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Comparison of Metals and Tetracycline as Selective Agents for Development of Tetracycline Resistant Bacterial Communities in Agricultural Soil

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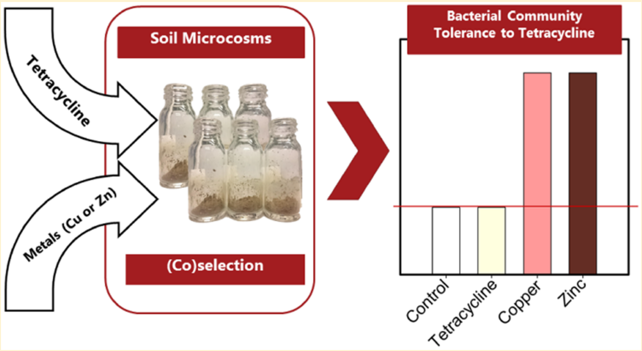
S *Supporting Information*

ABSTRACT: Environmental selection of antibiotic resistance may be caused by either antibiotic residues or coselecting agents. Using a strictly controlled experimental design, we compared the ability of metals (Cu or Zn) and tetracycline to (co)select for tetracycline resistance in bacterial communities. Soil microcosms were established by amending agricultural soil with known levels of Cu, Zn, or tetracycline known to represent commonly used metals and antibiotics for pig farming. Soil bacterial growth dynamics and bacterial community-level tetracycline resistance were determined using the [^{3}H]leucine incorporation technique, whereas soil Cu, Zn, and tetracycline exposure were quantified by a panel of whole-cell bacterial bioreporters. Tetracycline resistance increased significantly in soils containing environmentally relevant levels of Cu (≥365 mg kg^{-1}) and Zn (≥264 mg kg^{-1}) but not in soil spiked with unrealistically high levels of tetracycline (up to 100 mg kg^{-1}). These observations were consistent with bioreporter data showing that metals remained bioavailable, whereas tetracycline was only transiently bioavailable. Community-level tetracycline resistance was correlated to the initial toxicant-induced inhibition of bacterial growth. In conclusion, our study demonstrates that toxic metals in some cases may exert a stronger selection pressure for environmental selection of resistance to an antibiotic than the specific antibiotic itself.



INTRODUCTION

The ongoing dissemination of antibiotic resistance in pathogenic bacteria constitutes a global challenge to mankind, and an environmental dimension to the antibiotic resistance crisis is increasingly being recognized.[1−3] In this regard, agricultural soils are important as they provide rich sources of novel types of antibiotic resistance determinants yet-to-be recruited by human pathogens. Hence, antibiotic resistance genes (ARGs) are common in soil that is known to harbor a plethora of antibiotic producers and degraders,[4,5] and extensive transfer of ARGs between bacteria in soil and pathogenic bacteria in humans has been indicated.[6,7]

There is growing evidence that the human use of antibiotics has caused a vast enrichment of ARGs in agricultural soils.[8−10] Less is known about the relative importance of the underlying processes responsible for this expansion of the soil bacterial resistome. These processes may include dispersal of antibiotic resistant bacteria or ARGs from animals to soil via manure, horizontal gene transfer, and environmental selection of antibiotic resistance caused by antibiotic residues or other selecting agents such as toxic metals that can coselect for antibiotic resistance.[2,8] Various coselection mechanisms (i.e., coresistance, cross-resistance, and coregulation) have been known for decades, but only little is known about their relative importance in soil.[11]

In order to perform a human health risk assessment for the environmental development and transfer of antibiotic resistance in agricultural soil, the individual factors responsible for the dissemination of antibiotic resistance will need to be distinguished.[2] This implies that we need to predict the degree to which selection of antibiotic resistance is taking place in agricultural soils and to identify the selective agents. Widespread selection of antibiotic resistance above background levels in agricultural soils requires that antibiotic resistant bacteria have a selective advantage over antibiotic susceptible bacteria; in other words, a selection pressure must be present. Both antibiotics and metals are used in animal farming, where

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copper (Cu) and zinc (Zn) are widely used to complement antibiotics in order to promote animal growth and alleviate postweaning diarrhea.[12] Manure-amended soils will thus contain elevated levels not only of antibiotic residues but also of Cu and Zn making it relevant to compare their abilities to select for antibiotic resistance. As opposed to antibiotics, Cu and Zn are elements and thus persist in soil where they consequently accumulate in agricultural soils receiving animal manure.[13] These metals may also accumulate to toxic levels due to the use of Cu-based pesticides or industrial pollution.[14−16] In contrast, antibiotics probably only rarely accumulate to toxic levels in agricultural soils,[17,18] and metals are thus more likely than antibiotics to exert toxic effects in agricultural soils.

