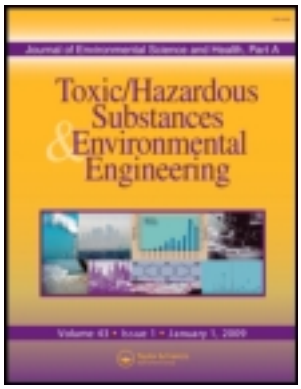

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An initial screening of antibiotic effects on microbial respiration in wetland soils

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Antibiotics are biologically active compounds that are routinely detected in the environment and usually associated with treated wastewater discharge. Due to their high biological activity, antibiotics may have more environmental impacts than other pharmaceuticals. Wetlands are often used to treat or polish wastewater, with the goals of reducing nutrient and carbon loading into the environment. Nitrogen and carbon processing in wetlands is largely associated with microbial activity, however impacts to microbial activity due to antibiotic loading into treatment wetlands is relatively unknown. Two wetland soils (mineral and peat) were individually spiked with ciprofloxacin, sulfamethoxazole or tetracycline ranging from 1–1000 ppb to examine effects on microbial mediated evolution of CH_4, CO_2 and N_2. The antibiotics both positively and negatively affected microbial respiration (a proxy for microbial activity) rates in the two wetland soils depending on soil properties and concentration. Sulfamethoxazole reduced CO_2 and N_2O respiration rates at higher concentrations (500, 1000 ppb) in the mineral soil. However, the CO_2 rates recovered within 48 hours, while N_2O suppression continued through the end of the incubation. Ciprofloxacin and sulfamethoxazole also demonstrated the ability to suppress respiration at low spiking concentrations (1, 50 ppb) for several treatments. The results demonstrate the ability of antibiotics to impact soil respiration at environmentally relevant concentrations. Parameters that appear to affect the impacts of antibiotics were sorption, length of exposure and soil carbon content. Future studies are needed to provide further insight into antibiotic effects to microbial community structure.

Keywords: Wastewater treatment, denitrification, sulfamethoxazole, carbon dioxide.

Introduction

Since Alexander Fleming's discovery of penicillin in 1928, antibiotics have become a cornerstone of our healthcare system and are also used to maintain high levels of livestock and aquaculture production.[1] These compounds are valued for their ability to interrupt the proliferation of specific bacteria. While antibiotics can occur naturally in the environment, a wide range of synthetic pharmaceuticals are now regularly detected in soil and water from various sources.[2–4]

In wetlands, microbes facilitate many biogeochemical transformations, such as organic matter decomposition and a wide variety of nutrient transformations.[5,6] Many ecosystem functions rely on microbial activity to break down organic matter in wetlands, providing bioavailable nutrients. As a by-product of their activity, heterotrophic bacteria produce carbon dioxide (CO_2), and methane (CH_4). Additionally, denitrifying bacteria facilitate the gaseous removal of nitrogen from wetlands by converting nitrate (NO_3^-) to nitrogen gas (N_2) and in some systems, N_2O.[7,8]

Nitrogen is a limiting nutrient in many aquatic ecosystems and therefore, it is important to reduce N loading to prevent eutrophication.[9] In some Louisiana communities and many other places around the world,[10,11] wetlands are used to treat wastewater. Wastewater is known to contain low concentrations of a wide range of pharmaceuticals, including antibiotics.[12] It is therefore important to determine if the presence of antibiotics in wastewater and subsequently in treatment wetland soil has an adverse impact on the biogeochemical function of the natural microbial communities, particularly the denitrifying bacteria responsible for removing nitrate from wastewater. Several studies have examined soil or sediment microbial respiration in the presence of pharmaceuticals at environmentally relevant concentrations, however there is relatively little information on their effects in wetland soils. Costanzo [13] found that some antibiotics reduced the rates of denitrification (erythromycin, clarithromycin, amoxicillin), yet amoxicillin/clavulanic acid showed no effect when soils were loaded with 1000 ppb. Ciprofloxacin was also tested over a concentration gradient from 0.1 to 1000

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ppb and no effects were noticed.[13] Fountoulakis [14] examined the influence of pharmaceuticals on methanogenesis and determined that propranolol hydrochloride, diclofenac (sodium), carbamazepine and ofloxacin all inhibited rates to some degree, while sulfamethoxazole and clofibric acid showed no significant effects on respiration rates. However, in the study by Fountoulakis,[14] samples were tested over a concentration gradient of 10 to 400 ppm, which is 2 to 6 orders of magnitude higher than environmentally relevant concentrations.[14] Kotzerke [15] examined manure containing sulfadiazine, which exhibited the ability to decrease CO_2 respiration, denitrification and nitrification in manure amended soils. A study by Liu [16] discovered that sulfonamides and trimethoprim caused significant short-term decreases in respiration in an agricultural soil. Overall, previous studies indicate that microbial respiration processes in soil are variably impacted by antibiotics.

