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A converging subset of soil bacterial taxa is permissive to the IncP-1 plasmid pKJK5 across a range of soil copper contamination

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ABSTRACT

Stressors like metals or antibiotics can affect bacterial community permissiveness for plasmid uptake, but there is little knowledge about long-term effects of such stressors on the evolution of community permissiveness. We assessed the effect of more than 90 years of soil Cu contamination on bacterial community permissiveness (i.e. uptake ability) towards a *gfp*-tagged IncP-1 plasmid (pKJK5) introduced via an *Escherichia coli* donor. Plasmid transfer events from the donor to the recipient soil bacterial community were quantified and transconjugants were subsequently isolated by fluorescence activated cell sorting and identified by 16S rRNA gene amplicon sequencing. Transfer frequency of plasmid pKJK5 was reduced in bacterial communities extracted from highly Cu contaminated (4526 mg kg^{-1}) soil compared to corresponding communities extracted from moderately (458 mg kg^{-1}) Cu contaminated soil and a low Cu reference soil (15 mg kg^{-1}). The taxonomic composition of the transconjugal pools showed remarkable similarities irrespective of the degree of soil Cu contamination and despite contrasting compositions of the extracted recipient communities and the original soil communities. Permissiveness assessed at the level of individual operational taxonomic units (OTUs; 16S rRNA gene 97% sequence similarity threshold) was only slightly affected by soil Cu level and high replicate variability of OTU-level permissiveness indicated a role of stochastic events in IncP-1 plasmid transfer or strain-to-strain permissiveness variability.

Keywords: Conjugal plasmid transfer, copper, horizontal gene transfer, plasmid permissiveness, soil bacterial community, transconjugants

Introduction

Horizontal gene transfer (HGT) is a driving force for bacterial evolution and accelerates genomic diversification by incorporating new genomic elements (Frost *et al.* 2005; Sørensen *et al.* 2005; Harrison and Brockhurst 2012). Bacteria can acquire foreign DNA through transformation, transduction or conjugation. Conjugation refers to genetic material typically in the form of plasmids being transferred from bacterial donor cells to recipient cells during direct cell-to-cell contact to form transconjugants (Norman, Hansen and Sorensen 2009). Conjugation is generally considered the most important HGT mechanism for transfer of antibiotic resistance (von Wintersdorff *et al.* 2016; Enault *et al.* 2017), but conjugation also contributes to ecosystem services via environmental dissemination of catabolic genes for biodegradation of organic pollutants (Wang *et al.* 2010).

In soil, many abiotic and biotic factors may affect bacterial conjugation processes, including availability of nutrients or chemical stressors, spatial bacterial distribution and physiological status of bacteria potentially engaging in plasmid transfer (van Elsas and Bailey 2002; Sørensen *et al.* 2005; Aminov 2011; Heuer and Smalla 2012; Klümper *et al.* 2017; Fan *et al.* 2019). Soil metal contamination can impair bacterial growth, activity and survival and selects for metal tolerant bacterial communities with altered composition and an increased incidence of metal resistant bacteria (Berg *et al.* 2010, 2012; Brandt *et al.* 2010). Adaptive evolutionary responses to metal stress in soil can occur via adaptive point mutations in individual bacteria, but conjugal plasmid transfer allows for rapid dissemination of metal resistance determinants in bacterial communities (Sobecky and Coombs 2009; Heuer and Smalla 2012). Many large self-

transmissible genetic elements are equipped with multiple genes encoding simultaneous resistance traits towards antibiotics, metals and biocides (Aminov and Mackie 2007). Transfer of conjugal plasmids between bacteria in metal polluted soil is therefore of particular concern due to the risk of metal induced co-selection of antibiotic resistance (Baker-Austin *et al.* 2006; Ashbolt *et al.* 2013; Pal *et al.* 2017; Zhao *et al.* 2019). This concern is further supported by the recent observations that metals such as Cu and Zn may constitute stronger selective agents for antibiotic resistance than antibiotic residues in soil (Song *et al.* 2017).

Previous studies of metal impacts on conjugal plasmid transfer have largely been performed using cultivation based studies relying on model bacteria (Aminov 2011; Heuer and Smalla 2012). However, the permissiveness for plasmid uptake varies dramatically between individual recipient bacterial strains and studies relying entirely on model bacteria thus have little predictive value for monitoring the transfer of plasmids in highly diverse bacterial communities (De La Cruz-Perera *et al.* 2012; Klümper *et al.* 2015). Further, only a small fraction of all bacteria is readily culturable, and the transfer frequency, as well as the host range of the plasmid may be greatly underestimated (Musovic *et al.* 2006).