To the best of our knowledge, no previous controlled studies have directly compared the ability of metals and antibiotics to (co)select for antibiotic resistance. Hence, we aimed to directly compare the abilities of Cu, Zn, and tetracycline to (co)select for tetracycline resistance in soil bacterial communities operationally defined as an increased ability of extracted soil bacteria to grow in the presence of tetracycline. To this end, we performed strictly controlled experiments employing soil microcosms spiked with known amounts of Cu, Zn, or tetracycline. Importantly, manure was not included as part of the experimental design in an attempt to fully resolve causal relationships. Tetracycline was chosen as the model antibiotic as tetracyclines represent one of the most commonly used classes of veterinary antibiotics[19] and as it is listed by the WHO as a critically important antibiotic.[20] Comparison of selection pressures for tetracycline resistance was carried out by a cultivation-independent pollution-induced community tolerance (PICT) approach with broad bacterial community coverage and no requirement for a priori knowledge on resistance mechanisms in individual members of the soil bacterial community. PICT analysis was performed after a selection phase of 9 weeks and was complemented by studies of in situ bacterial growth dynamics and determination of Cu, Zn, and tetracycline bioavailability over time using an array of whole-cell bacterial bioreporters.

MATERIALS AND METHODS

Soil Sampling and Experimental Setup. An agricultural soil (sandy loam) was representatively sampled by multiple coring in February, 2014, Taastrup, Denmark from the unfertilized control plots of a field experiment that was initiated in 2002 at an experimental farm of the University of Copenhagen. Detailed physicochemical and microbiological soil characterization are available from multiple investigations of the same soil plots.[21−23] Total Cu and Zn contents were 15 and 57 mg kg^{-1}, respectively. After 4 weeks of storage at 4 °C, the soil was air-dried for 5 h at 22 °C, sieved (2 mm mesh size), and preincubated (5 days) at 15 °C in the dark. Replicated microcosms ($n = 3$) were subsequently set up with 50 g (dry wt) of soil in 324 mL serum vials sealed with Parafilm. Different concentrations of $CuSO_4$ (CAS no: 7758-99-8, Merck), $ZnSO_4$ (CAS no: 7446-20-0, Merck), or tetracycline (tetracycline hydrochloride, CAS no: 64-75-5, Sigma) were spiked in soil by pipetting droplets (2.5 mL), resulting in a final soil moisture of 16.0% (dry wt). Soils were repeatedly gently mixed during the spiking process to ensure homogeneous distribution of the contaminants. Using freshly prepared solutions, tetracycline was amended in soil to increase nominal concentrations by 0, 1, 10, or 100 mg kg^{-1} dry soil. Cu was added to increase total soil Cu concentrations by 0, 33.3, 100, 333, or 1000 mg kg^{-1} dry soil, whereas Zn was added to increase total soil Zn concentrations by 0, 165, 500, 1650, or 5000 mg kg^{-1} dry soil. Total soil Cu and Zn concentrations were quantified by atomic absorption spectroscopy (AAS PerkinElmer PinAAcle 500) for dose confirmation (see Table S1 for dose confirmation). All soil microcosms were incubated at 15 °C in the dark and sampled (see below for quantities used) after 3 h, 1 day, 1 week, 4 weeks, and 9 weeks for determination of bacterial growth dynamics and bioavailable Cu, Zn, or tetracycline (see below).

Detection of Pollution-Induced Community Tolerance (PICT). Environmental selection pressures for development of tetracycline resistant bacterial communities was determined by a cultivation-independent PICT approach based on the use of the [^{3}H]leucine incorporation technique[24] for PICT detection. PICT detection was performed after 9 weeks of microcosm incubation as described previously.[22] In brief, soil bacterial communities were extracted with MES buffer (CAS no. 4432-31-9, Sigma, 4-morpholineethanesulfonic acid, 20 mM, pH = 6.4) using a soil/water ratio of 1:10 (w/v), and 1.5 mL aliquots of soil bacterial suspensions were preincubated at various concentrations (0, 0.25, 1, 4, 8, 16, 32, or 64 mg L^{-1}) of tetracycline ($n = 3$) for 30 min at 22 °C. Incubations were subsequently started by addition of [^{3}H]Leucine (2.59 TBq mmol^{-1}, 35 MBq mL^{-1}, Amersham, Hillerød) and non-radiolabled leucine to give a final radioactivity of 6kBq per microtube and leucine concentration of 200 nM and terminated after 2 h by addition of trichloroacetic acid (TCA). Tetracycline dose−response curves for bacterial community tolerance were fitted using the four parameter logistic function in the drc-package in R version 3.0.1. Bacterial community tolerance was also quantified as a tolerance index (TI) for each soil microcosm: $TI = L_{inc\_TC}/L_{inc\_control}$, where $L_{inc\_control}$ equals the average [^{3}H]leucine incorporation rate in replicated soil bacterial suspensions amended with Milli-Q water (absence of tetracycline) and L_{inc_TC} equals the average [^{3}H]leucine incorporation rate in replicated soil bacterial suspensions amended with tetracycline (0.25, 1, or 4 mg L^{-1}). Threshold concentrations were selected on the basis of complete dose−response data.

Determination of Bacterial Growth Rates. Soil bacterial growth in each microcosm was measured by the [^{3}H]leucine incorporation technique.[24] In brief, soil bacteria were extracted from 1 g of moist soil with 10 mL of Milli-Q water and relative [^{3}H]leucine incorporation rates were measured as described previously.[25] All growth rates were normalized relative to the growth rate in control soil after 3 h of incubation.

