Title:

Different metal stressors consistently modulate bacterial conjugal plasmid uptake poten-

tial in a phylogenetically conserved manner

Short title:

5   Metals affect conjugal plasmid uptake

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Abstract

The environmental stimulants and inhibitors of conjugal plasmid transfer in microbial

30   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

35   recipient community, and a previously described cultivation-minimal solid surface 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 could be isolated and phylogenetically described at high

resolution via FACS and subsequent 16S rRNA amplicon sequencing. Metal stress

40   consistently decreased plasmid transfer frequencies to the community vs reference

conditions, 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 modulated plasmid permissiveness

45   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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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.*, 2014c) 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 serve 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, bacteria might increase their plasmid uptake potential, or individual permissiveness, as a stress response.

Earlier single strain experiments have shown modulation in gene uptake as a result of stress exposure. In *Streptococcus pneumonia*, exposure to antibiotics caused increased competence and increased promiscuity towards foreign DNA (Slager *et al.*, 2014). In *Pseudomonas putida* pre-exposure to sodium dodecyl sulfate (SDS) increased its ability to receive and maintain plasmids, possibly by repressing restriction-modification systems (Pinedo & Smets, 2005). Similarly, in *Bacillus subtilis*, sub-inhibitory concentrations of ethanol activated the conjugative transposition of transposon Tn916 (Seier-Petersen *et al.*, 2013), involved in the spread of 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 effect composition and function of soil bacterial communities (Giller *et al.*, 1998; Gans *et al.*, 2005; Berg *et al.*, 2012). Selection for adaptive plasmids is known as a long term effect to metal exposure (Giller *et al.*, 1998; Campbell *et al.*, 1995). Thus, development of community tolerance upon metal stress is common even when no effects are observed at the level of activity or community composition (Brandt *et al.*,

2010). However, the existence and magnitude of short-term impact of metal stress on the

100   permissiveness of bacterial communities remains yet to be explored.

In this study, we combined a cultivation-minimal assay to measure community-wide permissiveness (Klümper et al., 2014c) with normalized stress exposure to five of the most environmentally relevant metal(oid)s (Cu^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+}, AsO_3^{3-}). This allowed for direct comparison of short-term metal stress effects on the permissiveness towards the

105   *gfp*-labelled, model broad host range IncP-1ε plasmid pKJK5 (Bahl *et al.*, 2007) introduced through an *E. coli* donor strain in a reference soil bacterial community. Plasmid transfer frequencies and the phylogenic composition of the transconjugal pools were compared to corresponding controls to assess stress-imposed modulation of permissiveness of the community and individual community members.

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Material & Methods

Soil sampling

Soil (16±1% clay, 15±1% silt, 43% fine sand and 26±1% coarse sand, with a pH(H$_2$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^2 mesh filter, homogenized and stored at 4˚C for up to a month before conducting the experiments.

Soil bacterial community extraction

Indigenous bacterial communities were recovered from 30 g sub-samples of homogenized soil by Nycodenz®-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^{-7} cells/mL to quantify metal induced stress (measured as growth rate inhibition) or to be used as recipients in the filter mating assay.

[^{3}H]leucine incorporation inhibition assay

A [^{3}H]leucine incorporation assay (SI Text 1) was used to estimate concentrations of individual metal(loids)s inhibiting soil bacterial growth rates by 20% (IC$_{20}$) and 50 % (IC$_{50}$), 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 *Escherichia coli* MG1655::*Km^R-Lpp-mCherry* (Klümper, *et al.*, 2014c) hosting the IncP-1ε broad host range plasmid pKJK5::*gfpmut3b* (Bahl *et al.*, 2007) were used as

donors. Plasmid pKJK5 has an extremely broad transfer range in the soil bacterial com-community covering both Gram negative and Gram positive phyla (Klümper *et al.*, 2014c). Additionally, pKJK5 does not encode for any known 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^q 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^q* 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.*, 2014c). The donor strain was grown overnight in LB-medium supplemented with trimethoprim (30 μg mL^{-1}), harvested by centrifugation, washed in 0.9% NaCl-solution, adjusted in cell density (OD$_{600}$) and used in filter mating assays as described previously (Klümper *et al.*, 2014a).

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) (SI Text 1). Filter matings were used 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).

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, 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 was performed based on bacterial size, green fluorescence and exclusion of red fluorescent donor cells as described earlier (Klümper, *et al.*, 2014c). Recipient community cells were sorted from the same samples using the same conditions, but excluding green fluorescence in the gating. Therefore, all red fluorescent donor cells were excluded, while green transconjugants and colorless recipient cells which did not engage in conjugation were sorted. 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.*, 2014c).

Sequence analysis

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. Sequences were classified based on the RDP classifier (Wang *et al.*, 2007). Sequences are submitted under accession number XXX-XXX.

