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## Metal stressors consistently modulate bacterial conjugal plasmid uptake potential in a phylogenetically conserved manner

U. Klümper<sup>1,2</sup>, A. Dechesne<sup>1</sup>, L. Riber<sup>3</sup>, K.K. Brandt<sup>4</sup>, A. Gülay<sup>1</sup>, S.J. Sørensen<sup>3</sup>, and B.F. Smets<sup>1</sup>

<sup>1</sup>Department of Environmental Engineering, Technical University of Denmark, Kgs. Lyngby, Denmark

<sup>3</sup>Department of Biology, University of Copenhagen, Copenhagen, Denmark

<sup>4</sup>Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg, Denmark

### Abstract

The environmental stimulants and inhibitors of conjugal plasmid transfer in microbial communities are poorly understood. Specifically, it is not known whether exposure to stressors may cause a community to alter its plasmid uptake ability. We assessed whether metals (Cu, Cd, Ni, Zn) and one metalloid (As), at concentrations causing partial growth inhibition, modulate community permissiveness (i.e. uptake ability) against a broad-host-range IncP-type plasmid (pKJK5). Cells were extracted from an agricultural soil as recipient community and a previously described cultivation-minimal filter mating assay was conducted with an exogenous *E. coli* donor strain. The donor hosted a *gfp*-tagged pKJK5 derivative from which conjugation events could be microscopically quantified and transconjugants isolated and phylogenetically described at high resolution via FACS and 16S rRNA amplicon sequencing. Metal stress consistently decreased plasmid transfer frequencies to the community, while the transconjugal pool richness remained unaffected with OTUs belonging to 12 bacterial phyla. The taxonomic composition of the transconjugal pools was distinct from their respective recipient communities and clustered dependent on the stress type and dose. However, for certain OTUs, stress in- or decreased plasmid permissiveness by more than 1000-fold and this response was typically correlated across different metals and doses. The response to some stresses was, in addition, phylogenetically conserved. This is the first demonstration that community permissiveness is sensitive to metal(loid) stress in a manner that is both partially consistent across stressors and phylogenetically conserved.

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**Corresponding author:** Barth F. Smets, Technical University of Denmark, DTU Environment, Miljøvej 113, 2840 Kgs. Lyngby, Denmark, Ph. +45 45 25 22 30, Fax +45 45 93 28 50. [bfsm@env.dtu.dk](mailto:bfsm@env.dtu.dk).

<sup>2</sup>Current address: European Centre for Environment and Human Health, University of Exeter Medical School, Royal Cornwall Hospital, Truro, UK

### Conflict of Interest

The authors declare no conflict of interest.

## Introduction

Horizontal gene transfer among bacterial species is recognized as a major evolutionary process (Zhaxybayeva & Doolittle, 2011). Mobile genetic elements can spread across diverse bacterial phyla (Klümper *et al.*, 2015) linking distinct genetic pools (Halary *et al.*, 2010; Norman *et al.*, 2009). A main parameter in assessing the ecology of plasmid transfer is community permissiveness, which refers to the ability of a community to receive a plasmid, both in terms of transfer frequency and transconjugant phylogeny (Musovic *et al.*, 2010).

The spread of plasmid-mediated antibiotic resistance across bacterial communities has recently been identified as a major threat to human health by the WHO (WHO, 2014). By carrying sets of genes coding for resistance, conjugal plasmids can facilitate bacterial community adaptation to stress imposed by antibiotics or by other toxicants such as metals and metalloids at elevated concentrations (Sørensen *et al.*, 2005; Heuer & Smalla, 2012).

Here, stress is defined as the exposure of bacteria to a toxicant at a dose that causes a transcriptional response (Cases & de Lorenzo, 2005) as a result of toxicant incompatibility with normal biological functions (Richardson, 1993). Low exposure levels might act as a stimulant or signal for the transcription of single genes (Pérez-Martín & de Lorenzo, 1996). An elevated dose of the stressor may cause the activation of entire regulons that are part of global stress-response mechanisms. Known stress-responses involve switching to slow growth, biofilm formation or the activation of efflux pumps (Mah & O'Toole, 2001). We hypothesize that, additionally, bacterial plasmid uptake potential, or individual permissiveness, might increase as a response to stress. This increased permissiveness could either be the direct or indirect effect of an evolutionary evolved stress response mechanism.

Single strain experiments have shown modulation in gene uptake as a result of stress exposure. In *Streptococcus pneumoniae*, exposure to antibiotics caused increased competence and increased promiscuity towards foreign DNA (Slager *et al.*, 2014). Heat-shock treatment of Gram-positive Corynebacteria enhanced their receipt of an IncP-type plasmid from a Gram-negative *E.coli* donor, likely due to restriction system inactivation (Schafer *et al.*, 1990). Similarly, in the Gram-negative *Pseudomonas putida*, pre-exposure to sodium dodecyl sulfate (SDS) increased its ability to receive and maintain plasmids, possibly by repressing restriction-modification systems (Arango Pinedo & Smets, 2005). Cells with a turned-off RM system can become hypersusceptible to foreign DNA or plasmid receipt (Schafer *et al.*, 1990; Corvaglia *et al.*, 2010; Roer *et al.*, 2015). Also, in *Bacillus subtilis*, sub-inhibitory concentrations of ethanol activated the conjugative transposition of transposon Tn916 (Seier-Petersen *et al.*, 2013) encoding tetracycline resistance (Franke & Clewell, 1981). In contrast, in *Escherichia coli*, cell-envelope targeted stress induced the expression of CRISPR associated (CRISPR-cas) genes which would serve as an intracellular defense against foreign DNA (Perez-Rodriguez *et al.*, 2011), thus decreasing permissiveness. While these mechanisms are described for specific strains under specific stress conditions, there have been no studies to date that evaluate modulation of permissiveness as a general bacterial stress-response mechanism using complex bacterial communities and multiple stressors.

A typical environmental stress for soil microbes are toxic metals accumulating to elevated concentrations due to agronomic practices, industrial activities, or atmospheric deposition (Nicholson *et al.*, 2003; Zhao *et al.*, 2015). Metal toxicity is mainly caused by metal ions disrupting iron-sulfur clusters of metallo-enzymes (Macomber & Hausinger, 2011; Macomber & Imlay, 2009; Xu & Imlay, 2012). Metals, at elevated concentrations, have been documented to affect the composition and function of soil bacterial communities (Giller *et al.*, 1998; Gans *et al.*, 2005; Berg *et al.*, 2012), although development of community tolerance is common even when no effects are observed at the levels of activity or community composition (Brandt *et al.*, 2010). Selection for adaptive plasmids within microbial communities has been described as a long term effect to metal exposure (Giller *et al.*, 1998; Campbell *et al.*, 1995). However, the existence and magnitude of short-term impact of metal stress on the permissiveness of bacterial communities remain to be explored.

In this study, we combined a cultivation-minimal assay to measure community-wide permissiveness (Klümper *et al.*, 2015) with normalized stress exposure to five of the most environmentally relevant metal(oid)s ( $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{AsO}_3^{3-}$ ). This allowed for direct comparison of short-term metal stress effects on the permissiveness towards the *gfp*-labelled, model broad-host-range IncP-1e plasmid pKJK5 (Bahl *et al.*, 2007) introduced through an *E. coli* donor strain in a reference soil bacterial community. Plasmid transfer frequencies and the phylogenetic composition of the transconjugal pools were compared to corresponding controls to assess stress-induced modulation of permissiveness of the community and of individual community members.

## Material & Methods

### Soil sampling

Soil (16±1% clay, 15±1% silt, 43% fine sand and 26±1% coarse sand, with a pH(H<sub>2</sub>O) of 7.16±0.13) was sampled from unfertilized control plots ( $n=3$ ) of the CRUCIAL agricultural field site (Dec 2013, Taastrup, DK) (Poulsen *et al.*, 2013; Lekfeldt *et al.*, 2014). Five hundred grams of soil was taken at 3 locations at each plot from a depth of 5-15 cm. The 9 soil samples were pooled, sieved through a 1 mm<sup>2</sup> mesh filter, homogenized and stored at 4°C for up to a month before conducting the experiments.

### Soil bacterial community extraction

Soil bacterial communities were recovered from 30 g sub-samples of homogenized soil by Nycodenz<sup>®</sup>-extraction (Lindahl & Bakken, 1995). Extracted cells were resuspended in sterile 0.9% NaCl solution, filtered, washed, quantified using a Thoma counting chamber and adjusted to 10<sup>7</sup> cells/mL to quantify metal-induced stress (measured as growth rate inhibition) or to be used as recipients in the filter mating assay.

