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Antibiotics and temperature interact to disrupt soil communities and nutrient cycling

Jane M. Lucas [a,b,*], Bronte S. Sone [a], Dana Whitmore [a], Michael S. Strickland [a]

[a] University of Idaho, Department of Soil and Water Systems, 875 Perimeter Drive, Moscow, ID, 83844, USA
[b] Cary Institute of Ecosystem Studies, Cary Institute of Ecosystem Studies, Millbrook, NY, 12545, USA

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ABSTRACT

Soils contain immense diversity and support terrestrial ecosystem functions, but they face both anthropogenic and environmental stressors. While many studies have examined the influence of individual stressors on soils, how these perturbations will interact to shape soil communities and their ability to cycle nutrients is far less resolved. Here, we hypothesized that when soils experience multiple stressors their ability to maintain connected and stable communities is disrupted, leading to shifts in C and N pools. To test this, we maintained soils across three temperatures representative of seasonal variability (15, 20 and 30 °C) and introduced high or low doses of the common livestock antibiotic Monensin. We monitored respiration and examined changes to microbial communities through amplicon sequencing and network analyses. We also examined soil C and N pools to understand how temperature and antibiotics shape ecosystem function. We found that antibiotics and rising soil temperatures interacted to disrupt bacterial assemblages and network structure, allowing for a rise in fungal dominance and change in soil nutrient stoichiometry. Antibiotics alone decreased bacterial diversity, abundance, total extractable N, and microbial carbon use efficiency, while increasing bioavailable C. Higher temperatures independently homogenized fungal community composition, decreased dissolved organic C and increased soil respiration rates. These results emphasize that as soils encounter multiple stressors, ecosystem efficiency, stability and resilience may be diminished.

1. Introduction

Soils are an essential component of terrestrial ecosystems, supporting agricultural industries, regulating nutrient cycles, and storing most of Earth's terrestrial carbon. However, soil communities and function are at risk because of increases in climatic and anthropogenic pressures (Montanarella et al., 2016). We are beginning to understand the influence of these pressures individually, but little is known about how soils respond to multiple concurrent stressors (Rillig et al., 2019). We must examine how the interactive effects of multiple pressures shape soil biota and their ecosystem functions to properly manage our soils and optimize agricultural practices.

Anthropogenic antibiotic use (i.e. human medicinal and veterinary use) is increasing while our understanding of their ecological consequences in soil systems remains limited (Wepking et al., 2017; Klein et al., 2018; Grenni et al., 2018). In 2017 alone, approximately 11 million kg of antibiotics were sold in the United States for livestock use (U.S. Food and Drug Administration 2018). Up to 90% of these administered antibiotics are excreted onto soils as un-metabolized and biologically active compounds (Kemper 2008; Ray et al., 2014) that can disrupt soil communities and the ecosystem processes they regulate (Wepking et al. 2017, 2019; Lucas et al., 2019). Monensin, the focal antibiotic in this study, is a prominent livestock antibiotic that helps prevent coccidiosis and bloat, but between 10 and 67% of the administered dose is excreted through waste with unknown ecosystem effects (EMA Committee for Medicinal Products for Veterinary Use, 2017). Antibiotic compounds change microbial community composition (Marti et al., 2013; Jia et al., 2014) and affect microbial networks by reducing the complexity and number of interactions (Gao et al., 2021). These community-level disruptions can lead to decreased microbial carbon use efficiency (i.e., efficiency with which microorganisms convert available organic substrates into stable, biosynthesized products, Geyer et al., 2016; Wepking et al., 2017). If microbial carbon use efficiency declines, antibiotic-laden soils may also have decreased organic matter formation

* Corresponding author. Cary Institute of Ecosystem Studies, Cary Institute of Ecosystem Studies, Millbrook, NY, 12545, USA.
E-mail addresses: lucasj@caryinstitute.org (J.M. Lucas), sone4469@vandals.uidaho.edu (B.S. Sone), dwhitmore@uidaho.edu (D. Whitmore), mstrickland@uidaho.edu (M.S. Strickland).

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and increased respiration (Schmidt et al., 2011; Bradford et al., 2013; Cortufo et al., 2013). Because most antibiotics given to livestock target bacteria, their introduction can alter ecosystem function by increasing fungal:bacterial ratios (Rousk et al., 2008; Wepking et al., 2019). In some instances, increased fungal dominance in soils is linked to increased nutrient cycling efficiency and plant growth (Bailey et al., 2002; Strickland et al., 2010), whereas other studies have found that increasing fungal dominance has little or negative effects on soil function (Strickland and Rousk 2010; Rousk and Frey 2015; Wepking et al., 2017).

Antibiotic introductions occur across a wide range of temperatures, which independently alter soil function. Higher temperatures increase soil CO_2 production, causing soils to store less C (Hagedorn et al., 2010) and increase nitrogen (N) mineralization rates (Melillo et al., 2011). Increasing temperatures can also exhaust labile C pools, which can briefly accelerate decomposition rates and change the microbial community consuming these resources (Dalias et al., 2001). Additionally, increased temperatures have been shown to increase mineralization of older soil organic matter, highlighting the range of responses nutrient pools in soils can have to rising temperatures (Ghee et al., 2013). As for biota, metabolic rates and microbial activity peak in summer months when temperatures are high. Higher metabolic rates and activity can decrease microbial carbon use efficiency (Allison et al., 2010; Zhou et al., 2013) and increases C loss from respiration (Lloyd and Taylor 1994; Lenton and Huntingford 2003; Sanderman et al., 2003). While changes in the microbial community can be difficult to predict, multiple studies demonstrate that temperature changes can lead to changes in composition, abundance, and network structure of microbiota in soils (Allison and Treseder 2008; Frey et al., 2008; Ding et al., 2015; Carrell et al., 2019; Jansson and Hofmockel 2020). Soil warming often promotes fungal growth (Zhang et al., 2005; Treseder et al., 2016), and fungal dominance is often associated with greater C storage (Adu and Oades 1978; Suberkropp and Weyers 1996). Whether the C gains from increased fungal growth can counteract C loss from increased respiration is poorly resolved. While variation in temperature is a natural regulator of soil function (Zhang et al., 2016; Durán et al., 2018), anthropogenic activity is warming soils beyond this natural variation making it one of the preeminent stressors affecting soils (Rillig et al., 2019).