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 recipients (R) and of the conjugation events (T) make it possible to measure permissiveness accurately. This is impossible at the OTU level, because only the relative abundance of an OTU in the recipient community (after mating incubation) of an OTU in the transconjugant pool can be measured (SI Figure 2). These values are not good estimators of the initial recipient number and of the conjugation events for a specific OTU because they are affected by that OTU's growth rate relative to other community members and by other biases (e.g. multiple transfers within a OTU microcolony). Hence, we can only calculate an apparent OTU-level permissiveness under a given condition:

$$AP_i = \frac{Abundance\ in\ transconjugal\ pool\ of\ OTU_i}{Abundance\ in\ recipient\ community\ of\ OTU_i} = \frac{T_i}{R_i}$$

Nevertheless, comparing changes in apparent permissiveness for an OTU between reference and stress conditions is possible because the biases present in the apparent individual permissiveness (AP) for each condition should largely cancel each other out.

We therefore calculate the ratio (δ) of an OTU's apparent permissiveness under stress ($AP_{i\,stress}$) and reference conditions ($AP_{i\,ref}$):

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

If the imposed stress has no effect on an OTU's permissiveness, δ would equal 1. A δ-value above or below 1 would indicate an increase or decrease in permissiveness in the examined OTU under the stress condition, respectively.

To analyze whether the variability of δ-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 δ-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 independently of activity reduction

Inhibitory metal concentrations (IC_{20} or IC_{50}) applied in the mating experiments were determined by dose-response modeling of [^{3}H]leucine incorporation data (SI Figure 1).

220   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 referred to as a combination of the elemental symbol and the inhibitory level (e.g. As20 *see* Table 1) and compared to the non-stressed reference mating.

Conjugation events were detected as green fluorescent cells and microcolonies in
225   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 the donor cell concentration was in all cases sufficiently high to ensure that each recipient microcolony was in contact with multiple donor cells.

230   Conjugation events were detected at high frequencies under reference conditions, with approx. 1 in 15,000 ($6.8 \times 10^{-5} \pm 3.8 \times 10^{-5}$) of the initial soil bacterial cells taking up pKJK5. This frequency decreased visibly 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
235   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 transfer frequency reduction.

Reduction in community permissiveness could result from a general decrease of transfer across all taxa or from taxon specific modulation of individual permissiveness. To investigate the possible reasons further, we FACS-sorted from each mating at least 20,000 transconjugant cells, which were subject to 16S rRNA amplicon sequencing.

All transconjugal pool sequence libraries showed coverage above 98%, and subsampling to a depth of 50,000, showed no significant dissimilarity (p>0.99) based on weighted UNIFRAC comparisons of 10 subsampled sets of sequences. 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 (α, β, γ and δ) 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 pools, in spite of its abundance in the corresponding recipient community. 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 abundance up to 8-fold under several stress conditions.

The average richness (α-diversity) of the transconjugal pools was 196.0 ± 14.4 OTUs, irrespective of transfer frequency reduction (Figure 4). While 38.5 ± 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: pKJK5 permissive OTUs survive better than non-permissive OTUs under metal stress. A total of 94-97% of the transconjugal sequences under metal stress conditions belonged to OTUs also found in the reference transconjugal pool. 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).

The phylogenetic composition of the transconjugal pools depended on the type and severity of the imposed metal stress. Replicate transconjugal pools and recipient communities consistently grouped together. Three main clusters were observed among the transconjugal pools (Figure 5). Cluster I comprised of the Cu50, Cd20, Ni20 and Ni50 replicate pools and 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 proposed mechanism, i.e. modulation of individual permissiveness, could be detected. Hence, we analyzed, for each of the 38 dominant transconjugal OTUs (Figure 3) and each metal, the change in permissiveness (δ) 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 most OTUs (263 of 342 tested combinations) increased or decreased more than two-fold in response to stress.

Under some conditions (As20, Cu20, Cu50, Ni20, and Zn20), individual permissiveness increased for most of the dominant OTUs (SI Figure 3). In contrast, arsenic at high concentration decreased the permissiveness of the majority of the OTUs. Individual OTUs tended to respond in consistent manner to different metal stresses. Of 15 pairwise correlations of the δ-values among the metal stresses (Cd20, Cu20, Cu50, Ni20, Ni50 and Zn20) 12 were significant (p<0.05) (Figure 6). The δ-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

-15-

metals; here all OTUs belonging to the Bacteroidetes phylum displayed a strong decrease

310 in permissiveness (Figure 6).

Despite 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 Rhodobacteriales family, responded to every applied stress with a significant decrease in plasmid uptake (always more than 100 fold and up to

315 5,000 fold) (Figure 7).

Phylogenetically similar OTUs responded similarly to metal stress. All Bacteroidetes OTUs responded similarly to the metal treatments (Figure 7): for all stresses except As50, plasmid transfer from the proteobacterial *E. coli* donor to the Bacteroidetes phylum increased by more than 10-fold. The only gram-positive OTU among the 38 most

320 abundant transconjugant OTUs, a member of the Firmicutes phylum, responded in a similar way. For most of the transconjugal OTUs in the Proteobacteria phylum, the stress response was more variable. Four of these OTUs, similar to Bacteroidetes, became increasingly permissive under stress conditions. Especially an OTU identified as Xanthomonadales responded with an increased permissiveness to most stresses and

325 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 δ-values across the community's phylogenetic tree was significantly correlated with OTU phylogeny (p<0.05) for two (Zn20, Ni20) or showed a tendency for

330 phylogenetic conservation (p=0.074-0.258) for six of the remaining seven stress conditions (SI Table 1).