### [<sup>3</sup>H]leucine incorporation inhibition assay

A [<sup>3</sup>H]leucine incorporation assay (SI Text 1) was used to estimate the concentrations of individual metal(oid)s inhibiting soil bacterial growth rates by 20% (IC<sub>20</sub>) and 50% (IC<sub>50</sub>), respectively (Lekfeldt *et al.*, 2014). The experimental data linking leucine incorporation to

metal dose were fitted with a four parameter log-logistic dose-response curve using the *drc* (Analysis of Dose-Response Curves) package for R (Knezevic *et al.*, 2007) (SI Figure 1).

### Plasmid and donor strain

Cells of *E. coli* MG1655::*Km<sup>R</sup>-Lpp-mCherry* (Klümper *et al.*, 2015) carrying the IncP-1e broad host range plasmid pKJK5::*gfpmut3b* (Bahl *et al.*, 2007) were used as donors. Plasmid pKJK5 has an extremely broad transfer range, including both Gram-negative and Gram-positive phyla (Klümper *et al.*, 2015). Additionally, pKJK5 does not encode metal related resistance mechanisms (Bahl *et al.*, 2007).

The plasmid was marked with an entranceposon (Bahl *et al.*, 2009) derived genetic tag that carries a *LacI<sup>q</sup>* repressible promoter upstream the conditionally expressed *gfpmut3b* gene, encoding the green fluorescent protein (GFP). The plasmid donor strain was chromosomally tagged with a gene cassette encoding constitutive red fluorescence, expressed by the *mCherry* gene, and constitutive *lacI<sup>q</sup>* production. As a result, *gfp* expression is repressed in the donor strain, but upon successful conjugal transfer to a soil bacterium, plasmid encoded *gfp* expression is de-repressed, resulting in green fluorescent cells or microcolonies, which can be retrieved by fluorescence activated cell sorting (FACS) or detected by fluorescence microscopy, respectively (Sørensen *et al.*, 2005; Klümper *et al.*, 2015). The donor strain was grown overnight in LB medium supplemented with trimethoprim (30 µg mL<sup>-1</sup>), harvested by centrifugation, washed in 0.9% NaCl solution, adjusted in cell density (OD<sub>600</sub>) and used in filter mating assays as described previously (Klümper *et al.*, 2014a). The viability and plasmid transfer ability of the donor strain under all metal stress conditions were verified in control mating assays with an untagged *E. coli* MG1655 recipient with microscopic observations.

### Filter matings

The extracted soil bacterial community was challenged with the exogenous plasmid via solid-surface filter matings (Musovic *et al.*, 2010; Klümper, *et al.*, 2014a) to maximize cell-to-cell contact, as physical barriers would limit the contact between freshly introduced plasmid donors and potential recipients in an intact soil matrix (Dechesne *et al.*, 2005). Compared to the original protocol (Klümper *et al.*, 2014a) the initial ratio of donor to recipient cells was increased to 10:1 to maximize recipient cell/microcolony contact with the donor cells during the 48 hour incubation period.

Filters were placed on an agar-solidified 10% soil extract medium after Musovic *et al.* (2010). The limited amount of nutrients resulted in monolayer cell coverage of the filter after 48h as confirmed with laser scanning confocal microscopy. With an initial density of approx. 300,000 cells per mm<sup>2</sup> and assuming an average cell surface area of 1µm<sup>2</sup>, the average number of cell doublings per inoculated cell during incubation was thus estimated at less than 5.

Filter matings were performed on medium without metal amendment (controls) or supplemented with CuSO<sub>4</sub>, NiSO<sub>4</sub>, ZnSO<sub>4</sub>, CdCl<sub>2</sub> or Na<sub>2</sub>HAsO<sub>3</sub> at concentrations corresponding to IC20 and IC50 based on [3H]leucine incorporation of the recipient community. Filters were incubated for 48 hours at 25°C, before 72 h storage at 4°C for GFP

maturation. Conjugation events were checked by epifluorescence stereomicroscopy and confocal laser scanning microscopy (CLSM) (Musovic et al., 2010).

### Transfer frequency quantification

Conjugation events were visualized by stereomicroscopy and quantified by automated image analysis (Image Pro Plus 7.1; Media Cybernetics, Silver Spring, MD) (SI Text 1) as previously described (Musovic *et al.*, 2010; Klümper, *et al.*, 2014b). The number of detected transfer events was scaled up to the total filter area and transfer frequency was calculated by dividing by the number of originally added recipient cells (SI Figure 2).

### Fluorescence activated cell sorting (FACS) and sequencing

For each mating condition cells from triplicate filters were combined in 2 mL 0.9% NaCl solution and detached by vortexing at 3,000 rpm for 30 s. Transconjugant cells and recipient community were sorted using fluorescent activated cell sorting (SI Text 1, SI Figure 2). Gating and sorting of transconjugants for each of the combinations were performed based on bacterial size, green fluorescence and exclusion of red fluorescent donor cells as described earlier (Klümper *et al.*, 2015).

Recipient community cells were sorted from the same samples using the same conditions, including both colorless recipient and green fluorescent transconjugal cells and excluding red fluorescent donor cells. In all cases, a minimum of 20,000 *gfp*-expressing transconjugal cells or recipient cells were sorted. Sorted bacterial cells were collected, lysed, and subject to tag-encoded 16s rRNA gene amplicon sequencing (SI Text 1) (Klümper *et al.*, 2015).

### Sequence analysis

All sequences documented in this study were deposited in the European Nucleotide Archive under study accession number PRJEB13628.

Sequence analysis was carried out using mothur v.1.32.1 (Schloss *et al.*, 2009) and the MiSeq SOP (Kozich *et al.*, 2013) as accessed on 01.12.2014 on [http://www.mothur.org/wiki/MiSeq\\_SOP](http://www.mothur.org/wiki/MiSeq_SOP). Sequences were classified based on the RDP classifier (Wang *et al.*, 2007).

Diversity was assessed based on observed OTUs at 97% sequence similarity. Phylogenetic trees were constructed using iTOL (<http://itol.embl.de/>) (Letunic & Bork, 2007).

NMDS plots showing the phylogenetic distance between samples were produced based on the theta Yue Clayton dissimilarity index (Yue & Clayton, 2005) using mothur software and clustering of samples was tested for significance by analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992). Based on NMDS clustering and because the number of replicates per condition differed, replicates were combined for subsequent statistical analysis.

### OTU-level permissiveness analysis

At the community level, precise estimates of the initial recipient abundance (R) and of the number of conjugation events (T) made it possible to measure permissiveness accurately. This was impossible at the OTU level, where only relative abundances of an OTU in the

recipient community (after mating incubation) and in the transconjugant pool were measured (SI Figure 2). These values are not solid estimators of the initial recipient abundance or of the number of conjugation events for a specific OTU as they are affected by the growth rate of that OTU relative to other OTUs, the possibility of multiple transfers within an OTU microcolony, and the permissiveness of other recipients. Increases or decreases in recipient OTU cell densities during the mating incubations per se (i.e., due to a competitive advantage or disadvantage compared to other OTUs) are possible, but equivalent effects would hold for the transconjugant cell densities of that OTU, as the pKJK5 plasmid does not encode for any growth-beneficial phenotype. Hence, an apparent OTU-level permissiveness can be calculated, based on the relative recipient and transconjugant abundances at the end of the mating incubation:

$$AP_i = \frac{\text{rel. abundance in transconjugal pool of OTU}_i}{\text{rel. abundance in recipient community of OTU}_i} = \frac{T_i}{R_i}$$

Although this apparent permissiveness is not only affected by transfer, but also by the fact that multiple transfers can occur in a microcolony and by recipient and transconjugant growth; these phenomena are expected to occur both under the reference and the heavy metal conditions. We therefore calculate the ratio ( $\delta$ ) of an OTU's apparent permissiveness under stress ( $AP_{i \text{ stress}}$ ) and reference conditions ( $AP_{i \text{ ref}}$ ) to describe stress-induced permissiveness changes in that OTU:

$$\delta_i = \frac{AP_{i \text{ stress}}}{AP_{i \text{ ref}}} = \frac{T_{i \text{ stress}} / R_{i \text{ stress}}}{T_{i \text{ ref}} / R_{i \text{ ref}}}$$

If the stress has no effect on an OTU's permissiveness,  $\delta$  would equal 1 while values above or below 1 would indicate an increase or decrease in permissiveness under the stress condition, respectively.