Recent technical advances now make it possible to study the effect of environmental conditions on permissiveness for plasmid uptake at the bacterial community level. Community permissiveness has two components as it 'refers to the ability of a community to receive a plasmid, both in terms of transfer frequency and transconjugant phylogeny' (Klümper *et al.* 2017). We thus consider bacterial community permissiveness as an inherent property of a an assemblage of bacteria (i.e. a community) and it must therefore be quantified in assays where the recipient community is in direct contact with donor bacteria. It has been demonstrated that an extremely diverse fraction of the soil bacterial community can be permissive towards the broad

host range IncP-1 plasmid pKJK5 when introduced via different bacterial donor strains (Klümper *et al.* 2014, 2015, 2017). In addition, one of these studies was the first to demonstrate a high sensitivity of bacterial community permissiveness towards short-term metal stress (Klümper *et al.* 2017). Hence, metal stress increased the permissiveness for some operational taxonomic units (OTUs, 97% sequence similarity) by more than 1000 times, whereas a strong opposite trend was observed for other OTUs.

This study raises the question of how the observed modulation of community permissiveness by short-term metal stress (48 h) compares to stress caused by long-term soil metal exposure. In other words, does long-term adaptation to toxic metals affect bacterial permissiveness towards the IncP-1 plasmid pKJK5 at the community or individual OTU level in a fashion similar to short-term metal stress? To address this question, we quantified, cell-sorted and taxonomically identified transconjugants to determine the permissiveness towards pKJK5 uptake in bacterial communities originating from two long-term copper contaminated soils as well as from a corresponding low Cu control soil. The sampling site, located in Hygum, Denmark, had been contaminated with $CuSO_4$ from 1911 to 1924 (Strandberg *et al.* 2006). Previous investigations on the same soils have demonstrated that differences in various bacterial community attributes (i.e. composition, diversity, metatranscriptome dynamics, and resistance to Cu and antibiotics) could be linked specifically to differential Cu exposure (Berg *et al.* 2010, Berg et al., 2012; Thorsen et al., 2013; Nunes *et al.* 2016; Jacquiod *et al.* 2018). Hence, this field site enabled us to study the legacy effects of Cu contamination without significant confounding effects caused by other toxic elements or other edaphic factors. Specifically, we used a previously developed high-throughput method for the quantification, isolation and taxonomic identification of transconjugants (Klümper *et al.* 2017) to investigate the impact of long-term Cu stress in soil on the ability of bacterial

community members to take up the broad-host-range IncP-1 plasmid pKJK5 from an *E. coli* model donor strain.

Materials and methods

Soil sampling and bacterial community extraction

Soil samples were collected at a depth of 0-20 cm from a well described Cu gradient field site in Hygum, Denmark (Strandberg *et al.* 2006; Berg *et al.* 2010, 2012; Arthur *et al.* 2012). Soil was sampled from each of three previously established field plots at the contaminated sites: MCC (moderately Cu contaminated; 458 mg kg^{-1} Cu), HCC (highly Cu contaminated; 4526 mg kg^{-1} Cu) contaminated sites and an adjacent control soil site (15 mg kg^{-1} Cu) (Nunes *et al.*, 2016). Soil samples were collected in December 2015 by taking nine soil cores per field plot (i.e. Control, MCC and HCC soil). The nine soil cores were divided into three replicate composite soil samples mixing three soil cores together for each replicate. The soil samples were sieved through a 2 mm^2 mesh, homogenized and stored at 10 ^oC for up to a month before the experiment.

Bacterial communities from each soil replicate (25 g) were extracted by Nycodenz density gradient centrifugation (Holmsgaard *et al.* 2011; Klümper, Dechesne and Smets 2014) (for details see supporting information; SI-1). Extracted bacteria were resuspended in sterile 0.9% saline solution, filtered through a 30 μm filter and quantified through viability staining (Propidium iodide/SYBR green I) (Grégori *et al.* 2001; Manti *et al.* 2008) to adjust the concentration of recipient and *E. coli* donor cells to 3×10^7 cells ml^{-1}.

Filter mating assay and quantification of transfer frequency

E. coli MG1655*::lacI*^{q}*::mCherry* (Klümper *et al.* 2015) hosting the broad host range plasmid *pKJK5-Plac-gfpmut3* (Bahl *et al.* 2007) served as the donor strain. The donor strain was chromosomally tagged with a Tn*7* based gene cassette encoding constitutive *mCherry* red fluorescence and a constitutive LacI repressor preventing expression of the plasmid encoded *gfp* in the donor strain. Hence, the *gfpmut3* gene only became expressed upon successful plasmid pKJK5 transfer to a recipient cell, resulting in green fluorescent transconjugal cells. The red fluorescent donor cell and the green florescent transconjugants were subsequently detected by fluorescence microscopy or flow cytometry and sorted by FACS (Sørensen *et al.* 2005; Klümper, Dechesne and Smets 2014).