Bioreporter Analysis of Bioavailable Cu, Zn, and Tetracycline in Spiked Soil. Bioavailable Cu, Zn, and tetracycline were measured in aqueous soil extracts which were derived from soil microcosm as described previously.[26] In brief, 1 g of moist soil was taken from each microcosm and mixed with 5 mL of Milli-Q water in a 15 mL Falcon tube (polypropylene),[27] followed by 2 h of shaking on a horizontal shaker (250 rpm, 22 °C). The supernatant was collected after centrifugation (10 000g, 22 °C, and 10 min) and stored at −20 °C for subsequent bioreporter analysis.

A panel of whole-cell bacterial biosensors (bioreporters) was used. Bioavailable Cu was determined using *Pseudomonas fluorescens* strain *DF57-Cu15*, which carries a chromosomal insertion of a promoter-less *Tn5::luxAB* cassette controlled by a Cu-inducible promoter.[28] Bioavailable Zn was determined using a *Pseudomonas putida KT2440* strain transformed with the

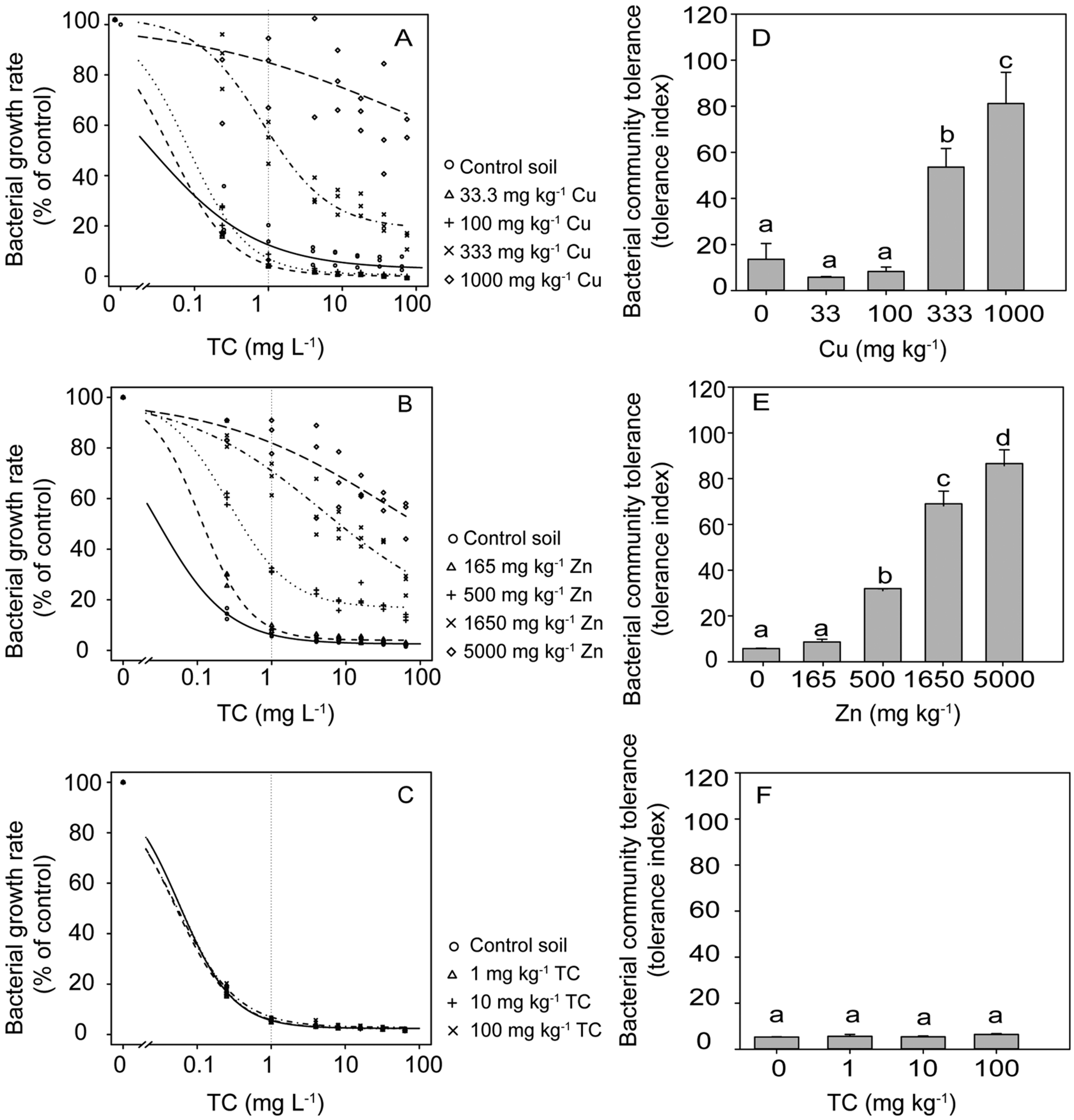

Figure 1. Bacterial community tolerance to tetracycline (TC) after 9 weeks of exposure to various levels of added soil Cu (Panels A and D), added Zn (Panels B and E), or added tetracycline (TC; Panels C and F) as measured by the [^{3}H]Leu incorporation method. Panels A−C depict full dose−response curves fitted with the four parameter logistic-logistic model (n = 3). Panels D−F depict the bacterial community tolerance index defined as Leu incorporation in the absence of stressors divided by Leu incorporation at a tetracycline test concentration of 1 mg L^{-1}. Tolerance indices were calculated independently for each soil microcosm. Error bars depict the standard deviation of the mean (n = 3). Different letters on top of columns indicate statistically significant differences between treatments (p = 0.05, n = 3).