To analyze whether the variability of  $\delta$ -values was phylogenetically conserved, we created a maximum likelihood tree of the dominant (average relative abundance in all transconjugal pools >0.05%) transconjugal OTUs based on similarity of their  $\delta$ -values across stresses. Phylogenetic conservation of permissiveness response for each stress condition was tested using the Analysis of Trait (AoT) module of Phylocom v4.2 (Webb et al., 2008).

Exploratory statistics were carried out in R using the package car (Companion to Applied Regression, version 2.0-25). The package psych (Revelle, 2014) was used to calculate Spearman correlations between parameters and test their significance, after adjusting the p-values for multiple testing (Hommel, 1988).



## Results

### Metal stress reduces transfer frequencies more than metabolic activity

Inhibitory metal concentrations ( $IC_{20}$  or  $IC_{50}$ ) applied in the mating experiments were determined by dose-response modeling of [ $^3H$ ]leucine incorporation data (SI Figure 1). Molar metal toxicity increased in the order (Table 1):  $AsO_3^{3-} < Zn^{2+} < Cd^{2+} < Cu^{2+} < Ni^{2+}$ .

Matings conducted with metal stress will subsequently be identified with a combination of the elemental symbol and the inhibitory level (e.g. As20 *see* Table 1) and compared to the non-stressed reference condition.

Conjugation events were detected as green fluorescent cells and microcolonies in microscopic images of the filter matings. Red fluorescent donor cells were detected in all matings, albeit with a slight decrease in density under stress conditions (Figure 1). Flow cytometric counts of intact red fluorescent donor cells confirmed that donor cell counts exceeded total recipient counts under each condition to ensure that each recipient microcolony was in contact with multiple donor cells.

Conjugation events were detected at approx. 1 in 15,000 ( $6.8 \times 10^{-5} \pm 3.8 \times 10^{-5}$ ) of the initial soil recipient bacterial cells under reference conditions. This frequency decreased under stress conditions for all metals (Figure 1). Community permissiveness significantly decreased ( $p < 0.05$ ) under conditions of metal stress in a dose-dependent manner (Figure 2). This decrease was metal specific and was more severe than the effect of the metal on community-wide metabolic activity (Table 1, Figure 2). Cu50, Zn50 (both >90% reduction) and Cd50 (no transfer detected) caused the largest permissiveness reduction.

Reduction in community permissiveness could result from a general decrease of transfer across all taxa or from taxon specific modulation of individual permissiveness. We FACS-sorted at least 20,000 transconjugant cells from each mating, which were subjected to 16S rRNA amplicon sequencing to analyze their phylogenetic composition.

All transconjugal pool sequence libraries showed coverage above 98%, and no subsampling effect was found at a depth of 50,000 sequences. 10 subsampled sets of each sample showed no significant dissimilarity ( $p > 0.99$ ) based on weighted UNIFRAC comparisons of when compared to the original sequencing library. Thus, further analysis of transconjugal pools was done at a subsampling depth of 50,000 sequences.

### Diversity of transconjugal pools is maintained in spite of transfer frequency reduction

We report an extremely diverse phylogenetic range of transconjugants. Across all pools, 206 permissive OTUs, distributed over 12 phyla, were identified (Figure 3A). These included the dominant Proteobacteria ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) and Bacteroidetes (Figure 3B), as well as other Gram-negative phyla (Acidobacteria, Nitrospira, Fusobacteria, Planctomycetes, Gemmatimonadetes, Verrucomicrobia), diverse Gram-positive phyla (Firmicutes, Actinobacteria, Chloroflexi) and the candidate phylum Cand. Saccharibacteria.



Plasmid transfer was detected both in abundant and rare taxa of the initial recipient community (Figure 3A). The phylogenetic composition of all transconjugal pools was similar at the phylum level (Figure 3B). The only exception was As50, where the Bacteroidetes phylum was nearly absent from the transconjugal pool, in spite of its abundance in the corresponding recipient community after incubation. Larger shifts were identified in the less abundant phyla with gram-positive Firmicutes (Figure 3B) and Actinobacteria (Figure 3C, SI Table 2) increasing in richness and up to 8-fold in abundance under several stress conditions.

The average richness ( $\alpha$ -diversity) of the transconjugal pools was  $196.0 \pm 14.4$  OTUs, irrespective of transfer frequency reduction (Figure 4). While  $38.5 \pm 2.2\%$  of the recipient community OTUs (identified after 48 hour incubation) were permissive to pKJK5 under reference conditions, this increased to 57-96% under metal stress (Figure 4). This increase resulted from a dramatic (more than 50%,  $p=0.0053$ ) decrease in the recipient community richness under metal stress conditions. As 94-97% of the transconjugal sequences under metal stress conditions belonged to OTUs also found in the reference transconjugal pool, the OTUs permissive to pKJK5 survived better under metal stress than the non-permissive OTUs. However, 86 additional OTUs, rare in abundance, were exclusively detected in transconjugal pools under metal stress conditions.

### Transconjugal pools cluster according to stress

The overall OTU richness and phylum level composition of the transconjugal pools remained unchanged under most stress conditions. However, at the OTU level their phylogenetic composition was significantly altered by stress. Some OTUs in the recipient communities were not permissive to plasmid pKJK5 under any of the tested conditions and were excluded prior to ordination (Figure 5). Transconjugal pools nevertheless clustered significantly apart from their respective recipient communities (AMOVA test,  $p<0.001$ ).

Replicate transconjugal pools and recipient communities consistently grouped together dependent on the type and degree of the metal stress. Three main clusters were observed among the transconjugal pools (Figure 5). Cluster I, comprised of the Cu50, Cd20, Ni20 and Ni50 replicate pools, grouped significantly apart ( $p<0.001$ ) from the reference transconjugal pools. Replicate transconjugal pools of Cu20 formed a separate Cluster II with a phylogenetic composition significantly different ( $p<0.05$ ) from pools obtained at any other stress condition. By contrast, the transconjugal pools of As20, As50 and Zn20 did not show a significant shift in phylogenetic composition compared to the reference pool ( $p>0.05$ ), with which they formed Cluster III.

### Stress specific responses at OTU level are resolved by transconjugal taxonomy

The relative abundance of transconjugal OTUs varied across metal treatments (Figure 5). Two factors may have caused these variations: a metal-specific shift in the composition of the recipient community, or a metal-specific modulation in the permissiveness of some OTUs. We investigated whether the second mechanism, i.e. modulation of OTU permissiveness, could be detected. Hence, we analyzed, for each of the 38 dominant

transconjugal OTUs (Figure 3) and each metal, the change in permissiveness ( $\delta$ ) between the stressed condition and the unstressed reference.

For some OTUs, under certain stress conditions, permissiveness increased over 1,000 fold or decreased up to 10,000 fold (Figure 6). The permissiveness of a high number of OTUs (138 of 342 tested combinations) in- or decreased more than ten-fold in response to stress. Under some conditions (As20, Cu20, Cu50, Ni20, and Zn20), permissiveness increased slightly for most of the OTUs (SI Figure 4). In contrast, arsenic at high concentration slightly decreased the permissiveness of the majority of the OTUs.

Individual OTUs tended to respond in a consistent manner to different metal stresses. Of 15 pairwise correlations of the  $\delta$ -values between the metal stresses (Cd20, Cu20, Cu50, Ni20, Ni50 and Zn20), 12 were significant ( $p < 0.05$ ) (Figure 6). The  $\delta$ -values for Zn50 correlated poorly with the others, except for Ni20, mainly caused by few Rhizobiales OTUs with a highly decreased permissiveness. The metalloid arsenic at As50 did not correlate well with the metals; here all OTUs belonging to the Bacteroidetes phylum displayed a strong decrease in permissiveness (Figure 6). Despite a general consistency across most metal stresses, almost none of the 38 most abundant OTUs responded similarly to all stress conditions (Figure 7). Only one OTU, a member of the alphaproteobacterial Rhodobacterales family, responded to every applied stress with a significant decrease in plasmid uptake (always more than 100 fold and up to 5,000 fold) (Figure 7).