Plasmid transfer was evaluated by exposing Nycodenz extracted soil bacteria to the *E. coli* donor strain in solid surface filter mating assays. The donor and the soil derived recipient cells were mixed at 1:1 ratio on black polycarbonate filter (0.2 μm pore size, 25 mm diameter) placed on top of a low-nutrient medium (Luo 2015) (for details see supporting information; SI-2) resulting in approximately 100,000 bacteria/mm^2 filter surface area. Five replicate filter matings were performed for each of the three biological replicates of all three soil types. In order to evaluate the effect of donor cells on recipient community composition, 'reference' filters exclusively hosting the recipient communities or recipient communities mixed with a plasmid free version of the donor strain *E. coli* MG1655*::lacI*^{q}*::mCherry* were also prepared. All filter mating plates were incubated at 25 °C in the dark for 48 hours.

Conjugation events were detected by stereomicroscopy and quantified by digital image analysis (Image Pro plus 7.1) as previously described (Klümper *et al.* 2014, 2017; Klümper, Dechesne and Smets 2014). The transfer frequency was defined as the number of conjugation events counted on the whole filter area divided by the number of introduced recipient cells at the start of the mating experiments.

Florescence activated cell sorting (FACS) and sequencing

Cells from each filter mating combination between the donor and recipient community were collected by transferring the filter into 2 ml of a 0.9% NaCl solution followed by vortexing at highest speed for 3 min. Transconjugant cells were isolated by triple gated fluorescence activated cell sorting (FACS). FACS was carried out using a FACS Aria IIIu (Becton Dickinson Biosciences, San Jose, CA) with the following settings: forward scatter (FSC) = 505 V, side scatter (SSC) = 308 V, and detectors for green (BP filter 530/30 nm) and red fluorescence (BP filter 610/20 nm) were set at 508 V and 500 V, respectively. Both cell counting and sorting were performed and analyzed with the software BD FACSDiva™ v6.1.3. The setup for gating and sorting was performed based on bacterial size, green fluorescence and exclusion of red fluorescent donor cells as described previously (Klümper, Dechesne and Smets 2014; Klümper *et al.* 2017) (for details see supporting information; SI-3). The corresponding recipient community cells from the same mating plates were sorted separately based on bacterial size and excluding red fluorescent donor cells. For each sample, a minimum of 20,000 recipient cells or *gfpmut3* expressing transconjugants were sorted. All sorted cells were collected in 0.9% NaCl solution, lysed (Lyse and Go PCR reagent, Thermo Scientific, Waltham, MA, USA) and followed by 16S rRNA gene amplicon sequencing as described previously (Klümper *et al.* 2017) (for details see supporting information; SI-4). Bacterial DNA was also extracted directly from the original soil

samples and from soil bacterial cells harvested by Nycodenz extraction using the FastDNA SPIN for soil kit (MP Biomedicals) as described by manufacturer's instructions and used for 16S rRNA gene amplicon sequencing to analyze the bacterial community structure and diversity of the recipient community (see SI-4 for details).

Sequence analysis

Raw fastq files were processed with BioDSL (https://github.com/maasha/BioDSL). Primers and diversity spacers were identified and truncated with trim_primer. Paired ends were matched with assemble_pairs and any sequences shorter than 100 bp were discarded, and finally dereplicated using dereplicate_seq. The sequences were clustered at 97% with cluster_otus, a wrapper around usearch 7.0.1090 (Edgar 2010). Chimeras were checked with uchime_ref that utilizes the same version of usearch with the Ribosomal Database Project (RDP) database as reference (Wang *et al.* 2007). The resulting sequences were classified using classify_seq against the RDP database (version 9). Align_seq_mothur (Schloss *et al.* 2009), a wrapper around mothur's align.seqs function for aligning OTUs against a RDP template. Finally, a phylogenetic tree was built from the alignment using write_tree, a wrapper function for FastTree (Price, Dehal and Arkin 2009). Phylogenetic trees displayed were constructed using iTOL v3 (http://itol.embl.de/) (Letunic and Bork 2007).