While antibiotic additions and temperature independently influence soil communities, their interactions are unknown. The combined stress of warmer temperatures and antibiotic additions could lead to additive negative effects on microbial communities as both factors decrease microbial carbon use efficiency (Wepking et al., 2017; Bradford et al., 2008). Because few taxa are likely to withstand both stressors simultaneously, their interaction could cause microbial communities to homogenize and network connectivity to collapse (Ding et al., 2015; Gao et al., 2021). By contrast, higher temperatures may benefit soil microbiota increasing microbial activity and causing rapid degradation of antibiotic compounds, therefore limiting the time of exposure (Bradford et al., 2008; Allison et al., 2010; Zhou et al., 2013; Ray et al., 2017). Fungi in particular may benefit from warm environments with bactericidal antibiotics due to the decrease in competition, ideal temperature and pulse of nutrients from bacterial necromass (Zhang et al., 2005; Treseder et al., 2016; Ullah et al., 2021).

Here we tested the hypothesis that antibiotic addition and higher soil temperatures interact to disrupt soil communities and the functions they control. Specifically, we applied the common livestock broad spectrum antibacterial, Monensin, in high or low doses to soils across a temperature range indicative of seasonal variation and future warming projections: 15, 20 and 30 °C. We predicted that the addition of antibiotics disrupts soil communities in five distinct ways: (1) Antibiotics shift microbial communities by decreasing overall bacterial richness and abundance. (2) The decrease in abundance and richness will also collapse bacterial network structure through a reduction in network members and their associations. (3) Additionally, the collapse of bacterial networks and decrease in bacterial abundance will allow fungal communities to dominate in antibiotic-laden soils. (4) Antibiotics increase bioavailable C by increasing microbial necromass. (5) Antibiotics decrease microbial carbon use efficiency by increasing microbial mass-specific respiration. As for temperature, we predicted that higher temperatures would interact with antibiotic additions by further decreasing microbial carbon use efficiency and disrupting soil communities. Specifically, we predicted that high temperature environments exposed to antibiotics will be the most compositionally homogenous as few species will be adapted to both stressors. They will also have the lowest network connectivity and complexity, and the highest fungal dominance. We expect this to cause disruptions in nutrient stoichiometry as the shift toward fungal dominance should increase microbial C:N ratios, while warming will accelerate C loss from soils.

2. Materials and methods

2.1. Study site and experimental set-up

We collected the top 10 cm of soil from remnant Palouse prairie adjacent to the Paradise Ridge reserve in Moscow, Idaho (46°40′39.8"N, 116°58′36.1"W). Paradise Ridge is classified as a warm-summer Mediterranean climate (*CSB*), with a mean annual high temperature of 15.2 °C and low of 2.3 °C and receives and average an average of 688 mm of rain annually (Weatherbase). This site was chosen as it contained tall-grass prairie similar to livestock rangelands but has not had any prior direct antibiotic inputs or agricultural activity. Palouse prairie primarily consists of perennial bunchgrasses growing in mesic Xeroll soils of the Schumacher silt loam type (McNab and Avers 1994). Soils were passed through a 4.75 mm sieve and screened for fine roots. Microcosms contained 160 g dry weight equivalent soil and were added at the same bulk density as field soils. Soils were maintained at 65% water holding capacity (WHC) throughout the experiment. Prior to microcosm construction, 5 g of soil was stored at −80 °C to allow us to assess starting microbial communities.

We assigned microcosms to a high antibiotic, low antibiotic, or control (no antibiotic) treatment at one of 3 different temperatures, 15, 20 or 30 °C (3 x 3 factorial design). We chose the 15 °C temperature as it represents the average soil temperature in mid to late May at our collection site. This time period is when cattle would pasture graze though no livestock have grazed our preserved collection site (Washington State University's AgWeatherNet database). The 20 °C temperature represents the average soil temperature at our collection site throughout the summer season and our high soil temperature, 30 °C, represents a ~3 °C increase in peak summer soil temperature at our field site (Washington State University's AgWeatherNet database).

We had 5 replicate microcosms per treatment per temperature. We used the antibiotic Monensin (Molecular weight = 670.871 g/mol, pKa = 4.24, water solubility = 0.00633 mg/ml), a broad-spectrum ionophore antibiotic that inhibits bacterial cell wall transport (Wishart et al., 2006). Monensin (trade name "Rumensin"- manufactured by Elanco Animal Health) is produced by *Streptomyces cinnamonensis*, and is one of the most common antibiotics used in beef and dairy as it improves feed efficiency, treats and prevents coccidiosis, and is not used in human medicine (Richardson et al., 1976; Goodrich et al., 1984; Duffield et al. 1998, 2002). It is typically administered to cattle at all life stages (Elanco Animal Health, U.S. Food and Drug Administration 2018). Monensin is excreted primarily in cattle feces, though it can also enter environments through urine (Kim et al., 2019). The recommended dosage of Monensin is 50–360 mg/head/day. Average excretion of unmetabolized Monensin in cattle can range from <10% to 68% (WHO--FAO Joint Expert Committee on Food Additives 2009). Antibiotics were added at a high (0.02 mg/g) or low (0.002 mg/g) concentration, far below the potential active-product excretion rate from a single cow per day (Kim et al., 2019). We chose these low levels because they represent Monensin contamination levels in soils not directly exposed to manure inputs (Boxall et al., 2004) and is comparable to other studies of

ecotoxicology of antibiotics (Carlson et al., 2006; Hamscher et al., 2004). Because Monensin needs to be dissolved in an organic solvent to be applied aqueously, dosages were dissolved in 15 ml of EtOH, diluted with 15 ml of H_2O and then applied to soils. Control soils received 15 ml of EtOH and 15 ml of H_2O, with no added antibiotic. Antibiotic treatments were applied to soil microcosms on days 1, 8 and 16. We maintained microcosms for 21 total days, in part because 21 days has been shown to be sufficient time for relic, "dead" DNA to degrade (Sirois and Buckley 2019).

2.2. Soil respiration

We used a LI-8100A infrared gas analyzer (IRGA) CO_2/H_2O gas-multiplexer system (LI-COR Biosciences, Lincoln, Nebraska, USA) to monitor CO_2 concentrations in the headspace of each microcosm. The flux of CO_2 was determined during a 60 s measurement period, followed by a 30 s flush to avoid carry-over between microcosms. We determined CO_2 flux for each microcosm every other day throughout the duration of the experiment (21 total days). In order to calculate the metabolic quotient for CO_2 (qCO_2), we took the final measurement of flux CO_2 and divided it by the amount of C found in the active microbial biomass pool (outlined below) as detailed by Anderson and Domsch (1993).