Phylogenetically similar OTUs responded similarly to metal stress. All Bacteroidetes OTUs responded similarly to the metal exposure: for all stresses except As50, the plasmid uptake ability of the OTUs belonging to the Bacteroidetes phylum increased by more than 10-fold (Figure 7). The only gram-positive OTU among the 38 most abundant transconjugant OTUs, a member of the Firmicutes phylum, responded in a similar way. For most of the transconjugal OTUs in the phylum Proteobacteria, the stress response was more variable. Four of these OTUs, similar to Bacteroidetes, became increasingly permissive under stress conditions. Especially an OTU classified as Xanthomonadales responded with an increased permissiveness to most stresses and showed the highest relative increase in permissiveness ( $> 1000$  fold for Cu50). Most Alphaproteobacteria, such as the Rhizobiales, displayed little permissiveness modulation under stress conditions.

The distribution of  $\delta$ -values across the community's phylogenetic tree was significantly correlated with OTU phylogeny ( $p < 0.05$ ) for two (Zn20, Ni20) of the nine stress conditions and additionally showed a tendency for phylogenetic conservation ( $p = 0.074 - 0.258$ ) for six of the remaining seven conditions (SI Table 1).

## Discussion

Broad host range plasmids introduced to microbial communities can spread among a wide variety of gram-negative and gram-positive bacterial species (De Gelder *et al.*, 2005; Shintani *et al.*, 2014; Musovic *et al.*, 2014; Klümper *et al.*, 2015). Here we demonstrate pKJK5 transfer to an extremely diverse fraction of a soil bacterial community both under reference and metal stressed conditions. The transconjugant pool included abundant as well

as rare species-level OTUs and covered 12 different phyla, thereby expanding the realized transfer range of IncP type plasmids in soil beyond our previous observations with two more phyla, *Nitrospira* and *Cand. Saccharibacteria* (Klümper *et al.*, 2015). 86 transconjugant OTUs were only detected under specific stress conditions, which might stem from a stimulation of permissiveness in these OTUs associated with metal stress exposure. Because of the low abundance of these rare OTUs (below 0.01% rel. abundance this possibility requires confirmation).

More importantly, we show, for the first time, that metal stress can modulate – increase or decrease - the permissiveness of different members of a soil bacterial community towards an IncP plasmid. Elevated levels of plasmids and plasmid encoded genes have been reported in soil communities subject to long-term anthropogenic selective pressure through agricultural use (Agersø *et al.*, 2006; You *et al.*, 2012; Heuer *et al.*, 2011), and exposure to long term metal stress was shown to increase plasmid mobilization capacity of a soil community (Top *et al.*, 1995). However, these long term effects offer no direct evidence for increased permissiveness towards plasmids because they may also be caused by indirect selection of specific beneficial phenotypes. By employing a plasmid (Bahl *et al.*, 2009) that does not encode a beneficial phenotype when exposed to the chosen stressors and examining transfer directly we aim at effectively uncoupling plasmid transfer from selective forces.

While earlier studies revealed that the plasmid uptake of individual strains can differ under stress conditions (Arango Pinedo & Smets, 2005; Slager *et al.*, 2014), this is the first assessment of OTU permissiveness in a community context. Taking advantage of our high-resolution approach, we demonstrated that stress-induced modulations of permissiveness are indeed unique for each OTU.

The composition and density of the transconjugal pools is affected by (1) transconjugant growth and (2) de novo transconjugant formation by plasmid transfer to recipient cells. Both processes can be affected by heavy metal exposure. While we employ a cultivation-minimal assay with a high initial bacterial density on the filters, growth may occur on the filters. However, transconjugant cells are expected to grow at the same rate as recipient cells of the same OTU, as pKJK5 plasmid carriage does not selectively affects growth. An artefactual underestimation of T/R due to recipient growth is, therefore, unlikely as similar growth is expected for the respective transconjugant. An overestimation of T/R due to transfer occurring before die-off of heavy-metal sensitive recipients OTUs, is unlikely as transfer can only be detected in transconjugant cells that maintain sufficient metabolic activity to ensure GFP production. Hence, T-to-R ratios can be compared across treatment conditions, to evaluate permissiveness differences across OTUs.

Artificial permissiveness changes could be inferred if the abundance of non-participatory (those with zero permissiveness) recipient OTUs would dramatically increase or decrease due to selective growth or death. However, 94-97% of all sequences within each recipient pool belonged to OTUs also found in the corresponding transconjugal pools, and an effect of changes in non-participatory OTU cell abundance was excluded by calculating alternative  $\delta$ -values based on relative abundances after excluding all non-participatory OTUs for each treatment. These corrected  $\delta$ -values are virtually identical to those calculated for the whole

recipient community (SI Figure 3) due to the low abundance of non-participatory OTUs. Additionally, such effects would manifest in a consistent in- or decrease in all  $\delta$ -values across all OTUs within one treatment. For all treatments  $\delta$ -values were distributed with a median insignificantly different from 1, the no-effect level on permissiveness if all OTUs would participate in plasmid transfer (SI Figure 4). Thus, changes in the recipient pool composition did not affect the measured  $\delta$ -values to a significant degree.

Our experimental conditions resemble the agronomic practice of manure application to soil. Pig manures (especially from piglets) often contain elevated levels of Cu and Zn (Nicholson et al., 2003) used as feed additives (Jondreville et al., 2003). These are introduced to soil alongside a high load of fecal bacteria hosting various antibiotic and metal resistance plasmids (Smalla et al., 2000; Zhu et al., 2013). We similarly introduce an enterobacterial exogenous donor strain to a soil bacterial community while simultaneously challenging it with metal stress. However, we remove the physical barriers that would limit the contact between freshly introduced plasmid donors and potential recipients in an intact soil matrix (Dechesne et al., 2005), thus assessing the full potential of the soil bacterial permissiveness.

Under reference conditions, approximately 1 out of 20,000 soil bacterial cells had the potential to receive and, at least transiently, host plasmid pKJK5, consistent with earlier permissiveness measurements of soil bacterial communities for IncP-1 $\alpha$ , IncP-1 $\epsilon$ , and IncPromA broad host range plasmids (Musovic *et al.*, 2014; Klümper *et al.*, 2015). Despite stress being commonly associated with increasing bacterial evolvability and promoting gene transfer (Gillings & Stokes, 2012), we here show that community-level plasmid acquisition decreased consistently and significantly (27-100%,  $p < 0.05$ ) with metal-stress independent of the metal, and even exceeded the degree of growth inhibition. This reduction in permissiveness might be the consequence of a decrease in metabolic status, the result of a specific response of plasmid transfer, plasmid replication or marker gene expression to metal exposure, or a combination of both. Higher stressor doses were furthermore associated with a further decrease in transfer frequency. Plasmid transfer frequency reduction, previously documented for Zn stress (De Rore et al., 1994), might be common across soil bacterial communities for a multitude of different metals.

In spite of the observed absolute decrease in community permissiveness, several OTUs had a markedly increased permissiveness under stress conditions. For several OTUs, e.g. many OTUs belonging to the Bacteroidetes phylum a more than 10 fold bigger proportion of recipient cells carried plasmid pKJK5 after stress treatment. Thus, the chance of gene transfer from the enterobacterial host across phyla to the soil indigenous community has the potential to significantly increase due to metal stress. This short-term increase might especially play a crucial role for the dissemination of plasmid encoded antibiotic resistance from manure as their original fecal hosts are quickly lost due to competitive exclusion (Estrada et al., 2004). In this way, the soil bacterial community can serve as a long-term reservoir for plasmids as elevated retention of plasmids can be observed under long-term metal stress, even for plasmids not coding for metal resistance (Smets et al., 2003).

Although studied for decades, our current knowledge of metal-bacteria interactions is insufficient to pinpoint the mechanisms that link metal exposure to the observed modulation

in permissiveness. Several generic and specific mechanisms of metal stress have been described. The cationic metals used in this study (Cd, Cu, Ni, Zn) as well as the anion arsenite share the toxic mechanism of disrupting iron-sulfur clusters of metalloenzymes (Macomber & Hausinger, 2011; Macomber & Imlay, 2009; Xu & Imlay, 2012; Hughes, 2002). Metal cations are excreted from bacterial cells as a stress response using efflux systems of similar types (P-type, RND, CDF) (Nies, 1999), while arsenite has its own type of efflux system (A-type) (Nies, 1999). All bacteria have a certain tolerance level to metal stress. Thus, for a given exposure, a gradient of stress levels ranging from sub-toxic to toxic or even lethal conditions can exist within a community (Rensing *et al.*, 2002). Due to the multitude of mechanisms of metal toxicity and resistance present in different bacteria, it is safe to assume that although introduced at the same community growth inhibition doses, each element caused diverse specific short-term stress responses in individual community members.