OTU level permissiveness analysis

The apparent permissiveness (AP) of an OTU was defined as follows (Klümper *et al.* 2017): $AP_i = \frac{T_i}{R_i}$, where T_i and R_i refer to the relative abundance of OTU_i in the transconjugal pool and the recipient community, respectively. In order to evaluate the change of permissiveness of individual OTUs after long-term Cu contamination, individual OTU permissiveness changes (δ)

were calculated as $\delta i = \frac{AP_{i\_Cu\ soil}}{AP_{i\_control\ soil}}$, where $AP_{i\_Cu\ soil}$ equals an OTU's apparent permissiveness in either HCC or MCC soil and $AP_{i\_control\ soil}$ equals the same OTU's apparent permissiveness in control soil. A total of 23 OTUs were selected for subsequent individual OTU permissiveness analysis based on the following two criteria: 1) OTU is present in all recipient communities and 2) average OTU relative abundance is above 0.01% across all transconjugant pools.

Statistics

Richness (α-diversity) of all sampled communities was calculated in R using the 'phyloseq' package (McMurdie and Holmes 2013) with at a minimum depth of 12,110 reads. Rarefaction curves were obtained using the PAST software ver.2.17 (Hammer, Harper and Ryan 2001). Differences of the taxonomic composition of transconjugant pools and recipient communities (phylogenetic distance between samples) were visualized by non-metric dimensional scaling (NMDS) analysis using weighted unifrac distances as input data in R with the package phyloseq (McMurdie and Holmes, 2013). The effects of long-term Cu contamination on the taxonomic composition of transconjugal pools and recipient communities were tested by permutational multivariate analysis of variance (PerMANOVA) in R using the 'adonis' function with 10,000 permutations in the 'vegan' package. Differences of transfer frequency and observed richness of recipient communities among control soil, MCC soil and HCC soil were tested by one-way ANOVA in Sigma plot 13.0.

Accession numbers

The partial 16S rRNA gene sequences have been deposited in the NCBI Sequence Read Archive (SRA) database under BioProject ID PRJNA559939.

Results

Decreased bacterial fraction is permissive in highly Cu contaminated soil

We determined the transconjugant per donor (T/D) ratio, as inferred from flow cytometric counts at the end of the 48 h mating, to compare transfer frequencies of plasmid pKJK5 between the three recipient communities. The T/D ratio was significantly lower (one way ANOVA, $p<0.05$; n=3; Figure S1) for the bacterial community extracted from the HCC soil (0.53% ± 0.26%) as compared to the corresponding communities from MCC soil (17.8% ± 5.5%) and control soil (12.8% ± 4.5%). Potential bias should be noticed here as the flow cytometric counts could not distinguish between real pKJK5 transfer events and subsequent preferential growth of transconjugants or donors. However, we find it highly unlikely that this preferential growth bias could solely explain the lower T/D ratio obtained for the HCC soil as compared to the MCC and control soils. Furthermore, estimates based on fluorescence microscopy data confirmed the trend obtained for T/D ratios (Figure S2).

The composition of transconjugal pools show higher similarity than their corresponding recipient soil bacterial communities

16S rRNA gene amplicon sequencing was performed to analyze the phylogenetic composition of the transconjugal pools; i.e. all FACS separated green fluorescent cells. Control experiments showed that the presence of *E. coli* donor cells did not affect recipient soil bacterial community composition (see Supporting Information SI-5 and Figure S3 for details). Transconjugant pools from all three soils encompassed 296 OTUs, including 105 abundant OTUs (> 0.01% relative abundance across all transconjugal pools) distributed across 10 different phyla (Figure 1). These

included the dominant *Proteobacteria* (Alpha, Beta, Gamma and Delta), other Gram negative phyla (*Bacteroidetes*, *Nitrospira*, *Planctomycetes*, *Gemmatimonadetes*, *Spirochaetes* and *Verrucomicrobia*), and three Gram positive phyla (*Firmicutes*, *Actinobacteria* and *Chloroflexi*). The investigated IncP-1 plasmid pKJK5 was transferred to all abundant phyla within the recipient community sorted from the filters (>0.01% relative abundance across all transconjugants pools) as well as to most rare phyla (<0.01% relative abundance across all transconjugants pools) except the phylum *Ignavibacteriae* (Figure S4).

At the phylum level, all transconjugal pools were dominated (>50%) by *Proteobacteria* (mainly Gamma-subgroup), while other rarer phyla were detected only at specific Cu contamination levels. For example, the phylum *Gemmatimonadetes* was detected in all recipient communities, but only permissive to the introduced plasmid when originating from MCC or control soil. By contrast, the phylum *Planctomycetes* was only detected in transconjugal pools from either the HCC or MCC soils. Further, the phylum *Spirochaetes* was only detected in transconjugal pools and the recipient community from the HCC soil.