2.3. Microbial community function and soil characteristics

We assessed active microbial biomass via substrate induced respiration ([SIR], Anderson and Domsch, 1973) following Strickland et al. (2010). We estimated microbial biomass C and N using a modified, chloroform-fumigation extraction method as described in Fierer and Schimel (2002, 2003). To better examine microbial physiology, we calculated SIR/FE-C to understand the glucose-responsive component of our total microbial biomass as outlined in Dilly (2006). Mineralizable-C (i.e., bioavailable C) was determined by measuring total CO_2 emissions over the course of a subsequent 60 d incubation, conducted after the 21 d experiment. Soils (8 g dry weight) were maintained at 65% water-holding capacity and 20°C, and we determined respiration across this time period using the same static incubation procedure as described by above for SIR measurements (Strickland et al., 2015); each soil sample was measured weekly for a total of 8 weeks. Cumulative mineralizable-C was estimated via integration. To estimate microbial C use efficiency (CUE), we calculated microbial mass-specific respiration as detailed by Wepking et al. (2017) and Bradford et al. (2008). A decrease in CUE is often associated with C loss from an ecosystem as organisms need to respire more CO_2 per unit biomass (Manzoni et al., 2012). We used the first measurement from our C-mineralization assay, which corresponds to soil respiration after 21 days of exposure to antibiotic and temperature treatments, divided by our estimate of active microbial biomass (i.e. SIR). We define a decrease in microbial efficiency as an increase in respiration per unit microbial biomass.

2.4. Microbial community composition, qPCR analysis of fungal:bacterial ratios and molecular ecological network analysis

Soil samples were taken from each microcosm at the end of the 21-day experiment and frozen at −80 °C until extraction. Microbial community composition was assessed using 16S/ITS metabarcoding protocol in accordance with the Earth Microbiome Project (www.earthmicrobiome.org, Caporaso et al., 2011). We assessed fungal-to-bacterial ratios from our extracted soil samples using quantitative PCR (qPCR) following Fierer et al. (2005) (Table S1). To examine the influence of antibiotics and temperature on bacterial interactions, we constructed phylogenetic microbial ecological networks (MENs) via a Random Matrix Theory (RMT) approach using the molecular ecological network analysis pipeline following the developers default settings (MENA, http://ieg2.ou.edu/MENA/; Deng et al., 2012; Zhou et al., 2010; Zhou et al., 2011). Detailed descriptions of our microbial sequencing techniques and network analyses are outlined in the supplementary methods.

2.5. Statistical analyses

We performed most analyses in the R statistical environment (R Core Team 2014), and the packages *mctoolsr* (http://leffj.github.io/mctoolsr/) and *phyloseq* were used to facilitate microbial sequencing data manipulation and analyses. We assessed the effects of treatments on substrate induced respiration (SIR), SIR/FE-C, qCO_2, soil pH, mass-specific respiration of soils, water holding capacity (WHC), the amount of "bioavailable" mineralized carbon (MinC), microbial alpha diversity metrics (Shannon (H) and richness; Shannon and Weaver 1949) and the relative abundance of taxonomic orders of microbiota (only those with >1.0% relative abundance) using linear models in R (function *lm* in base R and function *Anova* in car). We included antibiotic dosage and temperature and their interaction as fixed effects. We tested the significance of individual terms using F-tests and removed nonsignificant interaction terms. Because we monitored respiration across 3 weeks' time, we analyzed exponential flux CO_2 values using linear mixed-effects models (function *lmer* in the R package *lme4*, Bates et al., 2015). Antibiotic dosage and temperature and their interactions were included as fixed effects, and we accounted for repeated measures covariance with individual jar identifier as the subject and sampling time point as the repeated measure as outlined by Bates et al. (2015). We performed model reduction and report Chi-squared and P-values for our respired CO_2 measurements. Significance was determined when P-values were $\leq$ to 0.05. When significant, we tested for pairwise differences using likelihood ratio tests. We tested for normality using Shapiro–Wilk tests and we examined residuals to confirm appropriate model fit. For fungal:bacterial ratios, we used linear mixed effect models to account for PCR plate effects. Specifically, we included antibiotic dosage and temperature and their interaction as fixed effects, and PCR plate ID as a random effect. We evaluated the fixed effects using likelihood ratio tests.

We compared overall microbial community structure using the software Primer (Ver. 7.0.13) and R (package *vegan*, Oksanen et al., 2020). We square-root transformed the microbial community data before calculating Bray-Curtis dissimilarity. We used the square-root transformed Bray-Curtis community distance matrices to generate ordinations (non-metric multidimensional scaling or NMDS) for bacteria and fungi. We used PERMANOVA to compare community composition across dosages and temperature, and their interactions (Anderson 2001; Anderson et al., 2008). We also compared beta diversity across our samples using PERMDISP tests (Anderson 2006; Anderson et al., 2008). PERMDISP tests calculate within group dissimilarity in community composition and then compares the magnitude of dissimilarity among each group (9999 permutations). To confirm these results and examine how relative abundance may influence significance, we performed follow-up analyses using the Jaccard index on our presence-absence distance matrix. All analyses produced similar results, and therefore we present the Bray-Curtis values in text but provide values for both analyses in Table S2.

3. Results

3.1. Temperature and antibiotic additions decrease microbial biomass C:N ratios and shift DOC and extractable N

Temperature and antibiotic additions interacted to alter microbial biomass C:N ratios in microcosm soils ($F_{4,25} = 2.79$ $p = 0.02$; Fig. 1a). Soils incubated at 30 °C had lower microbial C:N ratios when antibiotics were added, with no difference between high or low antibiotic additions. However, microbial C:N ratios of soils incubated at 15 and 20 °C were not influenced by antibiotic additions. The change in microbial C:N ratios was driven primarily by changes in microbial biomass N (Fig. S1). Antibiotic additions interacted with temperature to increase microbial