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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.*, 2014c). 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 OTUs and covered 12 different phyla, thereby expanding the realized transfer range of IncP type plasmids in soil with two more phyla, *Nitrospira* & Cand. Saccharibacteria (Klümper, 2014c).

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, 1995). However, these long term effects offer no direct evidence for increased permissiveness towards plasmids because they may also be explained 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 effectively uncouple transfer and selection, thereby allowing mechanistic conclusions. As transfer is – furthermore - derepressed in pKJK5 (Bahl *et al.*, 2007), leading to constitutive pili expression, the observed effects were not controlled by donor activity, and viable donor cells were in excess under all conditions.

While earlier studies were able to show that the gene uptake of individual strains can differ under stress conditions (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. Transconjugants detected by flow cytometry and subsequent sequencing might either have stemmed from a primary plasmid transfer event, from a secondary transfer event, or from clonal growth with vertical replication. Thus, growth and vertical replication relative to other community members might interfere with the relative abundance of an OTU in the transconjugal pools and affect its apparent permissiveness. These biases cannot be measured but are, to large degree corrected in the δ-value by dividing the two apparent individual permissiveness values to evaluate the modulation in permissiveness (δ).

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

380   permissiveness measurements of soil bacterial communities for IncP-1α, IncP-1ε, and

IncPromA broad host range plasmids (Musovic *et al.*, 2014; Klümper, *et al.*, 2014c). De-

spite stress being commonly associated with increasing bacterial evolvability and promot-

ing 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

385   independent of the metal, and even exceeded the degree of growth inhibition. Higher

stressor doses were furthermore associated with a further decrease in transfer frequency.

Thus, plasmid transfer frequency reduction, earlier found for Zn stress (De Rore et al.,

1994) might be common across soil bacterial communities for a multitude of different

metals.

390   In spite of the observed absolute decrease in community permissiveness, several OTUs

had a markedly increased permissiveness under stress conditions. For several OTUs, this

increase is so large that we can conclude both the relative and the absolute abundances

of the transconjugants belonging to these OTUs increased upon stress exposure. For ex-

ample, we estimate that the absolute number of transconjugants belonging to the Bac-

395   teroidetes phylum more than doubled under specific metal stress conditions. Thus, the

chance of gene transfer from the enterobacterial host across phyla to the soil indigenous

community was significantly increased by metal stress. This short-term increase might

especially play a crucial role for the dissemination of plasmid encoded antibiotic re-

sistance from manure as their original fecal hosts are quickly lost due to competitive ex-

400   clusion (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 exact mechanisms that link metal exposure to the observed

405 modulation in permissiveness. However, several general and specific effect 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 a common main toxic mechanism by disrupting iron-sulfur clusters of metalloenzymes (Macomber & Hausinger, 2011; Macomber & Imlay, 2009; Xu & Imlay, 2012; Hughes, 2002). By contrast, metal cations are excreted from the

410 bacterial cell as a stress response using efflux systems of similar types (P-type, RND, CDF) (Nies, 1999), while arsenite has its own different 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 metal toxicity

415 and resistance mechanisms present in different bacteria, it is safe to assume that although introduced at the same community growth inhibition doses each element causes 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

420 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.

425 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 restriction-modification (RM) or CRISPR-cas systems in the specific phylogenetic groups. The expression of RM genes is not constant, but relies on environmental conditions (Bayliss *et al.*, 2006), therefore stress might very well play a role in their regulation. Cells with a turned off RM system have been shown to become hypersusceptible to foreign DNA or plasmid receipt (Corvaglia *et al.*, 2010) (Roer *et al.*, 2015). 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 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 of foreign DNA receipt changes as a response to stress for specific stressors (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 level.