This study expands on previous research by more thoroughly examining N_2O (as a proxy for denitrification), CH_4 and CO_2 production in an organic and mineral wetland soils containing antibiotic compounds commonly detected in the environment (ciprofloxacin: CIP, tetracycline: TET and sulfamethoxazole: SULF) (Table 1). By measuring the evolution rates of these three gasses in the presence of antibiotics, we will determine impacts to microbially facilitated biogeochemical processing of nutrients in these wetland soils.

Gas evolution in sample vials is indicative of specific biochemical properties present in soil. CO_2 production takes places under aerobic conditions, while N_2O is associated with moderately reduced environments and CH_4 production occurs in highly reduced environments. Generally CO_2 evolution occurs mostly near the soil surface and decreases with depth, while N_2O is produced when oxygen levels are depleted. Methane is produced at depth, where no oxygen is present in wetland soil.

Materials and methods

Study area

Mandeville, LA utilizes a system of lagoons and wetlands to treat wastewater, and is effective at reducing the concentration of many pharmaceuticals.[12] However, several of the detected compounds are still released, but at much lower concentrations into the adjacent forested wetland (Bayou Chinchuba) and ultimately Lake Pontchartrain, a large, shallow, oligohaline estuarine lake.[17] A nearby mineral wetland (relatively low organic matter: 60 g C Kg^{-1}) soil from Bayou Castine (BC, 15R 784949 E, 3361530 N) was chosen for this study due to its proximity to Bayou Chinchuba and similar soil classification as an arat silty clay loam, which is a fine silty, siliceous, non-acid, thermic typic hydraquent.[18] A peat soil (high organic matter: 232 g C Kg^{-1}), from the Davis Pond (DP) Freshwater Diversion (15R 0765814 E, 3307688 N) was also chosen for analysis to compare the effects of antibiotics on soils with a drastically different organic matter contents.[19] This wetland receives water diverted from the Mississippi River, which contains a wide range of pharmaceutical compounds.[20]

The Bayou Chinchuba mineral soil and the Davis Pond peat soil will be referred to as only the mineral and peat soil, respectively, for the remainder of the manuscript. Soils from the top 10 cm were collected, woody debris removed, homogenized and stored at 4°C. Soil characterization included total and extractable metals [21] and total carbon and nitrogen and organic matter.[22] Soil properties are shown in Table 2. All incubations were performed on field moist soil with rates expressed on a dry weight basis.

Antibiotic solutions

Tetracycline, ciprofloxacin and sulfamethoxazole (all > 98% purity) were obtained from Sigma-Aldrich (St. Louis, MO) (Table 1). Stock solutions of 20 ppm were prepared in deionized water for each compound, further diluted to a 5 ppm spike solution. The 5 ppm spike solution of each compound was then injected into the sample bottles or tubes containing soil and solution at specific volumes to achieve the pre-determined treatment concentration.

Methane and carbon dioxide determinations

For measurement of CH_4, and CO_2 production, 0.75 g dry weight of each soil was added to 27 mL anaerobic tubes, capped with a gas impermeable butyl rubber stopper and sealed with an aluminum crimp. Each sample tube was evacuated to a pressure of < -88 kPa and then flushed with 99.9% O_2 free N_2 for 5 min. Each vial was incubated at 25°C in an orbital shaker at 100 rpm for 72 h and the headspace was again flushed for 5 min prior to solution, substrate and antibiotic additions. Each vial received DI water to bring the total liquid volume of the vial to 15 mL, which includes the additions of one of five concentrations (1, 50, 100, 500, 1000 ppb) of CIP, TET or SULF. Basal (background nutrients and carbon levels) and substrate induced (carbon or carbon and nutrient additions) incubations (SIR) were prepared to determine baseline as well as the potential effects due to antibiotic introductions. Substrate induced incubations for CH_4, and CO_2 were identical to basal incubations, except sodium acetate (23 g C kg^{-1} dry soil) was added to CH_4 vials while glucose (30 g C kg^{-1} dry soil) was added to CO_2.