sensor plasmid *pDNPczc1 lux*, which contains the *luxCDABE* operon from *Photorhabdus luminescens* under the control of *czcCBA1* promoter from *P. putida*.[29] Bioavailable tetracycline was determined using *Escherichia coli K12* (*pTetLux1*) carrying the *luxCDABE* operon on a sensor plasmid under the control of the *tetA* promoter from *pASK75*.[30,31] Bioavailable Cu ($[Cu]_{bio}$), Zn ($[Zn]_{bio}$), or tetracycline ($[TC]_{bio}$) was operationally defined as water-extractable Cu, Zn, or tetracycline species that were able to induce reporter gene expression in bioreporter cells. Exponential phase bioreporter cells were harvested and mixed with equal volume soil−water extracts (see details on strain preparation and the bioreporter assay in the Supporting Information) before quantification of bioluminescence in a BMG FluoStar Optima plate reader (BMG Labtech, Offenburg, Germany). $[Cu]_{bio}$ was calculated as described previously assuming zero bioavailability of particle associated Cu,[32] whereas $[Zn]_{bio}$ and $[TC]_{bio}$ were determined essentially as described previously with full details given as Supporting Information.

Statistical Analyses. SigmaPlot Version 12.5 (Systat Software, Point Richmond, CA) was used for the correlation test and significance testing of treatments effects. Nonlinear correlations between bacterial community tolerance indices and bacterial growth rates were analyzed with the nonparametric Spearman rank correlation test. Significance testing of treatment effects on bacterial growth and bacterial community tolerance indices was performed by one-way ANOVA, and all multiple pairwise comparisons were performed by the Holm-Sidak method. When required, raw data was log transformed. The dose−response curves for bacterial community tolerance were fitted with four parameter logistic function with the drc-package in R version 3.0.1.

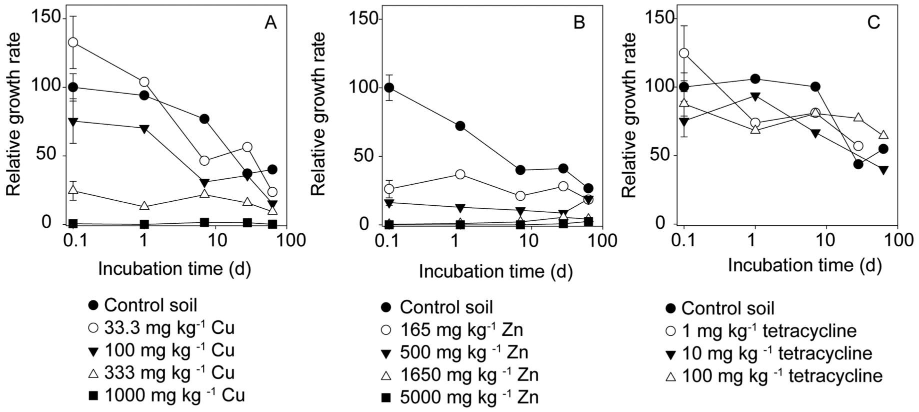

Figure 2. Bacterial growth dynamics measured with the [^{3}H]leucine incorporation technique during the first 63 days after spiking soil with different concentrations of Cu (Panel A), Zn (Panel B), or tetracycline (Panel C). All growth rates were normalized relative to the corresponding control treatment (spiking with water only) at the first sampling time. Means ($n = 3$) are shown, and average standard deviation of each treatment is shown at the first sampling time.