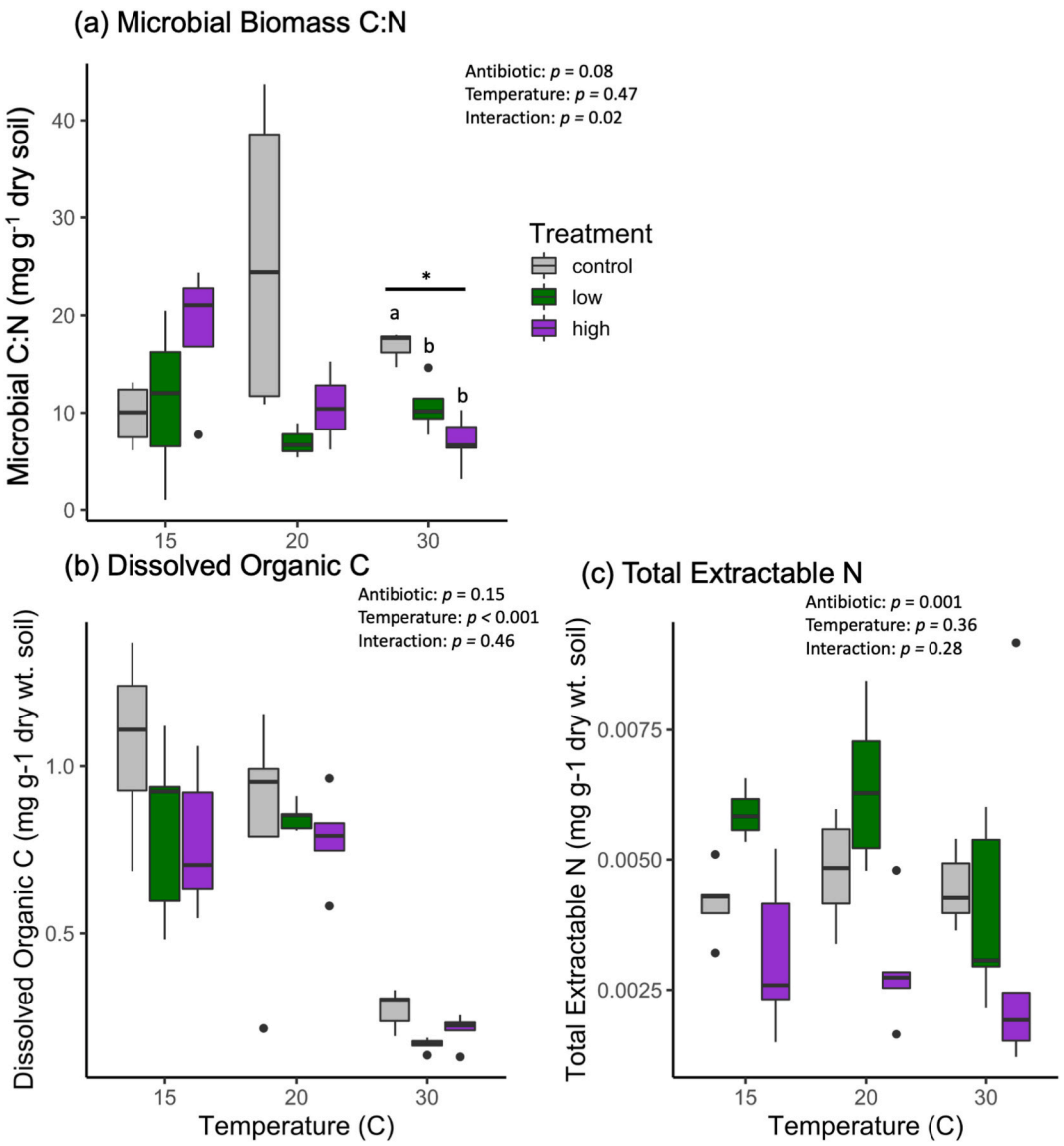

Fig. 1. The response of microbial biomass C:N (a), dissolved organic C (DOC) (b) and total N (c) levels in microcosms across antibiotic additions and temperature treatments. Antibiotics and temperature interacted to decrease microbial C:N at 15 and 30 °C ($F_{4,36} = 3.75$, $p = 0.01$). Dissolved organic C increased when microcosms were kept at 30 °C compared to soils at 20 °C ($F_{2,42} = 4.13$, $p = 0.02$), but was not influenced by antibiotic additions ($F_{2,42} = 0.27$, $p = 0.76$) nor was there an interactive effect ($F_{4,36} = 1.10$, $p = 0.37$). Antibiotic additions and temperature interacted to influence total N levels ($F_{4,36} = 3.47$, $p = 0.02$). Antibiotic additions had higher N levels than controls, but this effect was most exaggerated when the microcosms were kept at 30 °C. Boxes represent 95% confidence intervals, and all data points are represented by black points. Bars denote minimum and maximum values excluding outliers, letters denote significant differences across antibiotic treatments at each temperature.

biomass N at 30 °C (Fig. S1a). Microbial biomass C was greater at 30 °C than at 20 °C but there was no interactive effect of temperature and antibiotics, or antibiotic effect on microbial biomass C levels (Fig. S1b).

We also found that the dissolved organic C (DOC) and total extractable N (TN) were influenced by our treatments. Dissolved organic C decreased as temperatures increased, but was not affected by antibiotic treatments (Temperature: $F_{2,40} = 53.21$, $p < 0.001$; Antibiotics: $F_{2,40} = 1.98$, $p = 0.15$; Fig. 1b). In contrast, TN was influenced by antibiotic additions, but was not affected by temperature (Temperature: $F_{2,40} = 1.05$, $p = 0.36$; Antibiotics: $F_{2,40} = 7.87$, $p = 0.001$; Fig. 1c). Interestingly, TN was significantly decreased in high antibiotic additions environments compared to low antibiotic additions (pairwise: $t = 3.94$, $p = 0.001$), and trended towards a decreased compared to control soils, though this pattern was not statistically significant (pairwise: $t = -2.35$; $p = 0.06$; Fig. 1c). We did not see any interactions between temperature and antibiotics for either DOC or TN (DOC Interaction: $F_{4,36} = 0.93$, $p = 0.46$; TN Interaction: $F_{4,36} = 1.34$, $p = 0.28$).

3.2. *Antibiotic treatments, not temperature, increase bioavailable C post incubation*

High and low antibiotic additions increased bioavailable C (as determined by mineralizable C) by 17.3% and 13.5%, respectively, as compared to controls ($F_{2,40} = 4.38$, $p = 0.02$; Fig. S2e). High dosages were associated with an average increase of 20.89 mg of bioavailable C to each microcosm, while low dosages were associated with an average increase of 16.34 mg of C. The observed increase in bioavailable C cannot be explained solely by the addition of C from antibiotic treatments, as the amount of C added (from Monensin treatments) represents < 0.01% of the observed increase (0.039 mg in high dosages and 0.0039 mg in low dosages). Temperature did not have a significant effect on bioavailable C ($F_{2,40} = 1.15$, $p = 0.33$; Fig. S2f), nor was there an

antibiotic treatment by temperature interaction ($F_{4,36} = 0.57$, $p = 0.74$).