Gas samples for CO_2 basal respiration were collected and analyzed once during the first 48 hours and then a week later, followed by bi-weekly sampling for up to two months. Substrate induced respiration gas samples were analyzed daily for a week. Carbon dioxide samples were analyzed using a Shimadzu (Koyoto, Japan) GC-2014 fitted with a

Table 1. Compounds used to examine antibiotic effects on wetland soil microbial respiration and various properties of each drug.

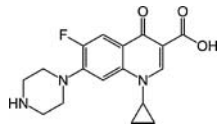
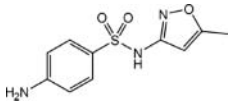
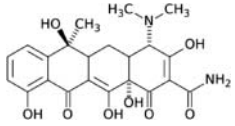
Compound		*Antibiotic effect*	*Targets*	*Human excretion*	*Treats*	*WWTP influent μg L^{-1}*	*WWTP effluent μg L^{-1}*	*Log K_{ow}*	*Log K_F*
CIP		Bactericidal	Gram − Gram +	35% in 24 hrs	Urinary Tract Infections	0.92 – 1.4[42]	0.27 – 0.45[42]	0.4[43]	4.01[32]
SULF		Bacteriostatic	Gram − Gram +	30% in 72 hrs	Urinary Tract Infections Chronic Bronchitis Pneumonia	0.72 – 2.8[42; 44]	0.21 – 0.68[42 44]	0.89[37]	0.04 – 1.27[37]
TET		Bacteriostatic	Gram − Gram +	80–90%[43]	Respiratory Tract Infections Skin and Tissue Infections	0.27 – 1.1[42; 44]	0.06 – 0.29[42; 44]	−1.19[43]	1.66 – 3.63[36; 45]

Table 2. Soil properties for the peat (Davis Pond) and mineral (Bayou Castine) soils.

	Davis Pond	*Bayou Castine*	
Moisture	87.9 ± 0.45	62.6 ± 0.23	*Weight%*
Loss on Ignition	44.1 ± 0.95	13.1 ± 0.21	*Weight%*
Total Carbon	232 ± 4.12	59.7 ± 0.92	g kg^{-1}
Total Nitrogen	15.9 ± 0.38	4.15 ± 0.07	g kg^{-1}
Potentially Mineralizable N	0.73	1.94	mg N kg^{-1} day
Total Phosphorus	619 ± 30.4	635 ± 26.1	mg P kg^{-1}

thermo conductivity detector operated at 160°C, utilizing a packed Poropak N (6 ft; 80/100 mesh) column, supplied by Sigma-Aldrich (St. Louis, MO), with an oven temperature of 80°C.

Gas samples for methane basal respiration were analyzed bi-weekly for up to 2.5 months. Substrate induced CH_4 samples were incubated and analyzed for 3 months. Samples were analyzed using a Shimadzu GC-2014 fitted with a flame ionization detector operated at 160°C, utilizing a packed Carboxyn 1000 (6 ft; 40/60 mesh) column, supplied by Sigma-Aldrich (St. Louis, MO), with an oven temperature of 110°C.

Denitrification methods

The acetylene block method was used to measure the denitrification rate.[23,24] In this procedure, N_2O production is an indicator of denitrification, as acetylene blocks the final reduction transformation of N_2O to N_2. Approximately 0.75 g dry weight of the two soils were added to 70 mL serum bottles, capped with a gas impermeable butyl rubber stopper and sealed with an aluminum crimp. Only SIR was measured for denitrification. Denitrification samples were prepared and incubated in the same manner as the CO_2 and CH_4. However, denitrification samples received acetylene (15% of headspace) and a carbon/nitrogen substrate (glucose: 597 g C kg^{-1} dry soil, potassium nitrate: 73.8 g N kg^{-1} dry soil). Headspace gas samples were collected within the first five hours after substrate addition and then daily for 4–7 days. Gas samples were analyzed using a Shimadzu GC-8A fitted with an electron capture detector operated at 150°C, utilizing a packed Poropak Q (6 ft; 80/100 mesh) column, supplied by Sigma-Aldrich (St. Louis, MO), with an oven temperature of 50°C.

All samples were prepared and incubated in triplicate, with a control consisting only of soil (Bayou Castine or Davis Pond), DI water and substrate (if SIR). Standard curves were run and continuing calibrations (within 5% of the previous curve) performed prior to each analysis.