■ RESULTS

Bacterial Community Tolerance to Tetracycline Induced by Metals and Tetracycline. Cu and Zn coselected for tetracycline resistant bacterial communities in a dose-dependent manner in soil microcosms during the 9 week long PICT selection phase. Hence, increased community tolerance to tetracycline was observed at high Cu (≥333 mg kg^{-1}, Figure 1A) and Zn levels (≥500 mg kg^{-1}, Figure 1B). Half-maximal effective concentrations (EC$_{50}$ values) could not be reliably determined from the dose−response curves due to insufficient inhibition of growth (i.e., due to high community tolerance) observed for high metal treatments. Community tolerance to tetracycline was therefore quantified as a tolerance index (TI) using a threshold concentration of 1 mg L^{-1} for tolerance determination (Figure 1D,E). Tolerance indices (TIs) increased progressively with elevated metal (Cu or Zn) exposure during the 9 week selection phase, and significantly increased community tolerance to tetracycline was observed at high Cu (≥365 mg kg^{-1}, $n = 3$, $p < 0.01$, Figure 1D; Table S1) and Zn levels (≥488 mg kg^{-1}, $n = 3$, $p < 0.01$, Figure 1E; Table S1), respectively. TIs calculated using threshold tetracycline concentrations of 0.25 and 4 mg L^{-1} showed comparable results except that significantly increased community tolerance to 0.25 mg L^{-1} tetracycline was even observed for the lowest soil Zn treatment tested (264 mg kg^{-1}, $p < 0.01$, $n = 3$, one way ANOVA; Figure S1; Table S1).

In stark contrast to metals, tetracycline did not select for tetracycline tolerance in tetracycline-spiked soil microcosms (Figure 1C,F). Hence, even extremely high concentrations of tetracycline (i.e., amendment with up to 100 mg kg^{-1}) did not cause development of tetracycline resistant bacterial communities during the 9 week selection phase.

Effects of Cu, Zn, and Tetracycline on Bacterial Growth Dynamics. Bacterial growth ([^{3}H]leucine incorporation) dynamics in soil microcosms was studied in an attempt to explain the surprising finding that environmentally relevant concentrations of metals (co)selected for tetracycline resistant bacterial communities whereas even extremely high, unrealistic levels of tetracycline did not. Hence, we hypothesized that bacterial growth would be inhibited more by Cu and Zn than by tetracycline. Indeed, bacterial growth was significantly affected by Cu and Zn exposure during the entire soil microcosm incubations as shown by progressively increasing growth inhibition with increasing metal amendment (Figure 2A,B, $p < 0.05$). In stark contrast, no clear toxic effect of TC on bacterial growth was detected even when spiking soil with extremely high concentrations of tetracycline (up to 100 mg kg^{-1}, Figure 2C). This observation is thus consistent with the lack of selection of bacterial community-level resistance to tetracycline in tetracycline-spiked soil (Figure 1C).

The relationship between bacterial growth inhibition and community-level resistance to tetracycline is depicted in Figure 3. It shows a highly significant correlation between bacterial growth inhibition during the first week of the microcosm study and tetracycline resistance. Hence, selection of community resistance only occurred in soil microcosms, in which bacterial growth was initially inhibited by more than 50% as a result of metal spiking, whereas selection was not apparent under soil conditions causing weaker growth inhibition.

Bioavailability of Cu, Zn, and Tetracycline in Spiked Soil Microcosms. To further improve our understanding of the key finding that environmentally relevant concentrations of metals (co)selected for tetracycline resistant bacterial communities whereas tetracycline did not, we used whole-cell bacterial bioreporters to quantify the bioavailability of the studied selective agents. $[Cu]_{bio}$ and $[Zn]_{bio}$ were consistently below the detection limit in the unamended control soil microcosms. As expected, both $[Cu]_{bio}$ and $[Zn]_{bio}$ progressively increased with increasing amendment of $CuSO_4$ or $ZnSO_4$, respectively (Figure 4), and metal bioavailability correlated strongly with bacterial community tolerance to tetracycline (Figure 5). For both metals, the relative bioavailability expressed as the percentage of total added metal increased with increasing metal loading. $[Cu]_{bio}$ ranged from 0.1% of total added Cu in the 33.3 mg kg^{-1} soil up to 2.9% in the 1000 mg kg^{-1} treatment (Figure S3), whereas corresponding values for $[Zn]_{bio}$ ranged from 1.2% in the 165 mg kg^{-1} treatment up to around 60% for the 1650 and 5000 mg kg^{-1} treatments (Figure S4). The increased relative bioavailability with increased metal loading could in part be explained by increased metal solubility as a result of decreased soil pH in metal spiked soils. Hence, the pH measured in soil bacterial suspensions decreased progressively with increasing metal loading from around 6.5−6.8 in the control treatments to around 5.5 for the highest Cu and Zn loadings (Figure S2).

$[Cu]_{bio}$ expressed as the percentage of total added Cu was not significantly affected by time after Cu spiking in the lowest

