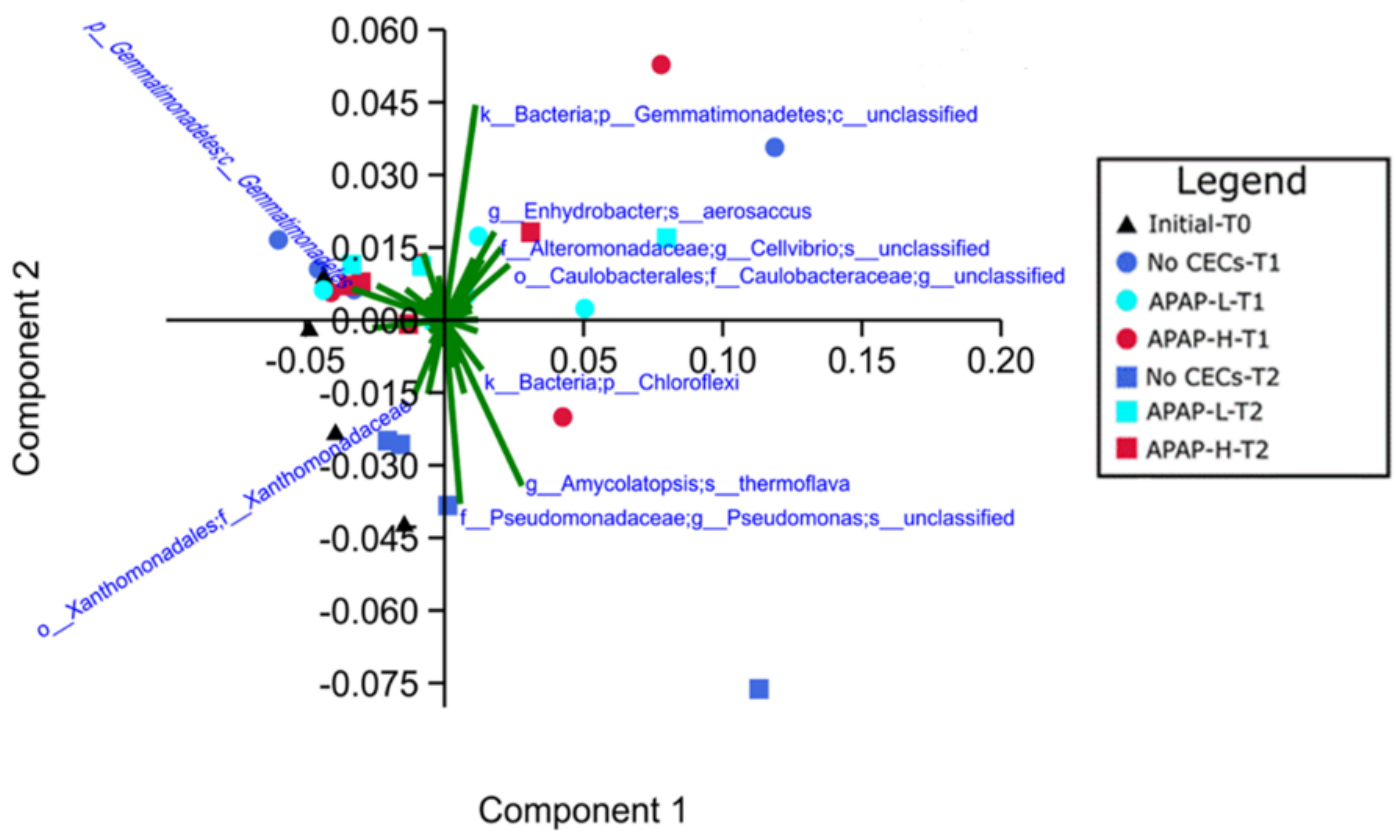

Figure 5

PCA graph of Illumina sequencing data with vectors plotted showing community members that contributed to the most variability among different soil communities.