Taking advantage of the high resolution identification of transconjugal OTUs, we demonstrated that stress-induced modifications of permissiveness are indeed unique for each OTU. However, the modified permissiveness of an OTU was generally consistent at least across most metal stresses. In contrast, the bacterial response to the metalloid arsenic was not as strongly correlated with the four metals. One potential explanation could stem from a connection between the regulation of efflux pumps, which are similar across metal ions but different for arsenite (Nies, 1999), and that of permissiveness.

Furthermore, phylogenetically similar OTUs tended to act similarly in their short-term response to different stresses. The stress-induced regulation of permissiveness thus seems to be taxon-dependent, potentially due to evolutionary conservation, and might well be connected to the regulation of the defense mechanisms against foreign DNA, such as CRISPR-cas or restriction-modification (RM) systems in the specific phylogenetic groups. While the different regulatory control systems for CRISPR-cas systems are not well understood (Mojica & Díez-Villaseñor, 2010), specific stress responses can lead to induced expression of CRISPR-cas genes, decreasing the plasmid receipt potential (Perez-Rodriguez *et al.*, 2011).

The expression of RM genes is not constant, but depends on environmental conditions (Bayliss *et al.*, 2006), and diverse stressors can play a role in their regulation (Schafer *et al.*, 1994). The observed phylogenetic conservation holds not only true across the highly correlated heavy metal stresses, but also for arsenic stress, thus further indicating that permissiveness towards plasmids might be directly coupled to different stress responses.

In pure cultures, the ability to uptake foreign DNA changes as a response to stress for specific stressors (Arango Pinedo & Smets, 2005; Pérez-Mendoza & de la Cruz, 2009). Due to the phylogenetic conservation of the stress response, results from single strain experiments might have predictive value based on their phylogeny and for diverse stresses of the same type. However, based on the highly diverse responses across different community members neither single strain, nor community averaged analyses suffice to predict the effect of stress on plasmid receipt across complex communities. Our results show that

understanding the effect of stress on the ecology of plasmid transfer can only be achieved by examination at both community and individual strain levels.

We demonstrated here that the stress-response mechanisms affecting the permissiveness among phylogenetically related bacterial groups are similar and consistent for a variety of different heavy metal stresses. Extrapolation of our results to phylogenetically related groups of bacteria and other stressors might therefore be valid. To further understand the stress induced modulation of permissiveness the exact cellular responses remain to be elucidated.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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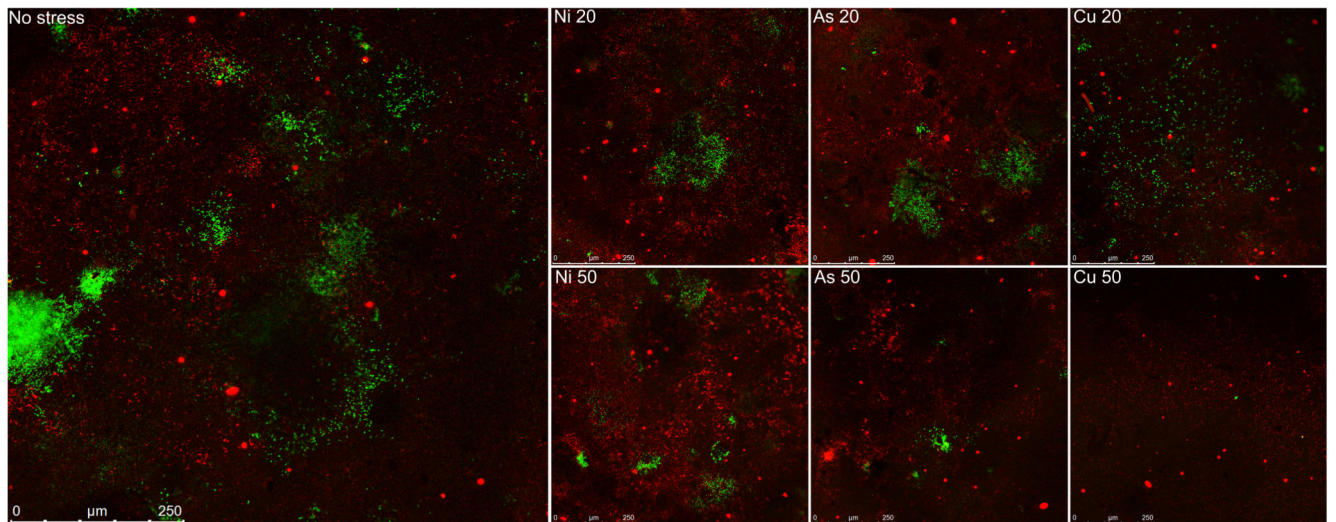