In order to analyze the Cu impacts on transconjugant pools at higher taxonomic resolution, further analysis was performed at the OTU level. All transconjugal pools showed coverage above 96% at a sampling depth of 12,110 sequences (see Figure S5 for rarefaction curves). The transconjugal pool OTU richness (α-diversity; mean ± SE; n=3) decreased from 86±9 OTUs in the control and 112±51 OTUs in the MCC soil to only 64±7 OTUs in the HCC soil. Likewise, observed OTU richness of the corresponding recipient community was significantly lower (78±5 OTUs) in the HCC soil ($p=0.013$, one way ANOVA, $n=3$) as compared to 103±5 and 89±8 OTUs in MCC and control soil, respectively. However, the fraction of total permissive OTUs in the

recipient community only decreased slightly from 68.5% in control soil to 66.5% in the MCC soil and 52.1% in the HCC soil.

Remarkably, the taxonomic composition within the transconjugal pools originating from the three different soils was highly similar (Figure 2). Although only 39 out of 263 OTUs in total were shared between all three soil communities, these shared OTUs represented 96.2% of the total sequences from the transconjugal pools. Dissimilarity analysis based on Bray-Curtis distance of transconjugal pools from different soils indicated that the transconjugal pools from the MCC soil and the control soil were more similar than the transconjugal pools from the HCC soil (Figure S6), but these differences were subtle and did not generate a significant soil clustering pattern in the non-metric 2-dimensional scaling (NMDS) plot (Figure 3). Hence, transconjugal pools from the three contrasting soils clustered together, whereas the recipient communities from the HCC soil clustered significantly apart from the MCC and control soils (Figure 3; Figure S7; Adonis test, $p<0.001$).

Limited long-term Cu effect on OTU-level permissiveness

In order to further investigate the long-term impacts of soil Cu contamination level on OTU level permissiveness, 23 abundant OTUs were selected based on the criteria that OTUs were present in all recipient communities with an average relative abundance above 0.01% across all transconjugant pools (Figure 4; Figure S8). OTU-level apparent permissiveness (AP_i) were consistent across soil Cu levels for most OTUs (Figure S8). Some OTUs including *Pedobacter* OTU 7, *Janthinobacterium* OTU 2, *Stenotrophomonas* OTU 120, and *Escherichia/Shigella* OTU 3 were consistently underrepresented in transconjugant pools for all soils, whereas only *Pseudomonas* OTU 0 was consistently overrepresented in all soils. Overall, there was no clear Cu impact on the permissiveness observed for the 23 selected OTUs. The only exception was

Pseudomonas OTU 14, which was more than 2-fold (average ± standard deviation, 2.60±0.63) over-represented in the transconjugal pool for control soil and more than 26-fold (156±134 p=0.091, t-test against 0) underrepresented for HCC soil with an intermediate effect observed in the MCC soil (Figure S8). When comparing the permissiveness of individual OTUs after long-term Cu exposure to their permissiveness in the control soil (Figure 4), OTUs generally did not show increased permissiveness (δ) with increasing soil Cu level across all three biological replicates. The only exception was *Pedobacter* OTU 7, which was exclusively permissive in the HCC and MCC soils, despite being abundant in the control soil. Across all three biological replicates, *Pseudomonas* OTU 401 displayed more than 37-fold (44±6, p=0.0036) and more than 3-fold (4.7±1.3, p=0.013) decreased permissiveness in HCC and MCC soils, respectively. *Pseudomonas* OTU 14 showed more than 69-fold (407±350, p=0.091) and more than 2-fold (5.3±5.0, p=0.056) decreased permissiveness in HCC and MCC soils, respectively. Across all three biological replicates, both *Janthinobacterium* OTU 2 (4.9±2.6 fold, p=0.043) and *Afipia* OTU 5 (4.5±1.6 fold, p=0.021) displayed more than 2-fold decreased permissiveness in MCC soil, whereas *Noviherbaspirillum* OTU1128 (28±29, p=0.12) and *Pseudomonas* OTU 2065 (21.4±25.4, p=0.14) showed more than 5-fold and more than 3-fold decreased permissiveness in HCC soils, respectively (Figure 4). In conclusion, we note a tendency for decreased OTU-level permissiveness with increasing soil Cu level, but also a considerable replicate-to-replicate variability.