Data analysis and statistics

Throughout the manuscript the use of the terms *basal, initial*, *potential*, and *delay* will refer to respiration rates occurring during specific time periods within each incubation. *Basal* respiration rates were calculated as the maximum rate observed during basal incubations. Substrate induced respiration rates were calculated for the early respiration rate (*initial*), prior to an exponential increase in gas production, and for the maximum (*potential*) rate observed. If there was a visible delay in the respiration rate with one or more of the treatments, an intermediate rate was also calculated, and is referred to as the *delay* rate (Fig. 1). Due to the addition of substrates to create non-limiting conditions, potential rates are significantly higher than what would be expected under normal environmental conditions. All rates are the average of the maximum rates for each individual sample vessel within that analysis. Significant differences between respiration rates were determined using a one-way ANOVA. A Latin Square Design (LSD; $p < 0.05$) model was used when equal variance was met, and a Dunnett's T3 test when equal variances were not met. ANOVA analysis was performed using SPSS 17 (SPSS Inc., Chicago,

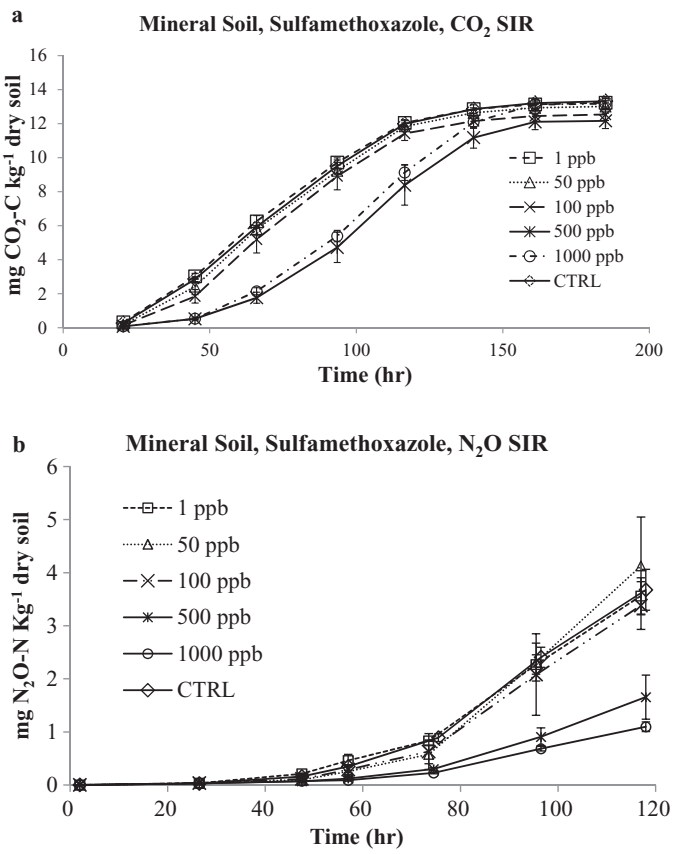


Fig. 1. CO_2 (a) and N_2O (b) substrate induced respiration with time in the mineral soil exposed to sulfamethoxazole.

IL), while correlations were determined using Excel 2007 (Microsoft Corp., Redmond, Wash).

Results and discussion

The total carbon content varied between the two soils, with the peat soil containing 232 g C kg^{-1} and the mineral soil containing 60 g C kg^{-1}. The CH_4 and CO_2 basal respiration (5.4× and 4.3×, respectively) and SIR initial (472× and 2.78×, respectively) rates in the peat soil were significantly higher than the mineral soil. However, there was no significant difference between soils when measuring the potential N_2O, CO_2 and CH_4. The removal of a substrate limitations lead to an increase in microbial activity as the microbial population increased. Therefore, under non-limiting conditions (substrate addition), both soils potentially have similar microbial activities.

Antibiotics decrease microbial activity and/or inhibit microbial replication. Consequently, it was expected that exposure to antibiotics in high (500, 1000 ppb) concentrations would stop, decrease or delay microbial respiration. No treatments resulted in complete cessation of respiration and only in the mineral soil exposed to SULF when measuring CO_2 and N_2O, did we observe a delay in respiration rates (Fig. 1a and b). While there were statistically significant results observed, 35 of the 48 total treatment combinations (basal, substrate induce – initial and potential, analyte – CO_2, N_2O, CH_4) demonstrated no correlation with regards to respiration rate and concentration or a significant difference from the control for any individual treatment. Each soil had 24 treatment combinations, with 18 and 17 combinations being insignificant for the peat and mineral soil respectively. Only one negative effect was observed in the peat soil, while several were detected in the mineral soil. The two soils were equally impacted by the antibiotics, however, most negative effects were observed in the mineral soil.