3.3. Antibiotics decrease microbial efficiency, while temperature increases microbial respiration

Antibiotics decreased microbial efficiency as determined by mass-specific respiration, (Antibiotics: $F_{2,40} = 8.07$, $p = 0.001$), but neither temperature nor its interaction with antibiotics changed microbial efficiency (Temperature: $F_{2,40} = 2.41$, $p = 0.10$; Interaction: $F_{4,36} = 0.94$, $p = 0.46$; Fig. 2). Low and high dosages of antibiotics increased mass-specific respiration by 36.61% and 54.68%, respectively, but the dosages did not differ from each other (pairwise $p > 0.4$; Fig. 2). While total CO_2 respiration rates stayed consistent across antibiotic additions (Chi-squared = 0.05, d.f. = 2, p = 0.97; Fig. S2c), microbial biomass decreased by 21.2% and 15.9% in high and low dose microcosms, respectively ($F_{2,42} = 7.62$, $p = 0.001$; Fig. S2a), contributing to the drop in microbial efficiency.

Although temperature had no effect on mass-specific respiration, it did influence respiration rates. Total CO_2 respiration increased by 69.0% and 255.2% when microcosms were kept at 20 and 30 °C as compared to 15 °C (Chi-square = 17.163, d.f. = 2, $p < 0.001$, Fig. S2d). Temperature had no effect on microbial biomass ($F_{1,41} = 1.51$, $p = 0.23$; Fig. S2b). There was also no interaction between antibiotic treatment and temperature for microbial biomass ($F_{2,39} = 0.27$, $p = 0.76$) or cumulative CO_2 respired ($F_{2,264} = 0.53$, $p = 0.59$). Additionally, there was no effect of treatment ($F_{2,39} = 0.08$, $p = 0.92$) or temperature ($F_{2,39} = 2.92$, $p = 0.07$) on SIR/FE-C, a measure of the glucose-responsive component of active microbial biomass. There was also no effect of antibiotics ($F_{2,32} = 0.1$, $p = 0.91$) or temperature ($F_{2,32} = 3.02$, $p = 0.07$) on the metabolic quotient for CO_2 (qCO_2).

3.4. Antibiotics and temperature interact to increase fungal:bacterial ratios and disrupt bacterial, but not fungal community composition

As predicted, temperature and antibiotics interacted to increase fungal:bacterial ratios ($X^2 = 14.27$, d.f. = 4, $p = 0.006$; Fig. 3a). Specifically, the addition of high levels of antibiotic caused fungal:bacterial ratios to be greater than control environments, and this effect was exacerbated at higher temperatures. Similarly, high antibiotic additions also have greater fungal:bacterial ratios as compared to low antibiotic addition treatments at 15 and 30 °C, but not at 20 °C. The change in fungal:bacterial ratios was driven by a decrease in 16S rRNA copy number when high doses of antibiotics were added at 20 °C, a consistent fungal copy number across treatments at 15 and 20 °C, and an increase in fungal copy number at 30 °C in antibiotic treatments (Fig. S3).

High doses of antibiotics decreased bacterial Shannon (H) diversity ($F_{2,42} = 6.73$, $p = 0.002$), but not bacterial richness ($F_{2,42} = 2.65$, $p = 0.08$), while temperature had no effect on either metric (Shannon (H): $F_{2,42} = 0.63$, $p = 0.45$; richness: $F_{2,42} = 0.43$, $p = 0.66$; Fig. S4). Soils receiving high doses of antibiotics had lower diversity compared to controls (pairwise test: $p = 0.04$), while soils receiving low dosages did not have lower diversity (pairwise test: $p = 0.08$). In contrast, temperature influenced both fungal diversity ($F_{2,42} = 39.39$, $p < 0.001$) and richness ($F_{2,42} = 26.0$, $p < 0.001$), but antibiotic additions did not (Shannon (H): $F_{2,42} = 0.94$, $p = 0.39$; richness: $F_{2,42} = 0.47$, $p = 0.63$; Fig. S5). Specifically, when soils were incubated at 30 °C, fungal diversity and richness decreased as compared to both 15 and 20 °C incubations (pairwise tests: $p < 0.001$).

Antibiotics and temperature interacted to shift bacterial community composition (PERMANOVA interaction: pseudo-$F_{4,36} = 1.44$, $p < 0.001$, NMDS stress = 0.13; Fig. 3b). Antibiotic additions led to significantly different communities as compared to controls, but high and low antibiotic dosages did not differ from each other (Table S2; Fig. S6a) and each temperature had a distinct bacterial community (Pairwise comparison: $p < 0.001$, Table S2; Fig. S6). Bacterial beta diversity, as determined by PERMDISP, did not differ across antibiotic treatments (PERMDISP: pseudo-$F_{2,42} = 0.19$, $P = 0.84$) or temperatures (PERMDISP: pseudo-$F_{2,42} = 0.32$, $p = 0.76$).

Temperature had a strong effect on fungal community composition and beta diversity (PERMANOVA temperature: pseudo- $F_{2,42} = 4.47$, $p < 0.001$, stress = 0.14; PERMDISP: pseudo-$F_{2,42} = 13.65$, $p < 0.001$, Fig. 3c), with communities at 30 °C having distinct and more homogeneous communities as compared to communities at 15 and 20 °C (Fig. S6f). Fungal community composition was unaffected by antibiotic additions and there was no temperature by antibiotic addition interaction (PERMANOVA antibiotic: pseudo-$F_{2,42} = 1.08$, $p = 0.19$;