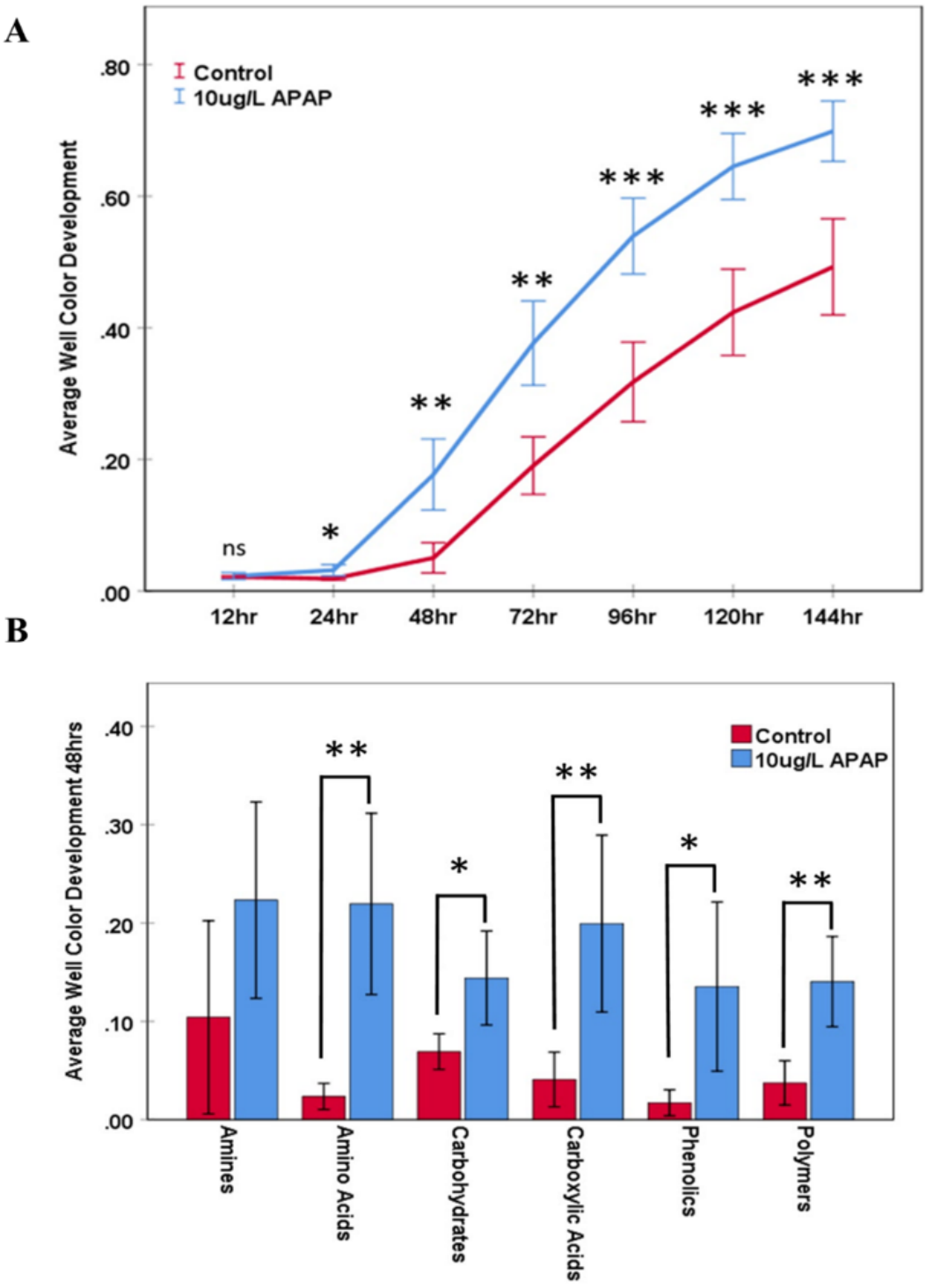

Figure 6

Average Well Color Development of Biolog EcoPlates A) Average Well Color Development of entire plates over the course of 144 hours. B) Average Well Color Development of each functional carbon group at 48hrs. For all graphs, error bars represent the standard error. Lines between treatments represent independent t-tests between control and 10µg/L APAP. Single asterisks represent a p-value <0.05, double asterisks represent a p-value < 0.01, and triple asterisks represent a p-value <0.001.