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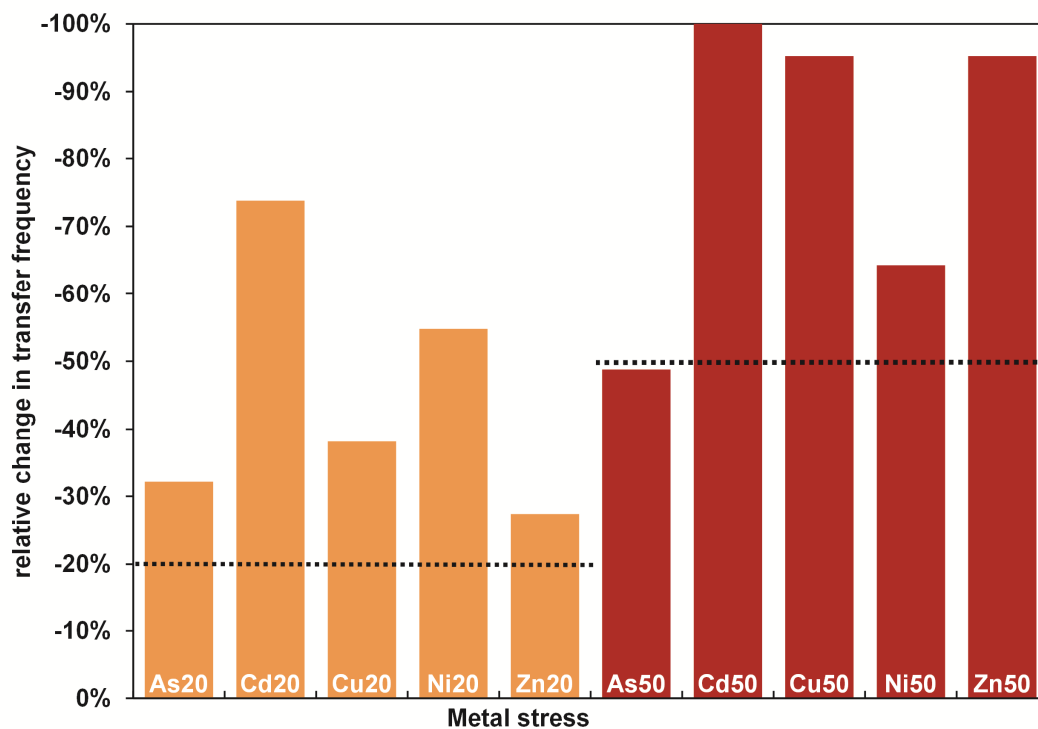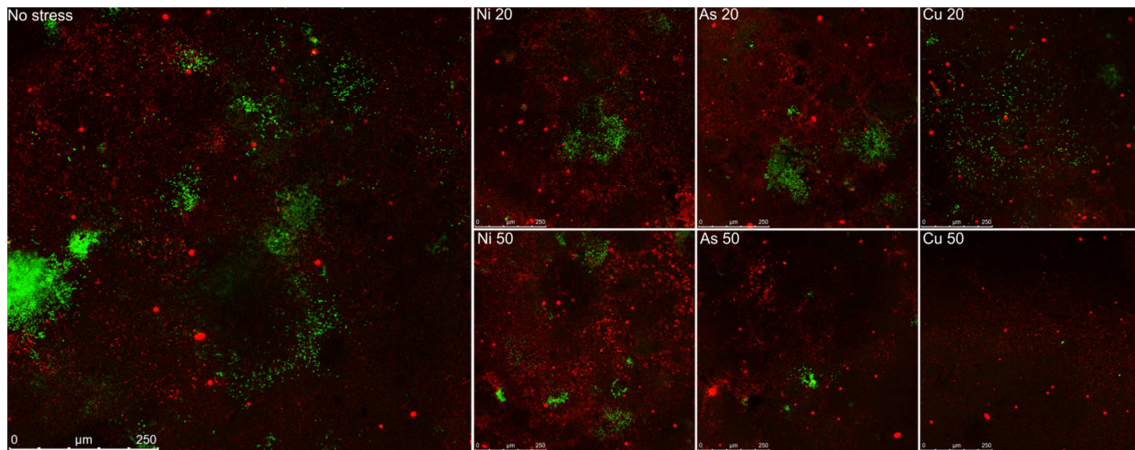
different heavy metal stresses. Extrapolation of our results to phylogenetically related groups of bacteria and other stressors might therefore be valid. To further understand the stress induced modulation of permissiveness the exact cellular responses remain to be elucidated.

Conflict of Interest

The authors declare no conflict of interest.

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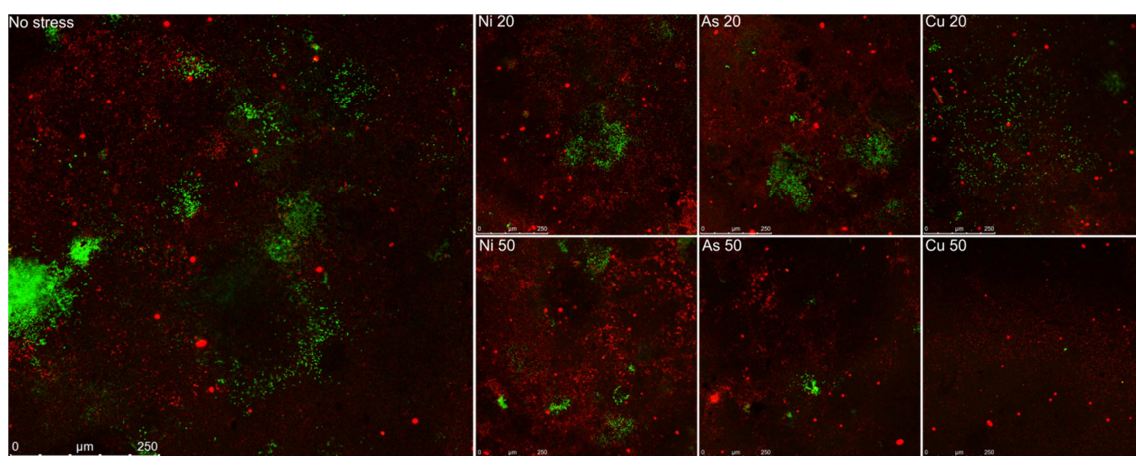Supplementary Information accompanies this paper on The ISME Journal website

640   (http://www.nature.com/ismej).