Discussion

Our study took advantage of a previously developed fluorescence marker gene based approach to detect IncP-1 plasmid pKJK5 transfer events and subsequently isolate and identify

transconjugants from the complex communities using fluorescence activated cell sorting (FACS) and subsequent 16S rRNA gene amplicon sequencing (Klümper *et al.* 2015, 2017). In accordance with recent studies applying the same experimental approach (Jacquiod *et al.* 2017; Li *et al.* 2018; Fan *et al.* 2019), we here demonstrate the transfer of the IncP-1 plasmid pKJK5 from *E. coli* to a remarkably wide taxonomic range (10 phyla) of both Gram negative and Gram positive soil bacteria during 48 hours of mating experiments. In this context it is important to differentiate between plasmid host range and plasmid replication range as plasmids initially taken up may subsequently be lost if the plasmid cannot replicate in its host. However, a very recent study specifically investigated the persistence of IncP-1 plasmid pKJK5 in bacterial communities derived from a sewage treatment plant and demonstrated extensive plasmid retention in diverse bacterial phylotypes during a 60-generation experiment even at conditions that were non-selective for plasmid retention and non-conducive for plasmid transfer (Li et al., 2020).

Even though large fractions of the recipient communities extracted from all soils remained permissive towards pKJK5, we also observed a substantially decreased transfer frequency of pKJK5 in the bacterial community extracted from the HCC soil as compared to corresponding communities from control and MCC soils (Figures S1 and S2). This effect is consistent with previous reports of reduced microbial activity in the most contaminated part of the studied soil Cu gradient (Arthur *et al.* 2012; Brandt, unpublished data).

Consistent with previous studies of the Hygum soil Cu gradient (Berg et al., 2012; Thorsen et al., 2013; Nunes *et al.* 2016), we observed contrasting composition and lower OTU richness of the recipient community in the HCC soil relative to the control and MCC soils. Nevertheless, highly similar taxonomic compositions of the transconjugal pools were observed in all three soils (Figure 3). This suggests that the realized host range for specific plasmids can remain remarkably

unaffected by long-term soil metal contamination. Our results also demonstrate that more than 90 years of differential field soil Cu exposure did not dramatically influence the fraction of permissive OTUs in the recipient community. Hence, the fraction of total permissive OTUs only decreased slightly from 68.5% in the control soil to 66.5% in the MCC soil and 52.1% in the HCC soil.

A possible explanation for this slight reduction in community permissiveness could be that some HCC soil bacteria already had acquired IncP-1 plasmids encoding Cu resistance thereby preventing them from taking up the pKJK5 from *E. coli*. This explanation is consistent with data from a Hygum soil strain collection demonstrating that isolates from the HCC soil contained significantly more plasmids than corresponding strains from the control soil (Luo 2015). Indeed, the percentages of isolates carrying detectable plasmids were 45.7% and 21.1% for the HCC and control soil, respectively. Interestingly, co-selection of Cu and antibiotic resistance has been demonstrated in two independent studies of the Hygum soil Cu gradient (Berg *et al.* 2010; Luo 2015). Environmentally relevant concentrations of Cu has also been shown to co-select antibiotic resistance in other soils (Hu *et al.* 2016; Song *et al.* 2017; Kang *et al.* 2018; Zhao *et al.* 2019) implicating that soil Cu contamination represents a risk for environmental development and transfer of antibiotic resistance to humans (Ashbolt *et al.* 2013).

It has been proposed that low, environmentally relevant concentrations of antibiotics and metals may increase rates of bacterial evolvability in part via stimulation of conjugal plasmid transfer (Gillings and Stokes 2012; Zhang *et al.* 2019). By contrast, our data indicate that soil Cu contamination decreased community permissiveness for the uptake of IncP-1 plasmid pKJK5. It should be noted that we studied the plasmid transfer process in the absence of growth benefit for transconjugal cells and that we did not monitor the long-term fate of the introduced plasmid in

soil (Fan *et al.* 2019). Hence, environmental selection of plasmids encoding metal resistance can probably 'compensate' for reduced plasmid transfer frequency thereby allowing for enrichment of resistance plasmids in severely metal contaminated soils. Finally, it should also be mentioned here that horizontal gene transfer may occur by other means than plasmid transfer and that these gene transfer processes can be affected differently by Cu. A recent metatranscriptomics study is of particular interest as it demonstrated an unexpected, Cu-dependent dominance of phage-related mRNA sequences accounting for ~30% of annotated mRNA in the HCC soil (Jacquiod et al., 2018).