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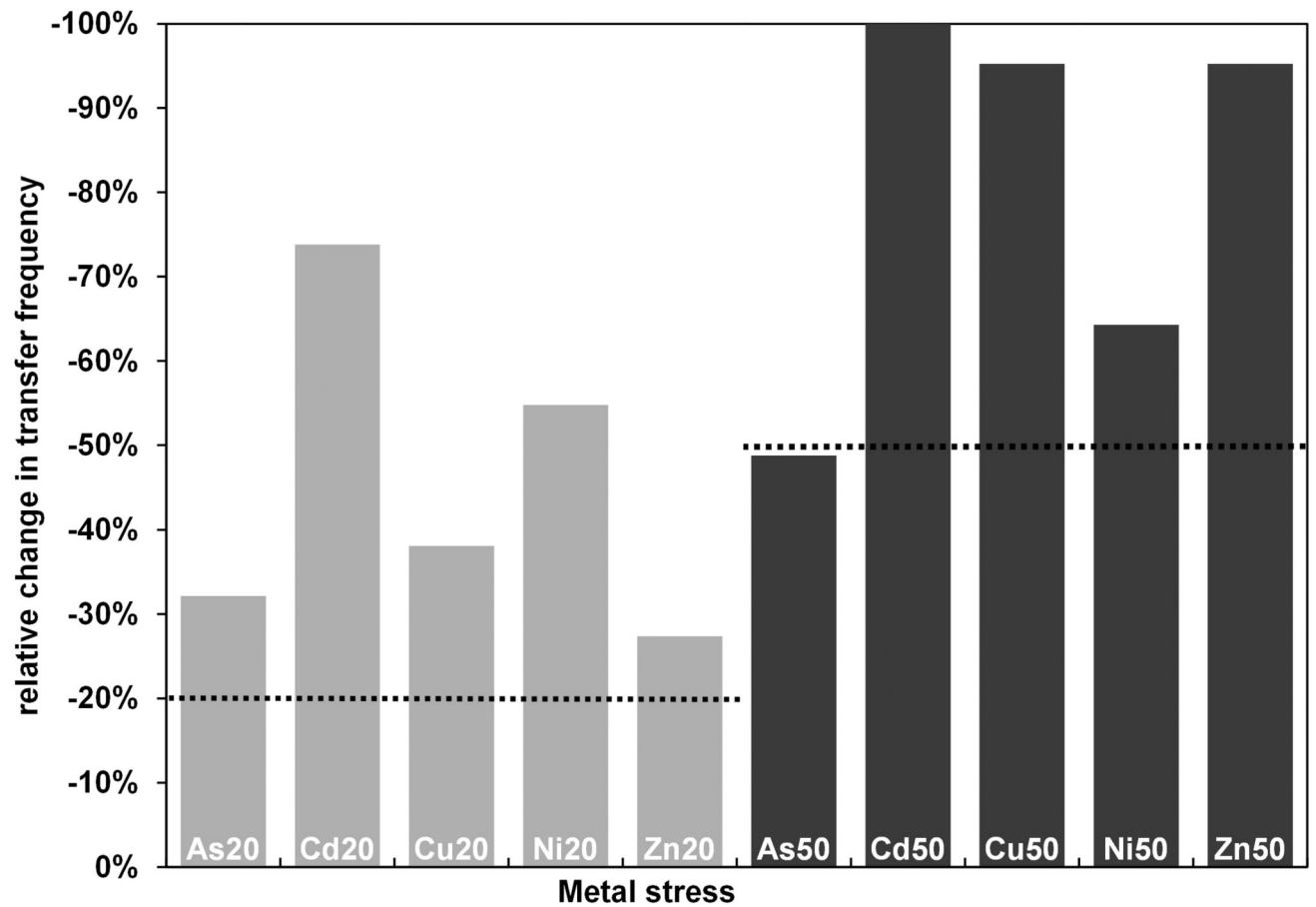
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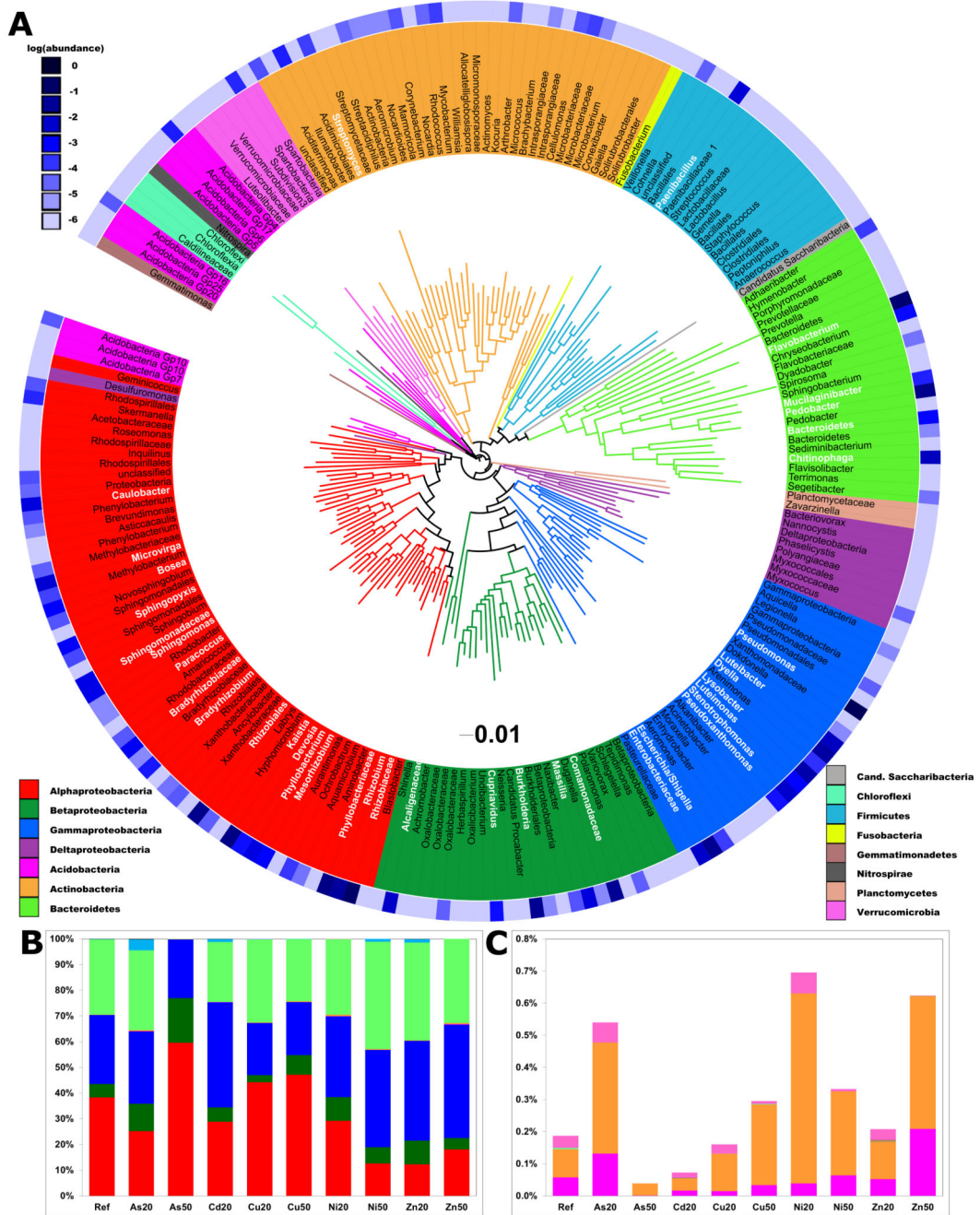
**Figure 1.**

Images of plasmid transfer from the *E. coli* MG1655 donor strain to soil bacteria at reference condition (no stress) (left panel) or as affected by metal stress. Type of metal (Ni, As or Cu) and degree of stress are indicated in the top left corner of the other panels; number refers to % inhibition of [ $^3$ H]leucine incorporation for the Nycodenz-extracted recipient bacterial community. Donor bacteria, chromosomally tagged with *mCherry* are red, recipient bacteria are colourless until successful plasmid receipt and subsequent *gfp* expression, transconjugants appear green.



**Figure 2.**

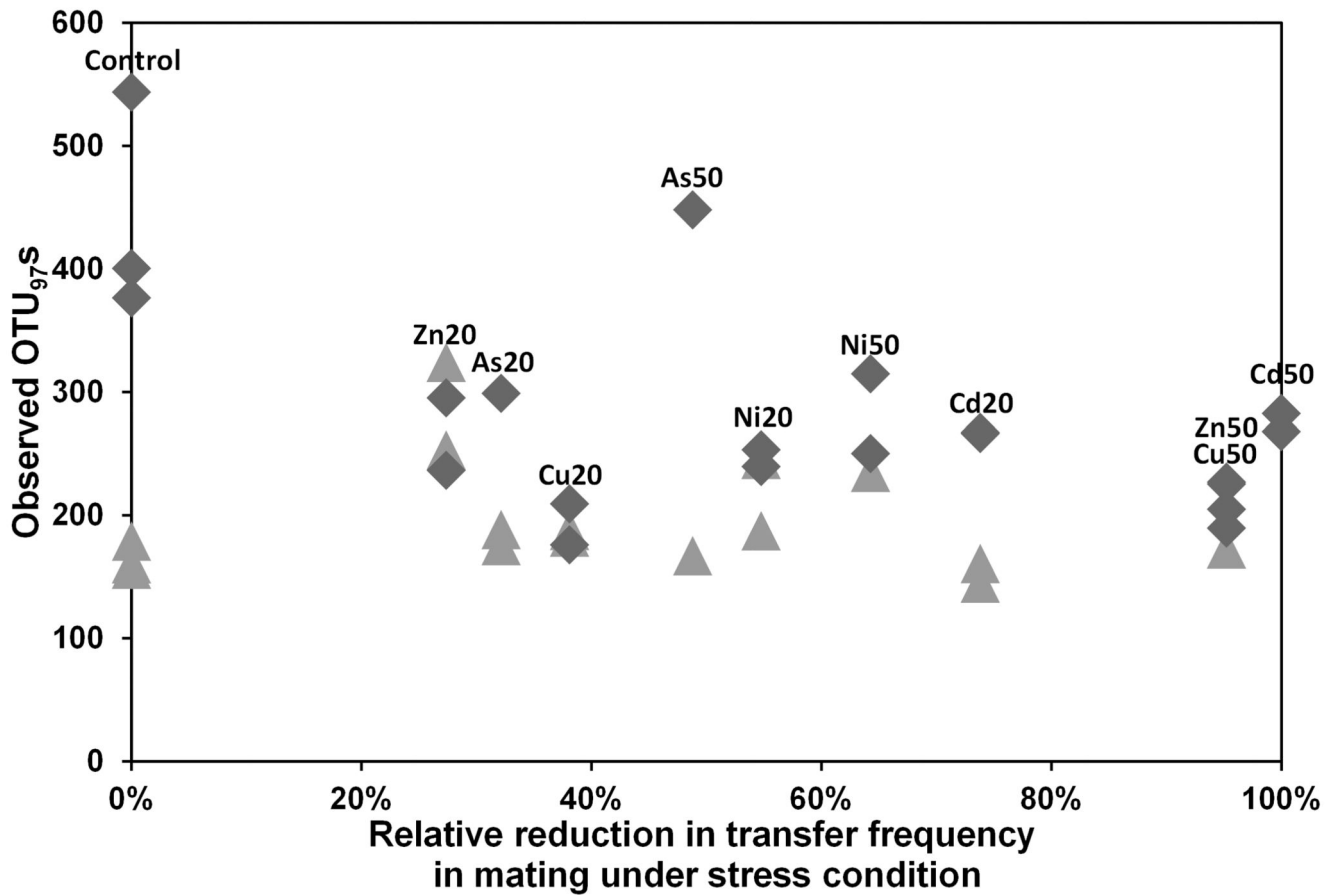
Reduction of pKJK5 transfer frequency under metal-induced stress conditions as compared to the reference condition (0% value). Type of metal (As, Cd, Cu, Ni, or Zn) and degree of stress are indicated on individual bars; numbers refer to inhibitory concentrations decreasing [ $^3\text{H}$ ]leucine incorporation rates of the recipient bacterial community by 20% (IC<sub>20</sub>, grey bars) and 50% (IC<sub>50</sub>, black bars). Plasmid transfer frequencies were calculated based on the analysis of 90-150 images per condition.