Table 1 Inhibitory concentrations causing 20% (IC_{20}) and 50% (IC_{50}) bacterial growth inhibition as inferred by dose-response modeling of [^{3}H]leucine incorporation data (SI Figure 1). Treatment names used throughout this paper are indicated in brackets.
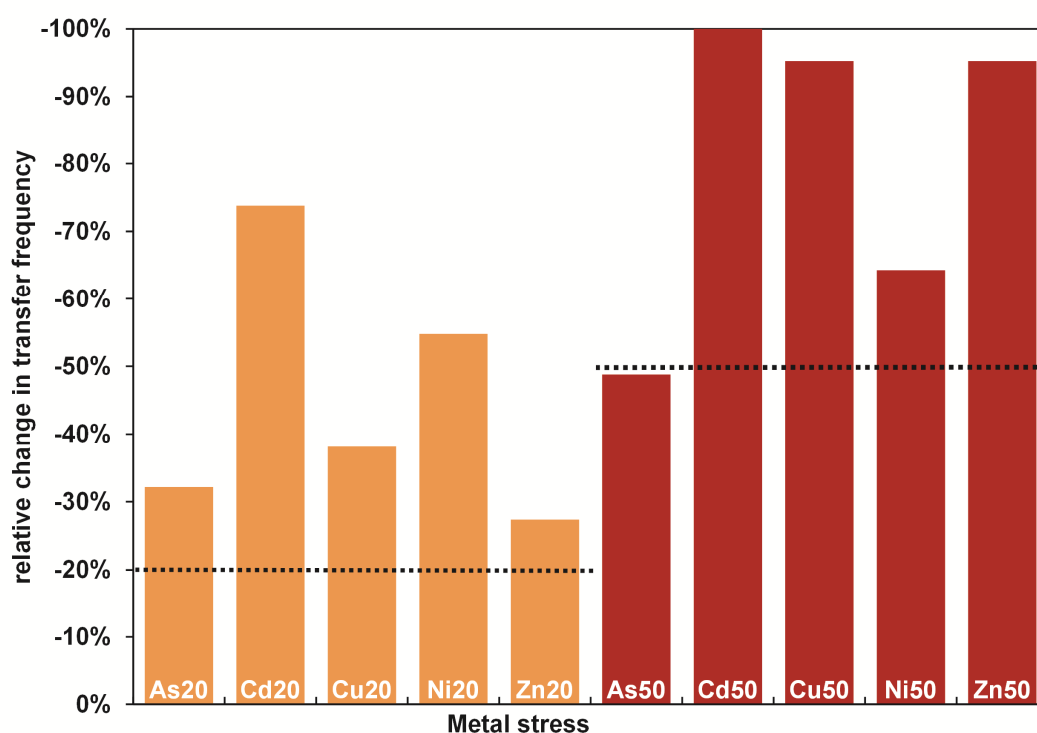
Table 1: Concentrations causing 20% (IC_{20}) and 50% (IC_{50}) bacterial growth inhibition as inferred by dose-response modeling of [^{3}H]leucine incorporation data (SI Figure 1). Treatment names used throughout this paper are indicated in brackets.

Metal	IC_{20} (µM)		IC_{50} (µM)	
AsO_3^{3-}	40.5	(As20)	125.2	(As50)
Cd^{2+}	12.6	(Cd20)	63.6	(Cd50)
Ni^{2+}	3.7	(Ni20)	11.5	(Ni50)
Zn^{2+}	24.7	(Zn20)	80.7	(Zn50)
Cu^{2+}	6.9	(Cu20)	28.9	(Cu50)