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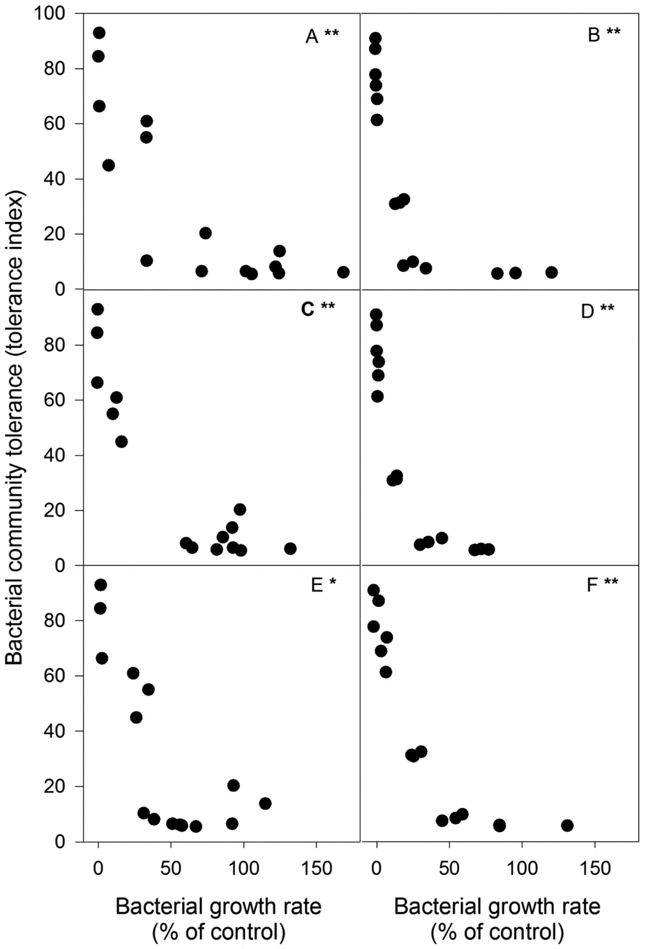

Figure 3. Bacterial community-level tetracycline tolerance in soils spiked with Cu (Panels A, C, and E) or Zn (Panels B, D, and F) plotted against relative bacterial growth rate 3 h (Panels A and B), 1 day (Panels C and D), or 7 days (Panels E and F) after metal spiking. Correlations between tolerance indices and growth rates were determined by the Spearman rank correlation test. Statistically significant correlations are indicated with * ($P < 0.01$) or ** ($P < 0.001$).

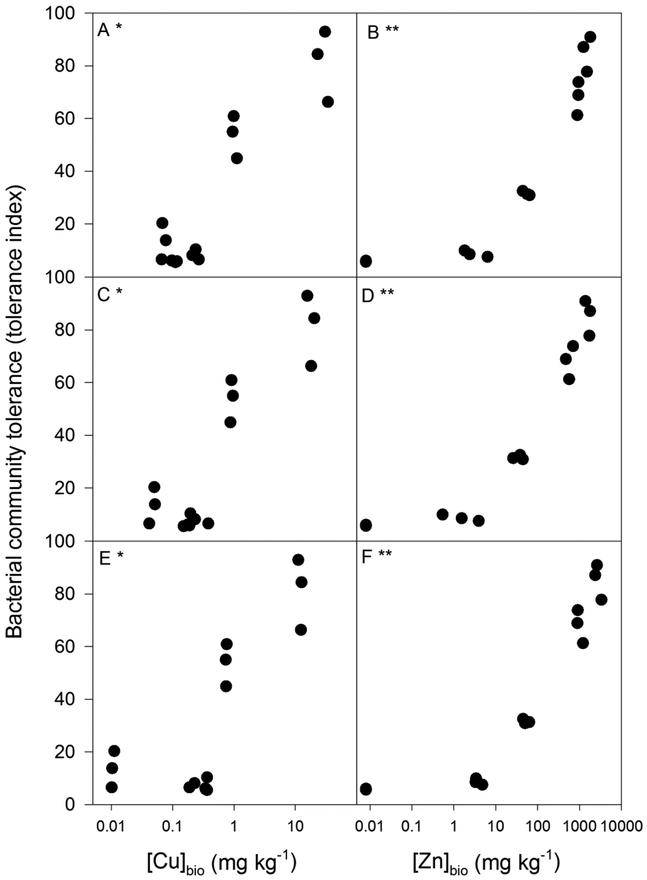

Figure 5. Bacterial community-level tetracycline tolerance in soils spiked with Cu (Panels A, C, and E) or Zn (Panels B, D, and F) plotted against bioavailable Cu ($[\mathrm{Cu}]_{\mathrm{bio}}$) or Zn ($[\mathrm{Zn}]_{\mathrm{bio}}$) 3 h (Panels A and B), 1 day (Panels C and D), or 7 days (Panels E and F) after metal spiking. Correlations between tolerance indices and $[\mathrm{Cu}]_{\mathrm{bio}}$ or $[\mathrm{Zn}]_{\mathrm{bio}}$ were determined by the Spearman rank correlation test. Statistically significant correlations are indicated with * ($P < 0.01$) or ** ($P < 0.001$).