Our study of long-term (90+ yrs) soil Cu effects on bacterial community permissiveness for IncP-1 plasmid pKJK5 uptake can be compared to a recent study employing the same model plasmid and bacterial communities from a low-Cu agricultural soil for studying short-term (48 h) metal stress effects on community permissiveness (Klümper *et al*., 2017). Both the short- and long-term study on Cu modulation of conjugal plasmid transfer showed that transfer frequencies of plasmid pKJK5 consistently decreased compared to the reference (i.e. control soil) conditions. However, the individual permissiveness of OTUs showed contrasting patterns in the long- and short-term studies. In the latter, Cu stress modulated the conjugation process by in- or decreasing the permissiveness for specific OTUs by up to 1000- and 10,000-fold, respectively (Klümper *et al.*, 2017). By contrast, the level of OTU-level permissiveness modulation was modest in our long-term study. Hence, when comparing the 23 core permissive OTUs, only one OTU (*Pedobacter* OTU 7) showed increased individual permissiveness in both MCC and HCC soils (Figure 4). Individual permissiveness of 6 OTUs from MCC or HCC soils decreased by more than 2-fold compared to the control soil and only for 2 OTUs did permissiveness decrease by more than 10 fold in the HCC soil (Figure 4). Collectively, these data clearly indicate different community

responses to short-term (48 h) and long-term (90+ yrs) Cu exposure. When discussing OTU-level permissiveness, it has previously been established that even closely related strains belonging to the same bacterial species can differ in permissiveness for plasmid uptake by orders of magnitude (Heuer et al., 2010). Hence, it should be mentioned here that the observed effects of Cu stress on OTU-level permissiveness will probably to some extent be masked by the presence of different strains differring in permissiveness within each OTU.

In conclusion, our results indicate that the transfer frequency of an IncP-1 plasmid (pKJK5) during filter matings were lower for bacteria extracted from HCC soil than for bacteria extracted from low-Cu control soil or MCC soil. However, the fraction of permissive OTUs was only marginally lower in HCC soil than in control and MCC soils. Further, transconjugant pools encompassed 10 different phyla and were remarkably similar for all studied soils even though they contained contrasting recipient communities. Finally, we observed considerable replicate variability for transconjugant pool compositon and especially for individual OTU-level permisiveness (Heuer *et al.,* 2010). Our results thus suggest that a defined subset of OTUs will be able to take up the IncP-1 plasmid pKJK5 across sites differentially contaminated with metals, but also that stochastic conjugation-related events (Delavat *et al.* 2017) or strain-to-strain variability (Heuer et al., 2010) may make it difficult to predict the fate of conjugal plasmids and their genes (e.g. antibiotic resistance genes). Finally, our results indicate that long-term Cu contamination does not select for bacterial communities with increased evolvability by virtue of increased plasmid permissiveness as suggested previously (Gillings and Stokes 2012). By contrast, bacterial plasmid transfer frequencies and the fraction of permissive OTUs are likely to be lower in highly metal impacted soils than in corresponding reference soils.