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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 [3H]leucine incorporation for the Nycodenz-extracted recipient bacterial community. Donor bacteria, chromosomally tagged with *mCherry* are red, recipient bacteria are colorless until successful plasmid receipt and subsequent *gfp* expression, transconjugants appear green.
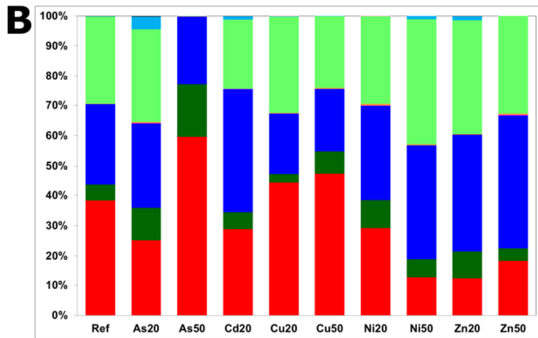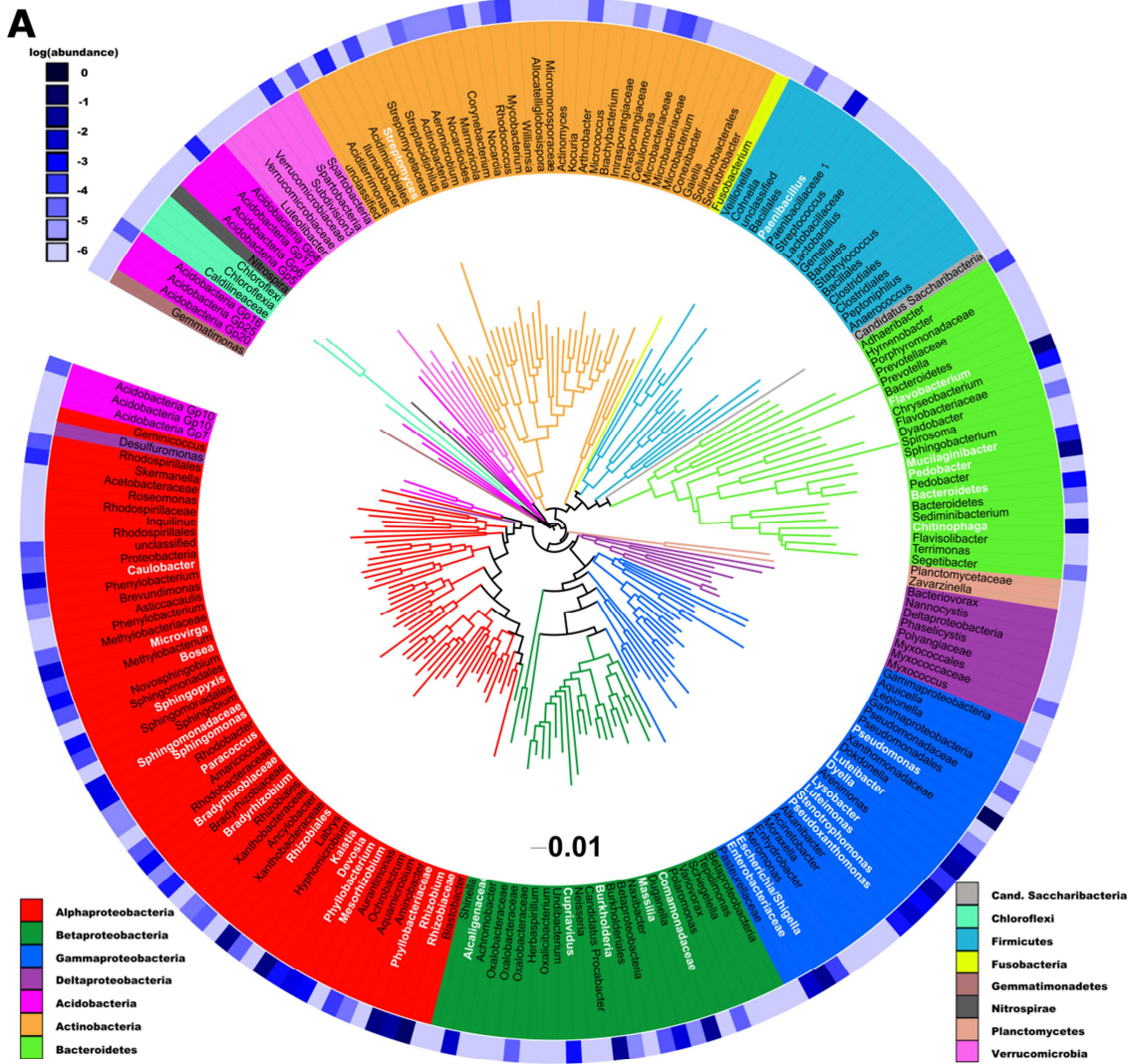
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Figure 3 Phylogenetic composition of transconjugal pools. A: Phylogenetic tree showing all 206 identified transconjugant OTUs. Colors of the branches mark different phylogenetic groups. The 38 most abundant OTUs (>0.05% relative abundance across all 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 [³H]leucine incorporation rates of the recipient bacterial community by 20% (IC$_{20}$) and 50 % (IC$_{50}$). 'Ref' denotes reference condition (unstressed control).