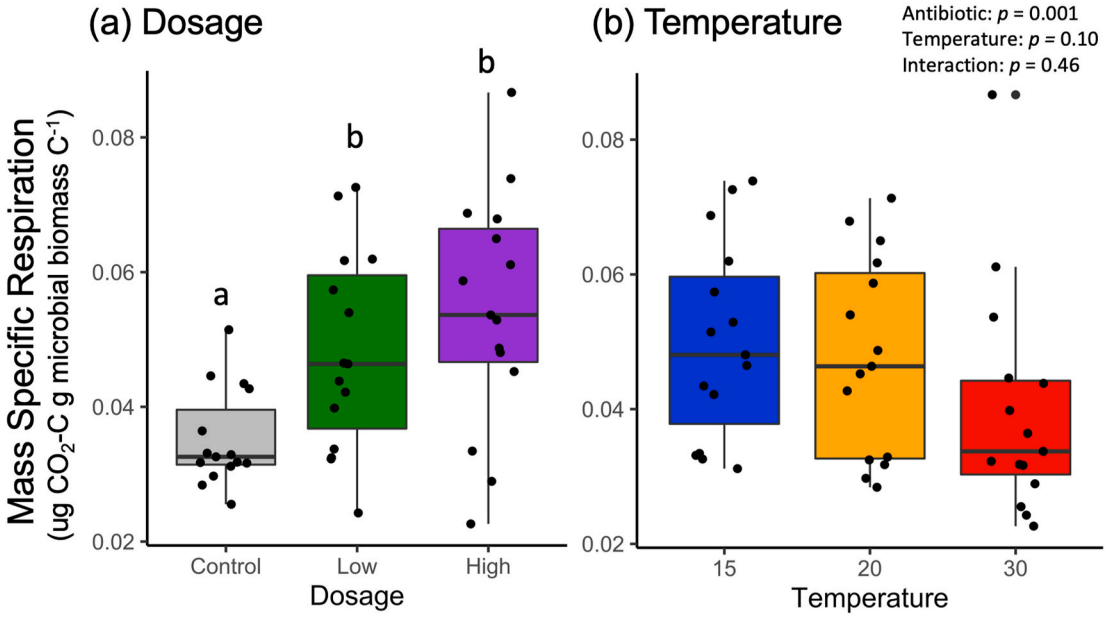

Fig. 2. The response of microcosm microbial mass specific respiration to antibiotic additions (a) and temperature (b). Antibiotic additions increased mass-specific respiration, while temperature had no effect (Antibiotics: $F_{2,42} = 7.05$, $p = 0.002$; Temperature: $F_{2,42} = 1.71$, $p = 0.19$). Boxes represent 95% confidence intervals, and all data points are represented by black points. Bars denote minimum and maximum values excluding outliers, letters denote significant differences across treatments.

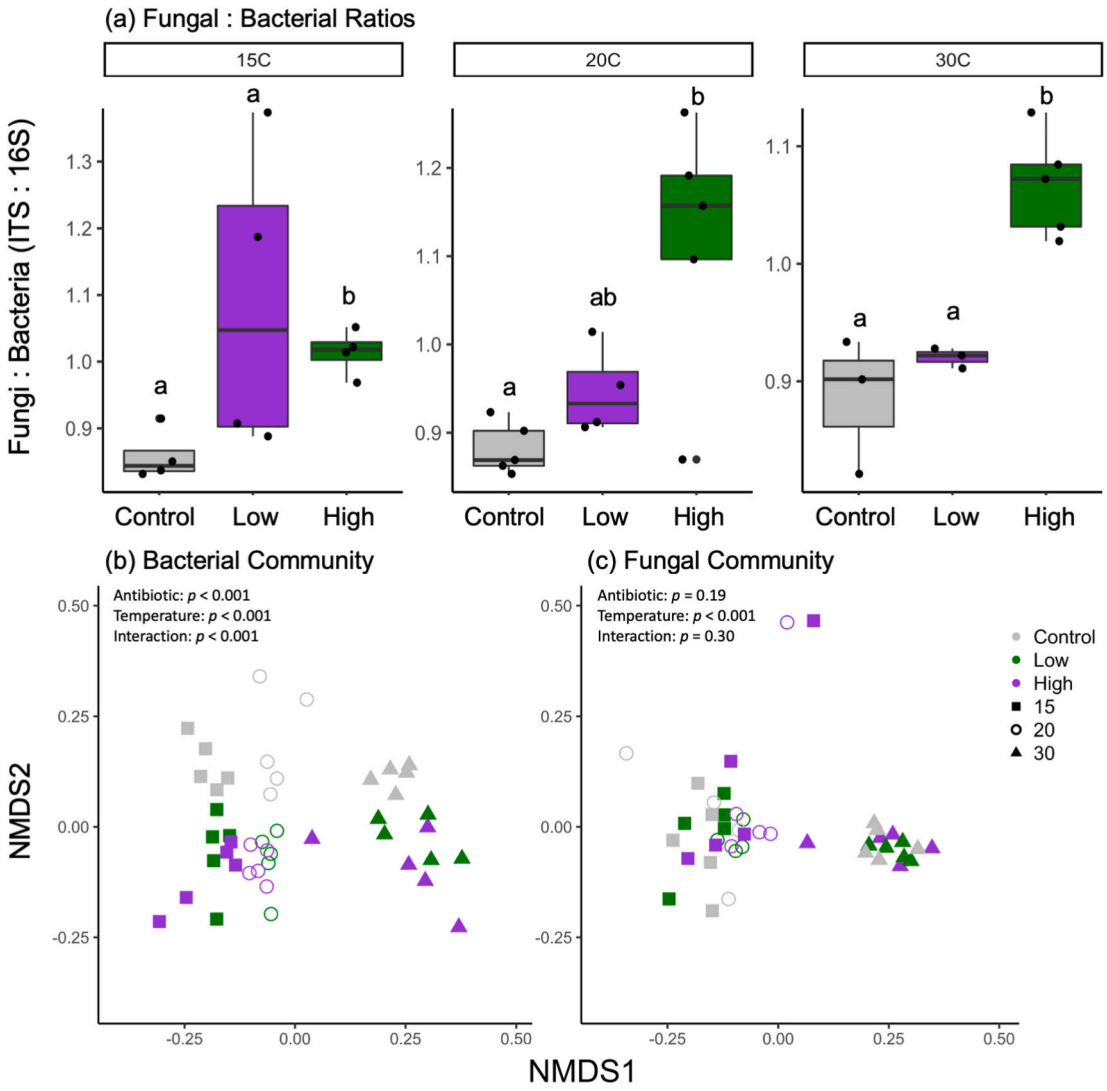

Fig. 3. Fungal:bacterial ratios (a), and bacterial (b) and fungal (c) community composition associated with antibiotic additions and temperature treatments. Panel (a) demonstrates the ratio of fungal ITS log copy number to 16S log copy number across each temperature. Panels (b) and (c) are ordination plots showing non-metric multidimensional scaling of Bray-Curtis distances between microbial communities. Colors denote antibiotic treatments (grey-control, green-low dose, purple-high dose) and shapes represent temperatures (square-10 °C, circle-20 °C, triangle- 30 °C). An interaction between temperature and antibiotic affected bacterial communities. Fungal communities only responded to temperature changes. Boxes represent 95% confidence intervals, and all data points are represented by black points. Bars denote minimum and maximum values excluding outliers, letters denote significant differences across treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

interaction: pseudo-$F_{4,36} = 1.03$, p = 0.30; Fig. S6). Fungal beta diversity did not change with the addition of antibiotics (PERMDISP: pseudo-$F_{2,42} = 0.56$, p = 0.58).

3.5. *Increasing temperature and antibiotic additions collapse bacterial networks*

Antibiotics and temperature altered bacterial network structure. Each combination of treatment and temperature had significantly different network structure using the same similarity threshold (Table 1, Fig. S7). Overall network average path length and clustering coefficient were significantly larger than the corresponding random networks, which suggests that all empirically produced networks had 'small world' properties (i.e., average distance between nodes is short, indicating closely related ASVs) (Table 1, Deng et al., 2012).