Negative impacts

There were five negative impacts due to antibiotics in the two wetland soils, with two instances of respiration rates negatively correlating with treatment concentration in the mineral soil (Fig. 2b and f). The most noticeable impact was an initial suppression in respiration rates associated with SULF for 100, 500 and 1000 ppb treatments (Fig. 2b). This resulted in a negative correlation between concentration and respiration rate.

However, the 100 ppb respiration rate recovered within 48 hours, while the 500 and 1000 ppb treatments recovered after ~60 hours, resulting in no significant differences for the potential respiration rates for this treatment combination. Increasing SULF concentrations were also negatively correlated with N_2O respiration rates in the mineral soil (Fig. 2f). The 500 and 1000 ppb treatments were significantly less than the control for the delay rate, while only the 1000 ppb treatment was significantly less than the control for the potential rate. The 500 ppb treatment was not significantly different than the control when determining the potential rate, which indicates that the microbial respiration may have started to recover (produce more N_2O) in the higher concentration treatments towards the end of the incubation. An extended incubation (<120 h) would be needed to further explore N_2O respiration recovery from SULF exposure in the mineral soil.

The negative impacts of SULF to CO_2 respiration in the mineral soil were relatively short-lived. A similar CO_2 suppression and recovery trend in the presence of SULF was also observed by Liu et al [16] in an agricultural soil. Possible explanations for the rapid recovery are: (1) there was a shift in the composition of the microbial community to SULF resistant microbes,[16,25] (2) the impact of the drug was short-lived and the microbes simply recovered within ~60 h of SULF introduction [16] and/or (3) the compounds were sufficiently sorbed to the soil to prevent further antibiotic impacts on microbial respiration.[15,16]

Thiele-Bruhn and Beck [26] theorize that when measuring basal respiration of soil microbes in the presence of sulfonamides and tetracyclines the bacteriostatic (preventing reproduction, but not killing bacteria) nature of the compounds will not influence microbial respiration unless microbial growth is occurring.[26] They argue that under basal respiration conditions, most microbes are dormant in the soil while still respiring. This may be why we observed few significant impacts on basal respiration of CO_2 with the mineral soil in the presence of SULF, but we did observe a negative impact when a substrate was added to the soil, activating the microbes.

The remaining negative impacts were observed for low treatment concentrations (1 and 50 ppb) (Fig. 2d, e and 3b). Ciprofloxacin significantly decreased respiration rates in the mineral soil for the initial rates of CH_4 (1 ppb) and N_2O (1 and 50 ppb). There were also several other instances where the mean values of the low concentration treatments were less than the other treatments and control, however they were not significant. Sulfamethoxazole also suppressed the respiration rate of CH_4 at 50 ppb in the peat soil (Fig. 3b). This treatment combination also resulted in a positive correlation between respiration rate and spike concentration.

Concentrations of the three compounds examined have been detected at 1 ppb within wastewater treatment systems (Table 1), while compounds may accumulate in soils at higher concentrations due to continuous loading and the sorptive properties of each compound. The low (1 ppb) treatment is environmentally relevant, while 50 ppb treatment may mimic some treatment wetland soil conditions. These results demonstrate that concentrations, which are "environmentally relevant", have the potential to affect microbial activity, at least in the short-term. The rationale for impacts at lower concentration and not higher