680 **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

685 [3H]leucine incorporation rates of the recipient bacterial community by 20% (IC_{20}) and 50
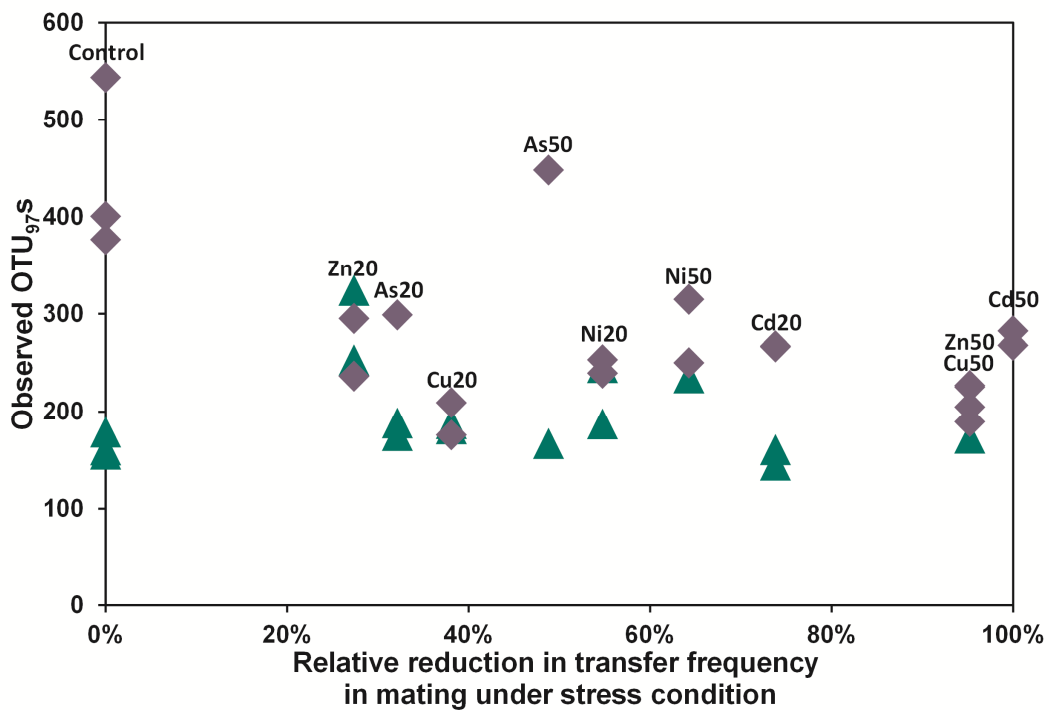
% (IC_{50}).