**Figure 3.** Phylogenetic composition of transconjugal pools. A: Phylogenetic tree showing all 206 identified transconjugant OTUs. Colors of the branches mark different phylogenetic groups. The 38 most abundant OTUs (>0.05% relative abundance across all pools), used in subsequent OTU permissiveness analysis are shown in white letters. The blue heatmap-circle at the periphery of the tree represents the log transformed relative OTU abundance in the reference recipient community. B: Phylogenetic composition of the transconjugal pools obtained at various stress conditions. C: Relative abundance of OTU identified as rare phyla

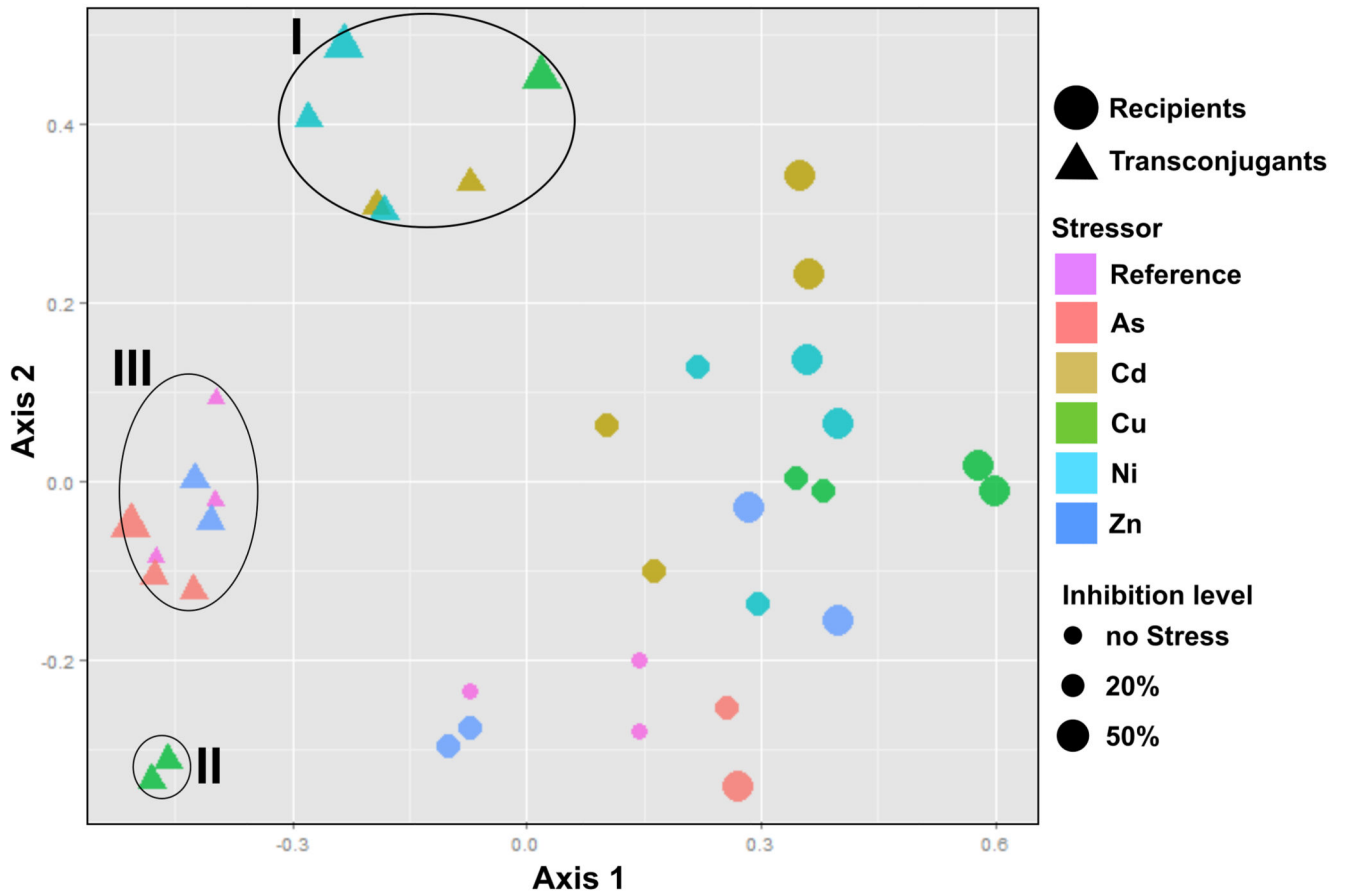


(<1% mean relative abundance) for the various stress conditions. Experimental treatments (i.e. type of metal and degree of stress) are indicated below the bars for panels B and C; numbers refer to inhibitory concentrations decreasing [<sup>3</sup>H]leucine incorporation rates of the recipient bacterial community by 20% (IC<sub>20</sub>) and 50 % (IC<sub>50</sub>). ‘Ref’ denotes reference condition (unstressed control).



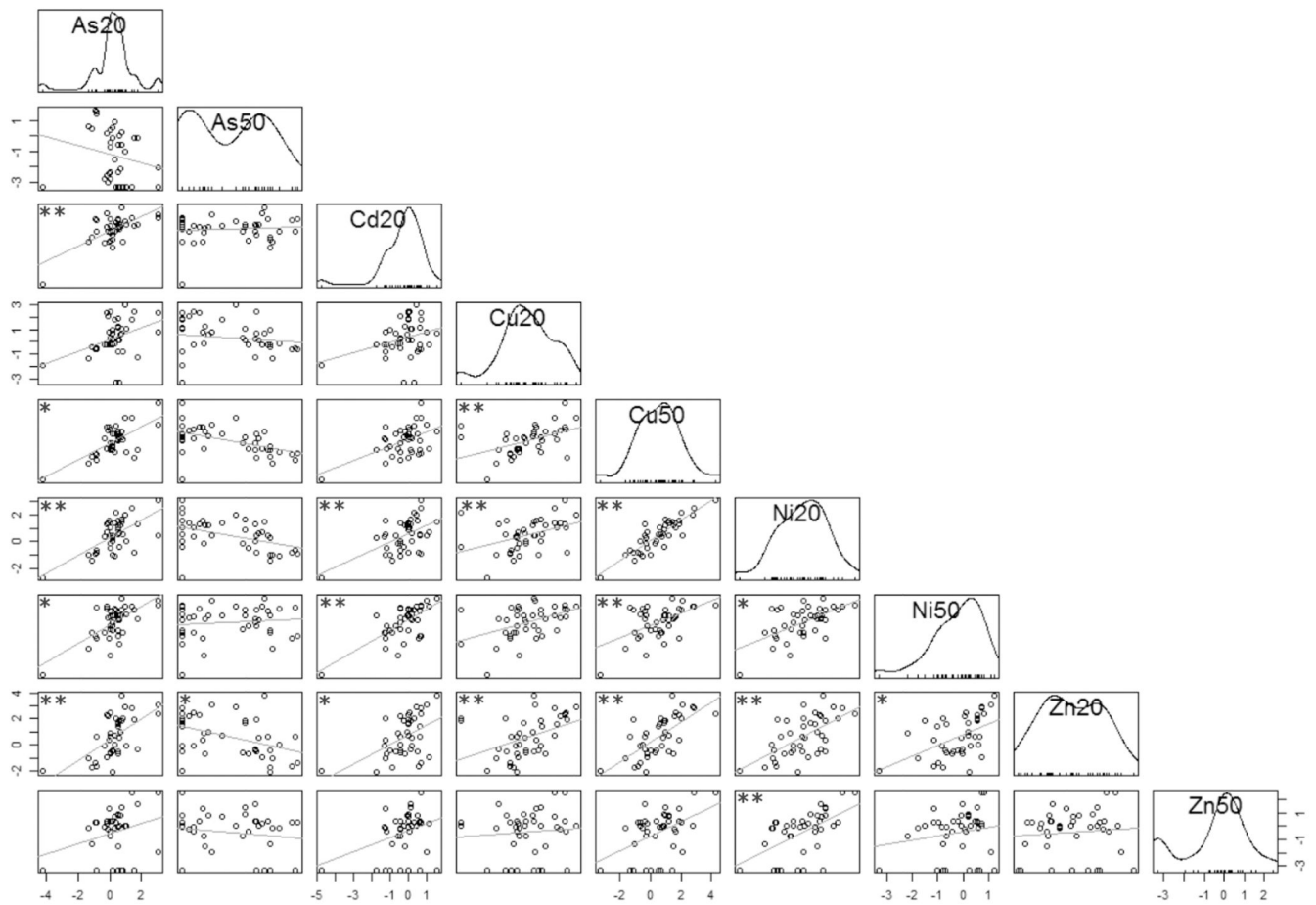
**Figure 4.** Observed number of unique operational taxonomic units (OTUs; 97% sequence similarity) as a function of reduction in plasmid transfer frequency caused by metal stress. The OTU richness of the transconjugal pools (triangles) and the corresponding sorted soil recipient communities (diamonds) is shown. Experimental treatments (i.e. type of metal and degree of stress) are indicated; numbers refer to inhibitory concentrations decreasing [<sup>3</sup>H]leucine incorporation rates of the recipient bacterial community by 20% (IC<sub>20</sub>) and 50% (IC<sub>50</sub>).