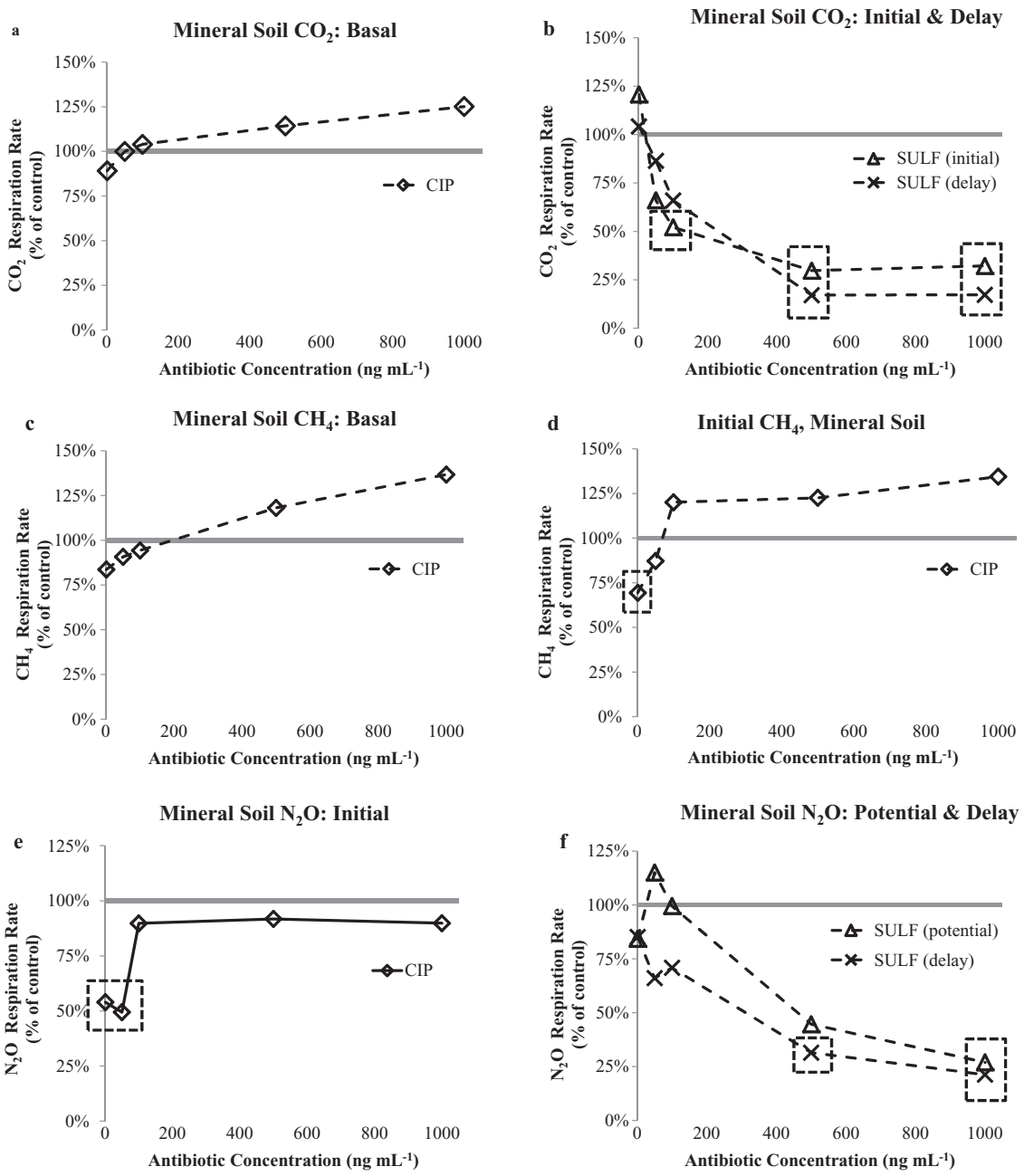


Fig. 2. Respiration rates for each treatment level as a percentage of the control in the mineral soil. Each figure presented has a significant correlation between respiration rate and treatment concentration (dashed line) and/or a treatment that is significantly different than the control (represented by a box around the significant data point(s)).

concentrations is not fully understood. This behavior resembles a "J-curve", which is used to describe phenomena in economics, political science and medicine, where a curve decreases from the initial starting point before rising to, or above the starting point.

The J-curve may be due to the low concentration treatments moderately impacting the microbial population, but not enough to significantly alter the microbial community dynamics. If this is the case, the soil microbes exposed to higher concentration treatments are capable of adapting or changing community structure without a detectable impact on respiration rates. However, this postulation requires testing microbial communities within the soil, which is outside of the scope of this study. Additionally, the presence of fungal respiration may also explain some variability in our results. However, under continuously flooded soils which are strongly reduced, bacterial communities dominate denitrification.[27]