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}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.
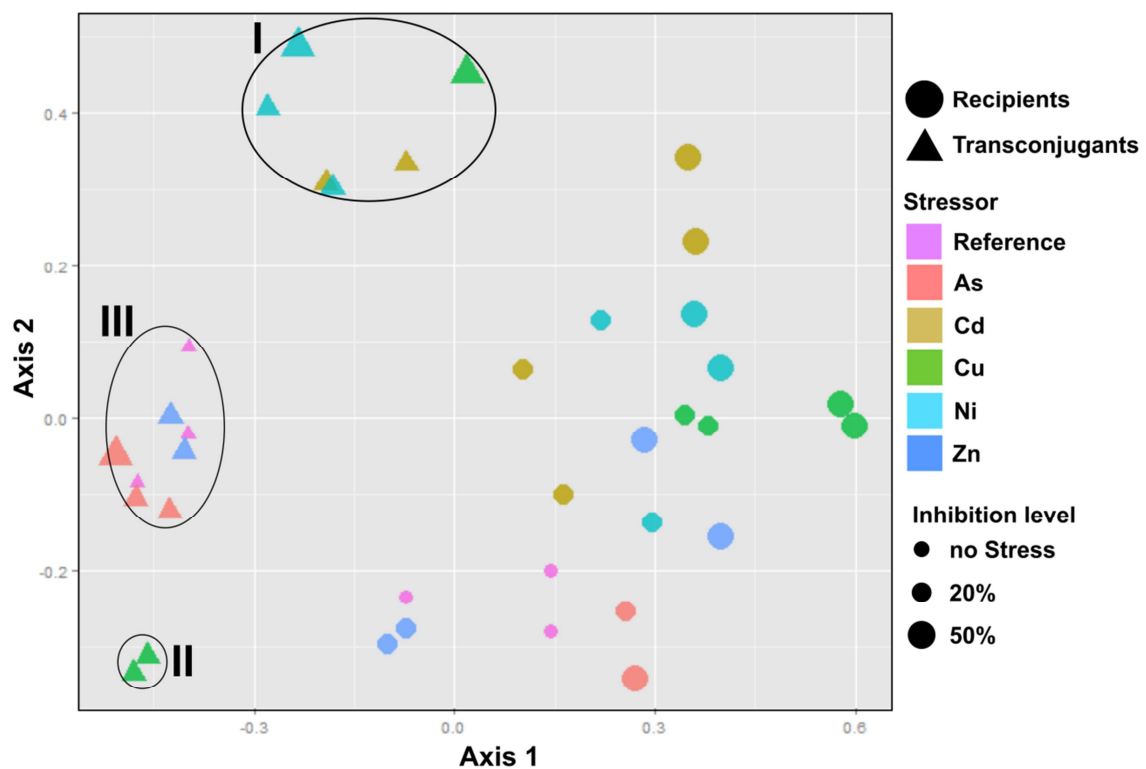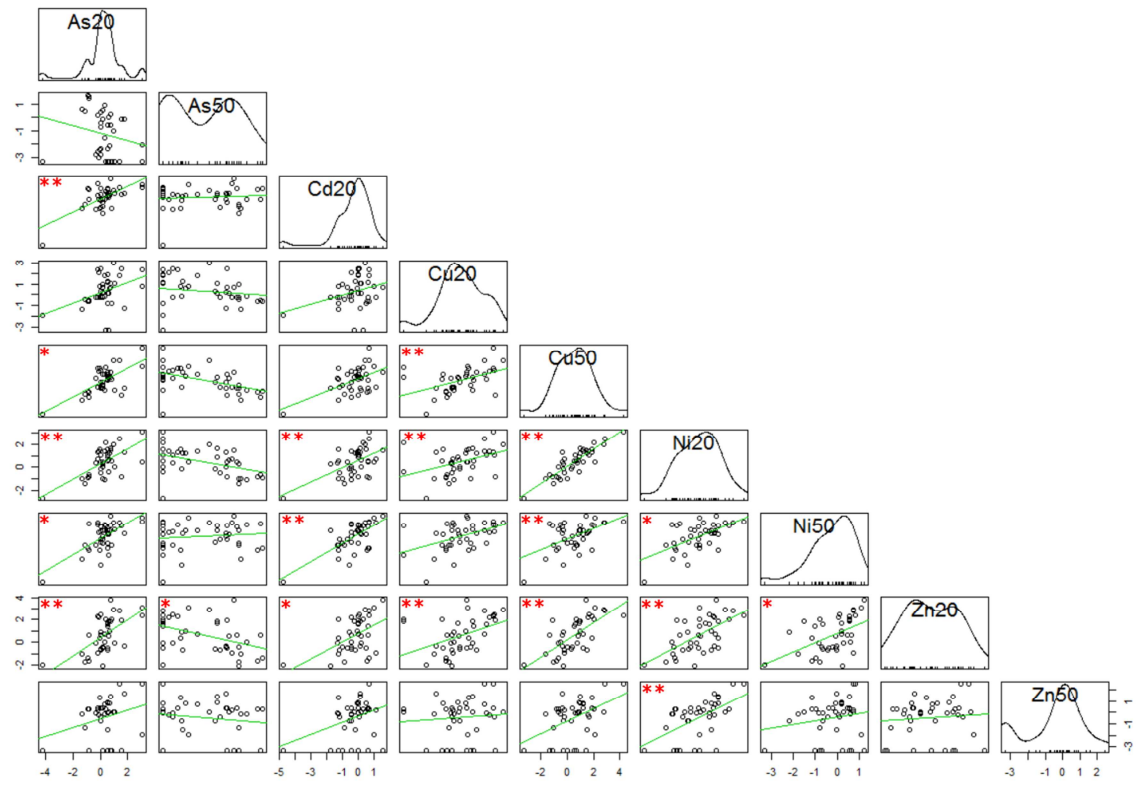
Figure 6 Pairwise correlation analysis of the modification of the permissiveness (log(δ)) across metal treatments for all 206 bacterial OTUs to explore whether two stress conditions resulted in similar response. Each circle symbol in the diagrams corresponds to one OTU plotted according to its log(δ) under the two compared conditions. Green 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 [3H]leucine incorporation rates of the recipient bacterial community by 20% (IC_{20}) and 50 % (IC_{50}).

710   **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 (δ 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

715   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}H]leucine incorporation rates of the recipient bacterial community by 20% (IC_{20}) and 50 % (IC_{50}).

Metal stress

| | As 20 | As 50 | Zn 20 | Zn 50 | Cu 20 | Cu 50 | Cd 20 | Ni 20 | Ni 50 |

Sphingobacteriales
Sphingobacteriales
Burkholderiales (β)
Sphingobacteriales
Sphingobacteriales
Flavobacteriales
Enterobacteriales (γ)
Bacillales
Xanthomonadales (γ)
Sphingomonadales (α)
Unclassified
Xanthomonadales (γ)
Rhizobiales (α)
Rhizobiales (α)
Sphingomonadales (α)
Rhizobiales (α)
Sphingomonadales (α)
Xanthomonadales (γ)
Caulobacterales (α)
Burkholderiales (β)
Xanthomonadales (γ)
Burkholderiales (β)
Pseudomonadales (γ)
Xanthomonadales (γ)
Burkholderiales (β)
Rhizobiales (α)
Rhizobiales (α)
Actinomycetales
Rhizobiales (α)
Xanthomonadales (γ)
Rhizobiales (α)
Rhizobiales (α)
Rhizobiales (α)
Rhizobiales (α)
Rhizobiales (α)
Rhizobiales (α)
Burkholderiales (β)
Enterobacteriales (γ)
Rhodobacterales (α)

Average
abundance
in T-pools

Log δ − value

4   2   0   −2   −4

Log relative abundance

−1   −1.5   −2   −2.5   −3   −3.5

Bacteroidetes
Proteobacteria
Firmicutes
Actinobacteria

720

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