Increasing temperatures and antibiotic additions caused bacterial network complexity to decrease. In control soils, the network complexity (i.e., number of nodes and interactions) increased with increasing temperature. However, when antibiotics were added to the soil, node numbers and interactions decreased as temperatures increased (Table 1). Specifically, in low antibiotic environments, the number of interactions (i.e., links) decreased by 38.92% at 20 °C and 46.24% at 30 °C, as compared to 15 °C soils. High antibiotic soils consistently had the fewest number of interactions, as well as decreased node numbers compared to the controls at each temperature (Table 1).

Antibiotics and temperature also altered patterns of bacterial co-occurrence (i.e., negative or positive pairwise associations) (Table 1, Fig. S7). At 15 °C, control environments were dominated by negative associations (only 37.77% positive associations), while low antibiotic networks were comprised of 75.18% positive associations and high antibiotics networks were 53% positive. However, at 20 °C, this pattern changed and the percent of negative interactions in control environments was 27.98% and 18.38% lower than low and high antibiotic networks, respectively. At 30 °C, control soils again had more negative associations than antibiotic soils, but the majority of the interactions were still positive (53.81%).

Table 1
Topological properties of the empirical molecular ecological networks (MENs) of bacterial communities across temperatures and antibiotic treatments, and their associated random MENs.

Network Indexes		Empirical Networks								Random Networks		
		No. of original ASVs	Similarity threshold (st)	Total nodes	Total links	R^2 of power-law	Percent positive interactions	Average degree (avgK)	Average clustering coefficient (avgCC)	Average path distance (GD)	Modularity (M)	Average clustering coefficient (avgCC)	Average path distance (GD)	Modularity (M)
15	**Control**	3852	0.93	252	1051	0.671	37.77	8.341	0.4^{a*}	6.268^{a*}	0.642^{a*}	0.094 ± 0.009	2.873 ± 0.025	0.282 ± 0.006
	Low	3852	0.93	283	1624	0.622	75.18	11.477	0.438^{b*}	4.04^{b*}	0.62^{b*}	0.094 ± 0.006	2.649 ± 0.017	0.234 ± 0.005
	High	3852	0.93	235	834	0.663	53	7.098	0.467^{c*}	5.105^{c*}	0.693^{c*}	0.062 ± 0.007	2.947 ± 0.020	0.326 ± 0.006
20	**Control**	3852	0.93	305	1521	0.485	62.39	9.974	0.498^{d*}	5.327^{d*}	0.73^{d*}	0.061 ± 0.005	2.784 ± 0.015	0.264 ± 0.005
	Low	3852	0.93	254	992	0.699	47.78	7.811	0.424^{e*}	5.717^{e*}	0.721^{e*}	0.061 ± 0.006	2.914 ± 0.019	0.307 ± 0.006
	High	3852	0.93	264	881	0.593	53.92	6.674	0.398^{f*}	5.592^{f*}	0.784^{f*}	0.039 ± 0.005	3.141 ± 0.020	0.346 ± 0.006
30	**Control**	3852	0.93	353	1498	0.315	53.81	8.487	0.429^{g*}	4.98^{g*}	0.736^{g*}	0.034 ± 0.004	2.975 ± 0.008	0.301 ± 0.005
	Low	3852	0.93	242	873	0.638	71.36	7.215	0.366^{h*}	5.081^{h*}	0.696^{h*}	0.050 ± 0.007	2.996 ± 0.020	0.324 ± 0.006
	High	3852	0.93	222	667	0.539	61.62	6.009	0.37^{i*}	5.05^{i*}	0.718^{i*}	0.039 ± 0.008	3.205 ± 0.027	0.370 ± 0.008

[a] Superscript letters represent significant differences between treatments.
* Asterix represent significant differences between empirical and random networks for each individual treatment and temperature.

The taxonomic composition of the nodes also shifted across temperatures and antibiotic treatments (Figure S8). Nodes belonging to Actinobacteria and Firmicutes were more abundant in control environments regardless of temperature. In contrast, Proteobacteria and Verrucomicrobia nodes were more abundant in antibiotic-laden environments than controls. This was particularly pronounced in the high antibiotic addition treatments. Nodes belonging to the phyla Elusimicrobia were only present at 30 °C, and they appeared in each antibiotic treatment.

4. Discussion

Antibiotics and higher temperatures disrupted soil microbial communities and function. When antibiotics were combined with higher temperatures, bacterial network structure collapsed, allowing for increased fungal dominance and decreased microbial C:N. Independent of temperature, antibiotics decreased bacterial diversity, abundance, total extractable N and microbial efficiency, while increasing bioavailable C. Higher temperatures independently homogenized fungal community composition, decreased dissolved organic C and increased soil respiration rates. These results demonstrate that soil response depends on the context of the stressor and that unique shifts in composition and function will occur as soils encounter multiple concurrent stressors.

4.1. Antibiotics and rising temperatures interact to disrupt soil communities and stoichiometry

The combined effects of antibiotics and temperature fundamentally restructured soil microbial communities with implications for their future functionality and resilience. The collapse of bacterial networks can have lasting implications, as lower network connectivity has been linked to decreased ecosystem stability and resilience (Olesen et al., 2007; Stouffer and Bascompte 2011; Coyte et al., 2015) and is indicative of increased land-use intensity (Creamer et al., 2016). Additionally, we saw a rise in the percent positive associations in high antibiotic environments as temperatures increased. This is also indicative of diminished ecosystem stability as positive associations represent high niche overlap and increased organismal interdependence (Hernandez et al., 2021). While antibiotic treatments generally had decreased connectivity and membership compared to controls, there was one exception: low dosage environments at 15 °C. These soils had a high number of associations, though the vast majority were positive (75.18%). It is possible that the introduction of low levels of antibacterials benefited resistant bacteria by decreasing competition, but these organisms were not able to handle the additional stress of warming. This suggests that soil microbial networks are potentially resistant to low intensity perturbations, but communities collapse if the intensity and number of stressors increases.

Indirect effects on fungal composition and biomass likely caused the observed changes in microbial stoichiometry. The collapse of bacterial communities allowed fungi to increase in dominance, likely due to a decrease in competition and an increase in nutrient availability from bacterial necromass. Fungal dominance was exaggerated under increased temperatures, indicating that bacteria were more sensitive to temperature changes. Interestingly, antibiotic additions caused microbial biomass C:N levels to drop at 30 °C. This can be attributed by the increase in copiotrophic fungi (i.e., Ascomycota and Murocomycota) that have lower C:N ratios and likely gained a competitive advantage from the pulse of N-rich bacterial necromass (Fierer et al., 2012; Ho et al., 2017; Zhang and Elser 2017).