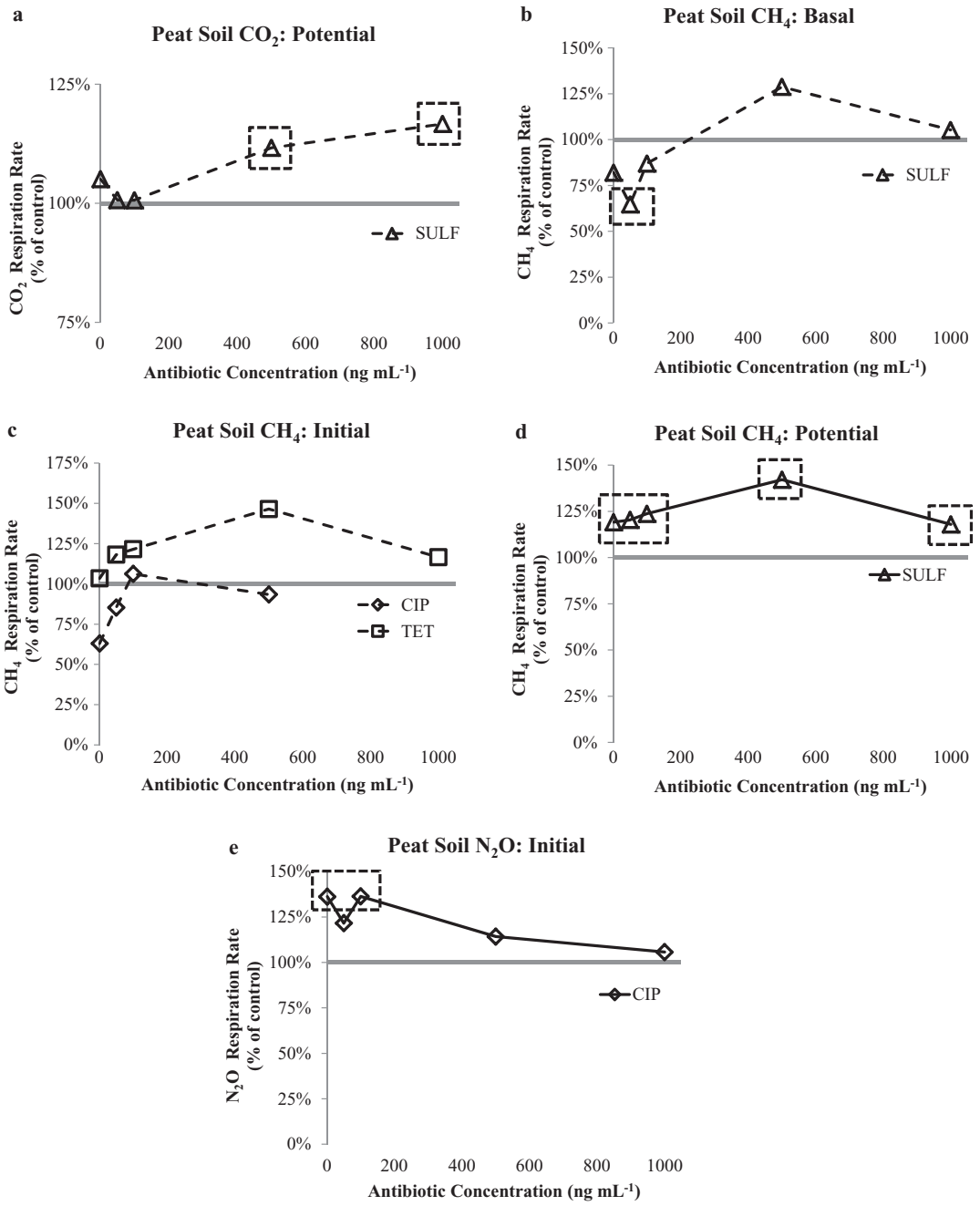


Fig. 3. Respiration rates for each treatment level as a percentage of the control in the peat soil. Each figure presented has a significant correlation between respiration rate and treatment concentration (dashed line) and/or a treatment that is significantly different than the control (represented by a box around the significant data point(s)).

Positive impacts

In several treatment combinations antibiotics appeared to have a stimulating effect on microbial respiration. The concentration of CIP positively correlated with basal respiration rates for CO_2 (mineral soil) and CH_4 (mineral soil), as well as the initial CH_4 (peat and mineral soil) respiration rates (Fig. 2a, c and d; Fig. 3c). Several instances were observed where CIP increased respiration under reducing (methane producing) conditions. Cordova-Kreylos and Scow [28] found that CIP increased microbial biomass when tested under anaerobic conditions and this result was explained as a decrease or complete loss of antibiotic activity in an anaerobic environment.[28–30] Ciprofloxacin may act as a substrate under reducing conditions, if its antibiotic activity is neutralized, and some bacteria have been found to subsist on antibiotics, [31] but this has not been studied in wetland or submerged soils.