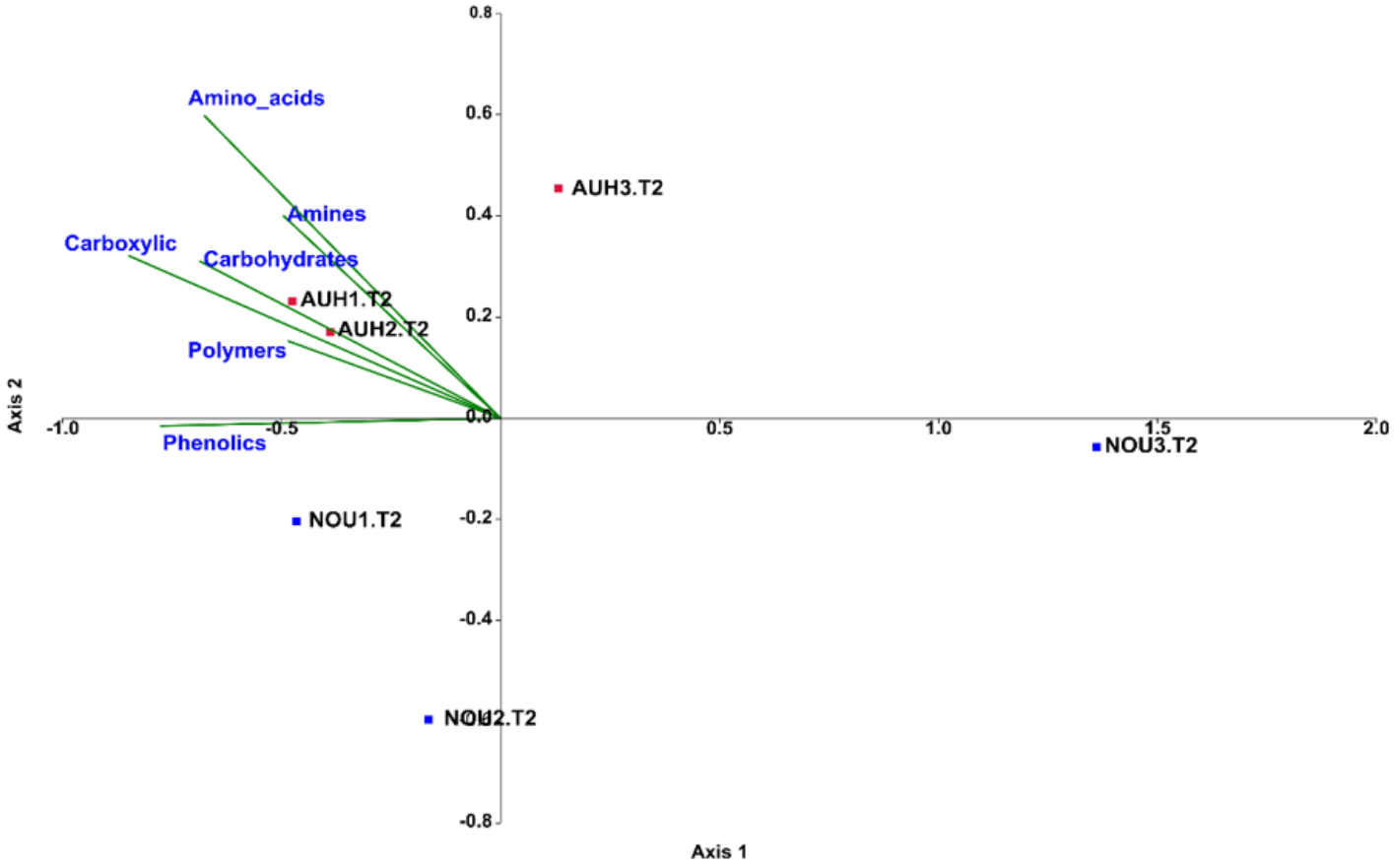

Figure 7

Canonical correspondence analysis in PAST of biolog plates that were incubated for 96 H. Showing the utilization of the 6 general carbon substrate groups among all treatments in T2.

Supplementary Files

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- SupplementarymaterialMcLainetal2021.docx