4.2. Antibiotics independently caused a decrease in microbial efficiency

Both bacterial and fungal responses to antibiotics likely contributed to the changes in soil carbon cycling. As predicted, antibiotics decreased microbial efficiency through a reduction in active microbial biomass.

Despite this drop in active biomass, soils exposed to antibiotics maintained high levels of CO_2 respiration throughout the 21-day experiment. One potential explanation for the sustained rate of CO_2 respiration is that the remaining bacterial organisms are resistant to antibiotics. Antibiotic resistance is metabolically costly (Zampieri et al., 2017), often accompanied by increased maintenance demands and reduced rates of bacterial replication (Melnyk et al., 2015; Zampieri et al., 2017). This result is also consistent with previous work demonstrating an increase in antibiotic resistant genes and mass-specific respiration when antibiotics are added to soil environments (Wepking et al., 2017). The increase in fungal dominance could have also contributed to decreased microbial efficiency in antibiotic environments (Bailey et al., 2002; Rousk and Frey 2015; Wepking et al., 2019). Though fungal-dominated ecosystems are generally considered to have higher C-use efficiency (Six et al., 2006; Keiblinger et al., 2010), our study demonstrates that antibiotic-induced shifts to fungal dominance do not support this supposition. Sustained decreases in microbial efficiency can slow the incorporation of microbially derived C into soils (Cotrufo et al., 2013), emphasizing the negative effect antibiotics can have on soil C storage.

The production of bacterial necromass by antibiotics could contribute to sustained CO_2 efflux and influence SOM formation. Bacterial necromass could increase decomposition rates and provide the observed pulse of bioavailable C. Ullah et al. (2021) reported similar patterns; biocide application increased respired CO_2 above levels that could be attributed to biocide nutrient enrichment alone. Traditionally, microbial necromass is considered an important source of soil organic matter (SOM); contributing up to 50% of all SOM (Miltner et al., 2012; Lian et al., 2019). Compared to plant detritus, C derived from microbial biomass remains in soil for longer periods of time (Sierra et al., 2018). While it is possible that the introduction of antibiotic compounds may provide an avenue to promote SOM formation, if the remaining microbial population has lower C-use efficiency (CUE) – as we observed here – then the ability for these soils to stabilize C may be offset (Manzoni et al., 2012).

4.3. Higher temperatures homogenized fungi and increased C loss

Higher temperatures simplified microbial communities with implications for soil carbon cycling. As predicted, warming homogenized fungal communities, increased CO_2 respiration and decreased DOC. This is consistent with previous work demonstrating that warming leads to an increase in soil C loss to atmospheric pools (Kirschbaum 1995; Schindlbacher et al., 2009; Chen et al., 2020). The homogenization of fungal communities at 30 °C aligns with previous studies demonstrating a loss in richness and beta diversity with ecosystem warming (Monchamp et al., 2018; Colossi Brustolin et al., 2019; Harrison 2020). When ecosystems lose their biodiversity their stability and resilience diminish (Hautier et al., 2015; Pennekamp et al., 2018) and C cycling processes are disrupted (De Graaff et al., 2015). This suggests that preserving microbial community diversity as soils warm is necessary to maintain full functionality of the soil C cycle.

4.4. Agricultural antibiotic use may disrupt soil nutrient cycling and C storage

Our findings have broad implications for agricultural soils. The antibiotic used in this study, Monensin, is one of the most administered livestock antibiotics (U.S. Food and Drug Administration 2018), partially because it is not used to treat human diseases (World Health Organization, 2017; U.S. Food and Drug Administration 2018). The WHO has deemed Monensin as “not medically important” and therefore not a threat to human health, but relevant trials do not consider its effects on ecosystems. Here we demonstrate that Monensin disrupts soil ecosystem function, and the magnitude of these effects are larger than those reported for other antibiotic compounds (Lucas et al., 2019; Wepking et al., 2019). The effects of Monensin on bacterial composition and microbial C:N were not dosage dependent, suggesting that even small amounts of these compounds have the potential to disrupt soil ecosystems. These findings suggest that the recent expansion in Monensin use could be profoundly altering soil function and carbon cycling. Field studies are needed to quantify the landscape-scale impacts of this common agricultural practice.

These results highlight additional avenues for future research. Because antibiotic additions often occur through excretion, simultaneous introductions of antibiotics and waste microbiomes are needed to fully understand the influence of agricultural antibiotic use. Future studies should also evaluate the consequences of repeated antibiotic additions from multiple cows. We simulated an acute introduction of antibiotics from the manure of a single cow, whereas antibiotic introductions in situ occur repeatedly from many cows. Finally, this study was performed over 21 days as it provides sufficient time for relic DNA to exit the ecosystem (Sirois and Buckley 2019), allowing us to observe changes in microbial community composition. However, additional studies should examine the longer-term consequences of interacting stressors in both field and controlled settings. Addressing these knowledge gaps is key to understanding the true consequences of Monensin additions across temperatures.

5. Conclusions

The combined effects of antibiotics and rising temperatures changed microbial community composition, decreased bacterial network structure, and altered nutrient stoichiometry with long-term implications for soil C storage. This suggests that when soils encounter multiple stressors, their ability to maintain diverse communities and function may be diminished. However, we also found that stressors can have differing and independent effects on soil ecosystems suggesting that there is not a single indicator for soil stress. This is an important result, as it emphasizes the need to examine multiple indicators of soil health to understand the influence of anthropogenic and environmental stressors. We also found that both independent and interactive effects of temperature and antibiotics will lead to decreases in soil ecosystem stability and resilience. This emphasizes that environmental variability needs to be considered when attempting to preserve soil health and C storage.

Data accessibility

All data acquired in the experiment are given in the main text and supporting information. Additionally, all data is archived at Lucas, Jane; Sone, Bronte; Whitmore, Dana; Strickland, Michael (2021): Antibiotics and temperature interact to disrupt soil communities and nutrient cycling. Figshare. Dataset. https://doi.org/10.6084/m9.figshare.14183042>.

Author contribution

JML conceived of the idea and together with MS designed the experiment. JML, BS and DH carried out data analysis and prepared the manuscript. MS contributed to the writing of the manuscript. JML, BS and DH conducted field work, collected samples, and ran analytical assessments. All authors contributed critically to the drafts and gave final approval for publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2021.108437.

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