Cu treatment but decreased linearly with log time for higher Cu treatments indicating an aging effect that became more

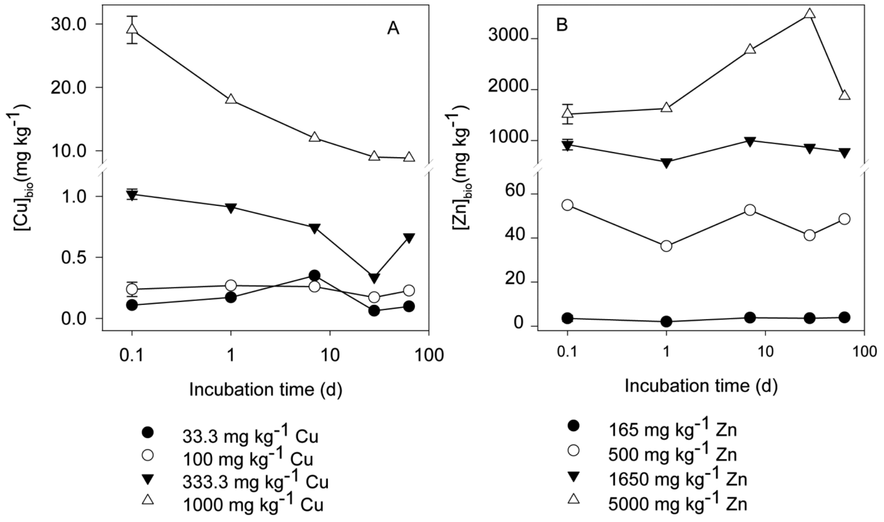

Figure 4. Bioavailable Cu ($[\mathrm{Cu}]_{\mathrm{bio}}$, Panel A) and Zn ($[\mathrm{Zn}]_{\mathrm{bio}}$, Panel B) during the 63 day soil microcosm experiment as determined by whole-cell bacterial bioreporters. Means ($n = 3$) are shown, and average standard deviations of each treatment are shown at the first sampling time.

apparent with increasing Cu loading (Figure S3). Hence, $[\mathrm{Cu}]_{\mathrm{bio}}$ constituted 2.9% of the total added Cu 3 h after metal spiking but only 0.98% after 63 days. The percentage of $[\mathrm{Zn}]_{\mathrm{bio}}$ relative to added Zn remained unaffected during the 63 days of incubation (Figure S4) demonstrating contrasting aging behavior of Cu and Zn in the studied soil.

In contrast to $[\mathrm{Cu}]_{\mathrm{bio}}$ and $[\mathrm{Zn}]_{\mathrm{bio}}$, ($[\mathrm{TC}]_{\mathrm{bio}}$) could only be detected for a short time even in soils spiked with high levels of tetracycline (data not shown). Hence, $[\mathrm{TC}]_{\mathrm{bio}}$ could only be detected in tetracycline amended soil (100 mg kg^{-1}) after 3 h (0.25 mg kg^{-1}) and 1 day (0.29 mg kg^{-1}). After 7 days, $[\mathrm{TC}]_{\mathrm{bio}}$ was below the bioreporter detection limit for all treatments.

■ DISCUSSION

A number of studies have implicated a role of metals in coselecting antibiotic resistance in manure-amended field soils,[10,11,33] but these studies are all complicated by difficulties associated with elucidation of causal relationships since correlations between the prevalence of metals and ARGs in manure-amended soil simply may be a result of their higher persistence relative to degradable antibiotics that could have selected for ARGs during animal passage.[34] Hence, strong evidence for the ability of metals to coselect for antibiotic resistance (including resistance to tetracyclines) in soil so far mainly comes from soils experimentally spiked with Cu[35,36] and from a study of an industrial site contaminated with $\mathrm{CuSO_4}$.[14] Support for a role of Cu in coselecting antibiotic resistance also comes from a field study demonstrating statistically significant correlations between Cu concentrations and selected ARGs in randomly selected soils from a Scottish soil archive.[37] Direct evidence for a role of Zn in coselecting antibiotic resistance is scarce. A recent long-term field study in Denmark investigated the effects of long-term cattle manure application.[7] Although metal accumulation patterns could not explain the observed ARG patterns, a possible role of Cu and Zn in selecting class 1 integron genes, known to play an important role for ARG dissemination, was proposed.

Our study is the first to demonstrate the ability of Zn to coselect antibiotic resistance in soil and corroborates the previous reports demonstrating an ability of Cu to coselect antibiotic resistance in soil bacterial communities.[14,35,36] The observed Cu and Zn coselection threshold concentrations (365 mg Cu kg^{-1} and 264−488 mg Zn kg^{-1}, respectively; Figure 1; Table S1) represent high, but environmentally realistic, metal levels sometimes attained in contaminated agricultural soils, e.g., below galvanized pylons or in vineyard soils which in extreme cases may contain more than 3000 mg kg^{-1} of Zn or Cu.[15,38] Even higher Cu and Zn levels may be present in industrially polluted sites.[39,40] The Cu coselection threshold is similar to the coselection threshold for development of tetracycline resistance in a previous study employing a similar Leu-PICT approach.[36] However, it should be emphasized that the used Leu-PICT methodology may be unable to detect coselection events in cases where coselection only involves a small fraction of the total bacterial community. This may explain why Cu threshold concentrations for direct selection (Cu resistance) were lower than corresponding Cu threshold concentrations for coselection of antibiotic resistance (including resistance to tetracycline) in the previous study.[36] Other studies have demonstrated that Cu resistant soil bacteria tend to be more resistant to antibiotics than their Cu-sensitive counterparts, irrespective of whether they were isolated from low-Cu or high-Cu soils.[14,35]