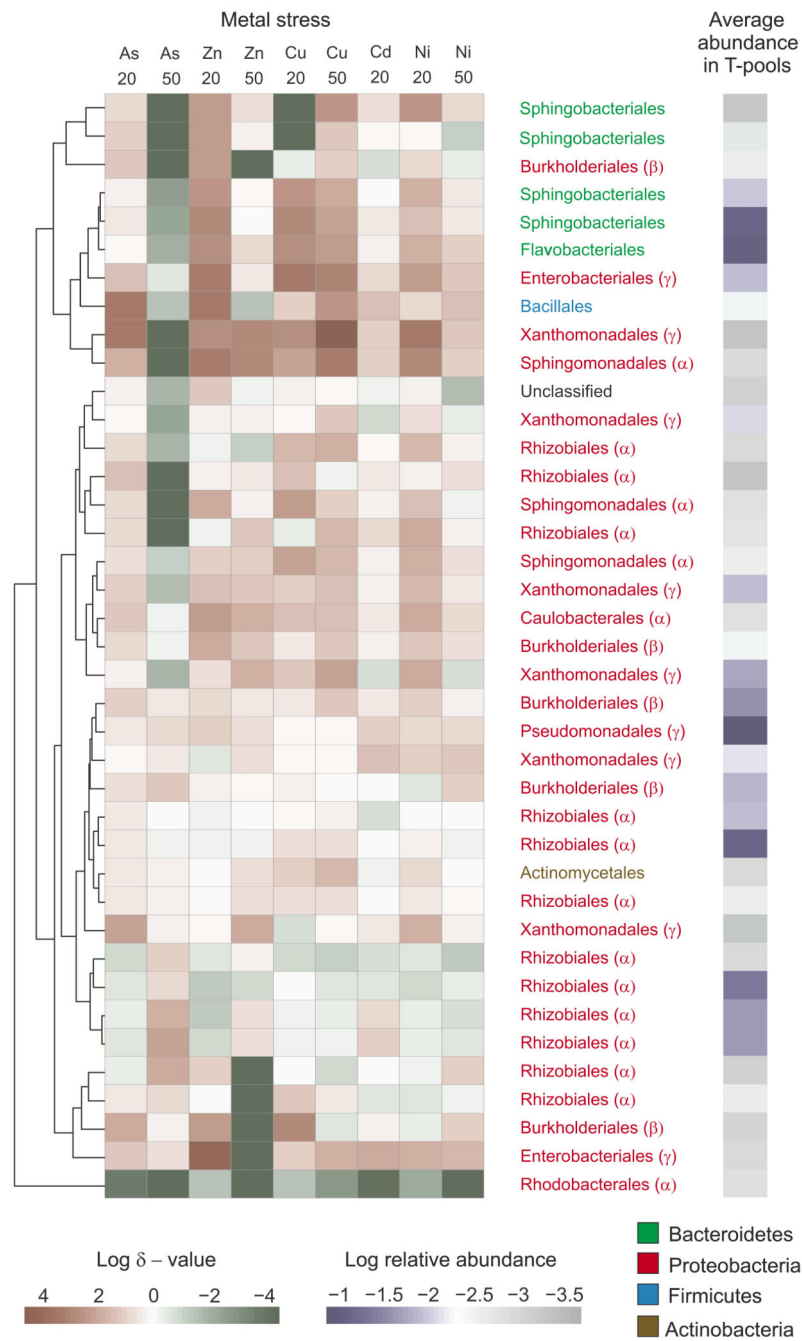
**Figure 5.**

Non-metric 2-dimensional scaling analysis (NMDS) revealing distinct clustering of transconjugal pools (circles) and recipient communities (triangles). Three transconjugal clusters are identified and named with roman numerals. Experimental treatments (i.e. type of metal and degree of stress as operationally defined by % inhibition of [ $^3\text{H}$ ]leucine incorporation rates) are shown by color and size of symbols, respectively. Amplicon pools were subsampled at 50,000 sequences. OTUs from the recipient pool that were not represented in any transconjugal pool were removed prior to the ordination based on weighted OTU abundance using the Theta Yue Clayton (Yue & Clayton, 2005) algorithm.



**Figure 6.**

Pairwise correlation analysis of the modification of the permissiveness ( $\log(\delta)$ ) across metal treatments for all 206 bacterial OTUs to explore whether two stress conditions resulted in similar response. Each circle symbol in the diagrams corresponds to one OTU plotted according to its  $\log(\delta)$  under the two compared conditions. Lines show linear regression. Star(s) in the top left corner indicate the significance of the Spearman correlation (\*,  $p < 0.05$ , \*\*,  $p < 0.005$ ) after correction for multiple testing (Hommel, 1988). Experimental treatments (i.e. type of metal and degree of stress) are indicated with numbers referring to inhibitory concentrations decreasing [ $^3\text{H}$ ]leucine incorporation rates of the recipient bacterial community by 20% ( $\text{IC}_{20}$ ) and 50% ( $\text{IC}_{50}$ ).



**Figure 7.** Maximum likelihood tree clustering of the 38 most abundant OTUs in the transconjugal pools (relative average abundance >0.05%) based on similarity in their changes in permissiveness under different stress conditions. The heatmap shows the relative change in permissiveness ( $\delta$  value) of each OTU: an increased plasmid receipt response is shown in red, a decreased one, in green. The average relative abundance of an OTU across all transconjugal pools is shown in violet (right). Their taxonomy is indicated by the color of the font (for Proteobacteria, the class is shown in brackets). Experimental treatments (i.e.

type of metal and degree of stress) are indicated above the heat map with numbers referring to inhibitory concentrations decreasing [ $^3\text{H}$ ]leucine incorporation rates of the recipient bacterial community by 20% ( $\text{IC}_{20}$ ) and 50 % ( $\text{IC}_{50}$ ).

**Table 1**

Inhibitory Concentrations causing 20% (IC<sub>20</sub>) and 50% (IC<sub>50</sub>) bacterial growth inhibition as inferred by dose-response modeling of [<sup>3</sup>H]leucine incorporation data (SI Figure 1). Treatment names used throughout this paper are indicated in brackets.

<b>Metal</b>	<b>IC<sub>20</sub>(<math>\mu</math>M)</b>		<b>IC<sub>50</sub>(<math>\mu</math>M)</b>	
AsO <sub>3</sub> <sup>3-</sup>	40.5	(As20)	125.2	(As50)
Cd <sup>2+</sup>	12.6	(Cd20)	63.6	(Cd50)
Ni <sup>2+</sup>	3.7	(Ni20)	11.5	(Ni50)
Zn <sup>2+</sup>	24.7	(Zn20)	80.7	(Zn50)
Cu <sup>2+</sup>	6.9	(Cu20)	28.9	(Cu50)