Although SULF exhibited the most negative effects on soil respiration rates in the mineral soil, multiple positive impacts on respiration rates were observed in the peat soil. Sulfamethoxazole respiration rates were positively correlated with concentration when measuring basal CH_4 as well as the potential CO_2 rate (500 and 1000 ppb treatments respiration rates were significantly higher than the control) (Fig. 3a and b). A positive correlation for CO_2 respiration is the opposite of what was observed in the mineral soil, where SULF initially suppressed respiration rates. While there was no significant correlation between respiration rate and concentration, SULF treatments for the potential CH_4 respiration rates were all significantly higher than the control treatment (Fig. 3d). With the peat soil, SULF stimulated microbial respiration when examining the potential respiration rates.

Previous research has determined that antibiotic effects can be time dependent due to rapid sorption to soils, which would reduce the bio-availability of the compounds.[15–16] Sorption of antibiotic compounds to soil (organic or mineral fraction) is a pathway for PhAC removal from the water column.[32–34] The magnitude of the effect of the antibiotic compounds studied on microbial communities is known to be inversely related to compound sorption to the soil. [26,28] Sorption causes a reduction in bioavailability and antibiotic potency of the compounds. [26,28] CIP sorption to the mineral soil produced a log K_F value of 4.01.[32] Log K_F values from the literature for TET and SULF range between 1.66 – 3.63 and 0.04 – 1.27, respectively.[35–37] Based on published K_F values, the general sorption potential follows this order: CIP > TET > SULF.[32; 36–38] A reduction of antibiotic activity due to lower sorption potential may explain why SULF exerts a stronger negative influence on the soil microbes compared to the other compounds in this study.

TET and SULF are broad spectrum antibiotics which target a wide range of bacteria, while CIP is designed to target specific bacteria.[39] Since CIP only targets a specific suite of bacteria, this compound could cause a shift in the microbial community dominance or alter the microbial activity.[39] High sorption and abiotic degradation of TET may explain why there are less pronounced effects on respiration rates than for this compound compared to what is found with CIP and SULF.[40] Many of the treatment combinations we examined did not produce significant results compared to the control or a conclusive respiration trends. A lack of antibiotic influence on many of the respiration rates measured indicates that antibiotics in the environment may not significantly impact overall biogeochemical cycling and hence, may not effect treatment wetland functioning. Therefore, treatment wetlands may be a potential solution to PPCP pollution in the environment.[41]

Conclusions

Overall, SULF exhibited the greatest influence on microbial respiration (negative in mineral soil and positive in the peat soil). Samples amended with TET demonstrated very little effects, with the exception of one positive correlation between concentration and respiration rate (Fig. 3c), which was measured for the initial methane rate in the peat soil. Several significant relationships were observed for CIP, where there was either a significant positive correlation between respiration rate and treatment concentration or a decrease in respiration rate compared to the control at low spiking concentrations.

In general SULF impacted both soils and the production of all three gasses (CH_4, CO_2 and N_2O). Suppression at high concentrations was observed in the mineral soils initial CO_2 respiration rates. However, no suppression was observed for the potential CO_2 respiration rates, meaning that the overall microbial activity recovered to that of the controls. The only instance of suppression observed for a potential rate occurred in the mineral soil with denitrification in the presence of SULF. The effects of each compound on microbial activity may to be tied to the sorption potential of each compound and the compounds overall stability. The general sorption potential is CIP > TET > SULF. Therefore, SULF having a lower sorption potential, had the most obvious impacts on microbial activity.

At environmentally relevant concentrations, antibiotics may negatively impact microbial activity in wetland soils. Release of CH_4, CO_2 and N_2O is a byproduct of microbial respiration/activity in wetland soils, and a decrease in respiration indicates that the collective microbial pool is less active. This finding has significant implications for wetland systems used to improve water quality, a common practice around the world. The efficiency of wetlands to treat and polish wastewater may be altered due to the presence of antibiotics. However, we also observed many instances where no impacts, whether negative or positive, on microbial activity were detected. Further research addressing community structure or even cell counts are needed to develop an understanding of the impacts to microbial communities within wetland soils to address the questions raised by this research.

Microbes are essential to the overall function of treatment and natural wetlands, through the transformation of stored nutrient to bioavailable forms linked to plant productivity. The bioavailable forms of nutrients are essential to wetland plants and higher trophic levels. A reduction in bioavailable nutrients due to antibiotic impacts on the microbial pool may decrease the overall productivity of treatment wetlands. Due to the mixed results from this study and the high complexity of the microbial consortia, molecular studies addressing the microbial community structure need to be undertaken. Long-term exposure and loading of compound mixtures also needs to be studied to gain a greater understanding of the effects of antibiotics on treatment wetland processes.

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