Our study is the first to directly compare metals (Cu and Zn) and an antibiotic (tetracycline) for their ability to select for antibiotic resistance in a soil bacterial community. The results showed that environmentally relevant levels of Cu and Zn coselected for antibiotic resistance (see Discussion above), whereas even unrealistically high concentrations of tetracycline (100 mg kg^{-1}) never previously measured in agricultural soils did not select for tetracycline resistance. Hence, according to a recently established database of measured environmental concentrations (MECs) of pharmaceuticals,[17] the highest MECs in soil were reported to be just 5.17, 0.82, and 0.53 mg kg^{-1} for oxytetracycline, chlortetracycline, and tetracycline, respectively. Although a somewhat higher concentration of tetracycline (2.45 mg kg^{-1}) has been measured in a Chinese soil heavily impacted by animal manure deposition,[33] these MECs are all significantly lower than the highest tetracycline concentrations applied in our study.

Although we did not record antibiotic resistance genes directly, we consider our results to be robust. Hence, the employed Leu-PICT approach is a valid tool for evaluation of bacterial community-level selection pressures irrespective of the selection mechanisms involved.[22,25] Leu-PICT has broad bacterial community coverage, and as a phenotypic approach, it does not suffer from artifacts such as the presence of unknown, and thus unrecognizable, ARGs or ARGs that may serve other functions than antibiotic resistance in environmental bacteria depending on the ecological and genomic contexts of ARGs.[41] Furthermore, the lacking ability of tetracycline to select for tetracycline resistant bacterial communities is also supported by our data showing negligible bacterial growth inhibition (toxicity) and bioavailability of tetracycline in spiked soils consistent with previous studies showing strong sorption or low bioavailability of tetracyclines in soil.[42,43] In contrast, both Cu and Zn induced a persistent selection pressure on the soil bacterial community as inferred from bacterial growth and bioreporter data (Figures 2 and 4) and strong correlations of both growth inhibition and bioavailability with bacterial community tolerance to tetracycline (Figures 3 and 5). Interestingly, there were no indications of reduced bioavailability over time (aging) for Zn (Figure 4B), whereas an aging effect was clearly visible at least for high concentrations of Cu (Figure 4A). The latter finding is consistent with a previous Cu bioreporter study performed in another agricultural soil.[44]

On the basis of our data and the available literature, we predict that metals such as Cu and Zn in many cases may constitute stronger selective agents for antibiotic resistance than antibiotic residues in agricultural soil. Hence, many antibiotics can be inactivated due to sorption, sequestration, or degradation processes once they reach the soil compartment,[42,45] and antibiotic residues probably only rarely reach toxic levels in agricultural soils.[18] The potential for metal-induced coselection in agricultural soils is likely amplified by current agricultural practices causing bacteria to be selected by high levels of Cu, Zn, and antibiotics (e.g., tetracycline) at the same time during animal passage.[10,11] This increases the likelihood for selection of mobile genetic elements conferring resistance to both metals and antibiotics.[46] In turn, manure amendment to agricultural soil can lead to a vast enrichment of ARGs and mobile genetic elements known to disseminate antibiotic resistance.[10] Manure may further modulate conjugal plasmid transfer and community-level adaption.[47,48]

In conclusion, our results call for an increased focus on metals if we are to mitigate the current expansion of the soil bacterial resistome in agricultural soils. In this context, it will be important to determine minimal coselective metal concentrations at field conditions, where metals typically accumulate slowly over extended time periods. It will also be relevant to focus on possible synergistic effects between selective agents (metals and antibiotic residues) and antibiotic resistance determinants present in manure-amended agricultural soils and on the ability of low concentrations of metals to modulate horizontal gene transfer processes in soil bacterial communities.[49] It has recently been proposed that risks for environmental selection of antibiotic resistance should be incorporated into environmental risk assessment of pharmaceuticals.[18,50] By analogy, we here propose that the ability of metals such as Cu and Zn to coselect antibiotic resistance should be taken into consideration when assessing environmental risks of metals either by in-depth quantification of antibiotic resistance determinants[10] or by targeted analysis of metal resistance in soil bacterial communities.[22] Finally, we propose that future research should unravel the dominating coselection mechanisms in soil to better evaluate the risk posed by soil metals for the environmental dissemination of ARGs from soil to pathogenic bacteria in clinical settings.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b05342.

> Detailed experimental procedures for bioreporter and total metal analysis; Figure S1, bacterial community tolerance indices as determined for tetracycline test concentrations of 0.25 and 4 mg L^{-1}; Figure S2, pH changes over time in soil microcosms; Figure S3, bioavailable Cu ($[Cu]_{bio}$) as the percentage of total added Cu; Figure S4, bioavailable Zn ($[Zn]_{bio}$) as the percentage of total added Zn; Table S1, total soil Cu and Zn in soil microcosms; Table S2, Spearman correlation coefficients between tolerance indices, bacterial growth rates, and bioavailable Cu or Zn (PDF)

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Notes

The authors declare no competing financial interest.

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