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Figure 1

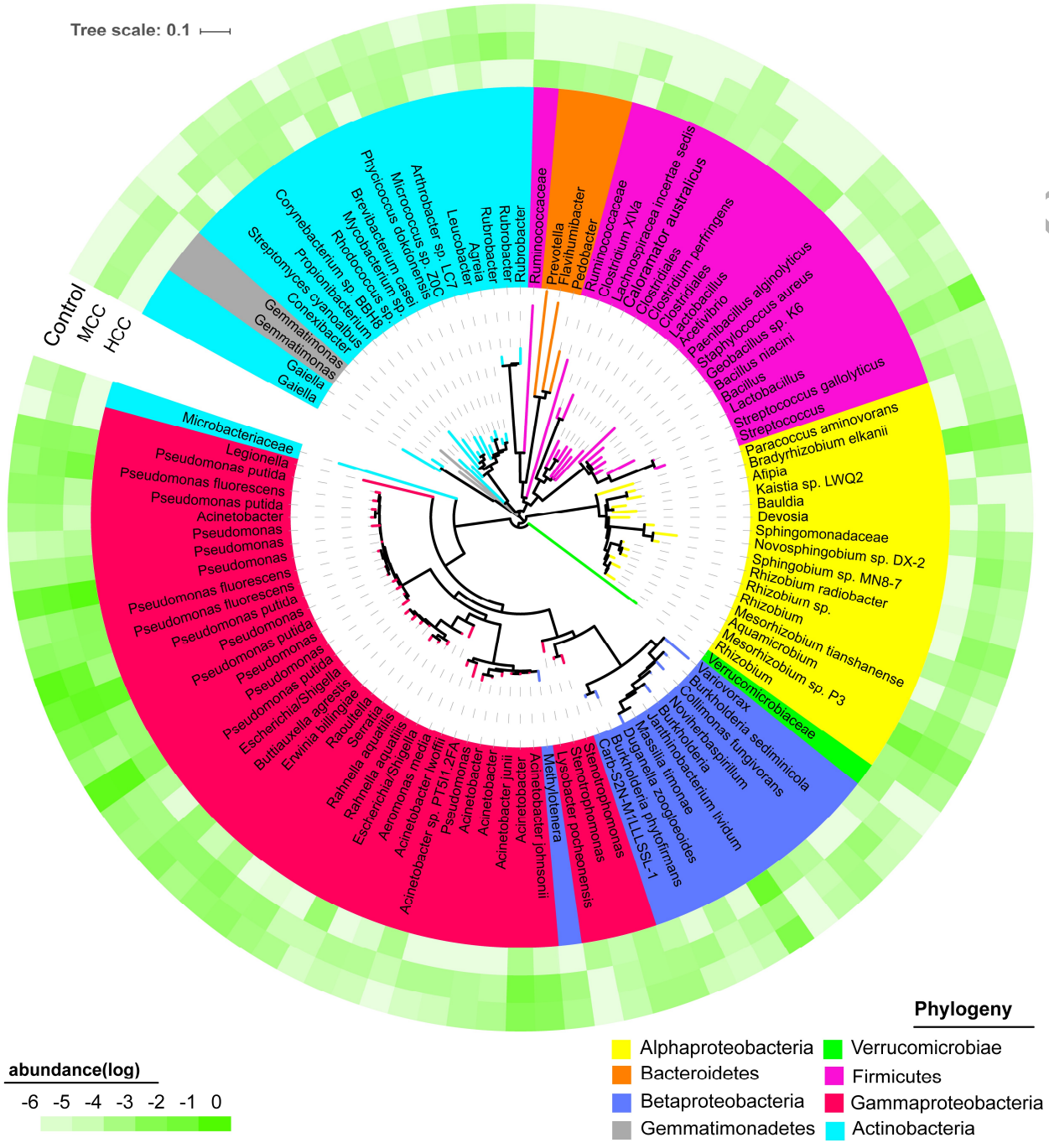

Figure 1. Phylogenetic tree (approximate maximum likelyhood) showing the 105 most abundant OTUs within the transconjugant pools (>0.01% relative abundance across all transconjugant pools). Colors of the branches mark the different phylogenetic groups. The three green heatmap-

circles at the periphery of the tree represent log-transformed relative OTU abundance from different transconjugant pools; i.e low-Cu control soil (Control), moderately Cu contaminated soil (MCC), and highly Cu contaminated soil (HCC).

Figure 2

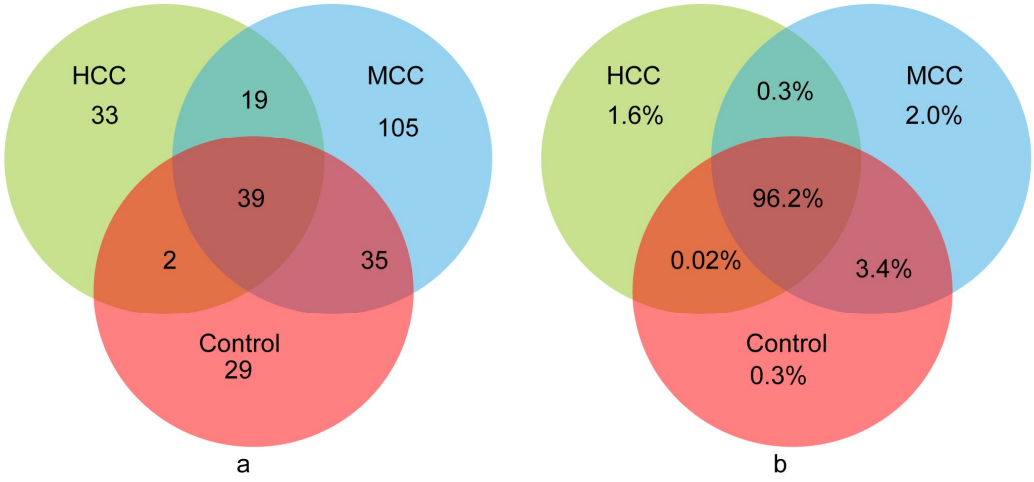

Figure 2. Venn diagrams of the transconjugal pools from the three different soils; i.e. low-Cu control soil (Control), moderately contaminated soil (MCC), and highly contaminated soil (HCC). Venn diagrams are presented for OTU incidence (a) and for OTU relative abundance (b). OTUs were defined at the 97% sequence similarity level and sequences from treatment replicates were pooled to get a population size of 24,200 sequences per treatment.

Figure 3

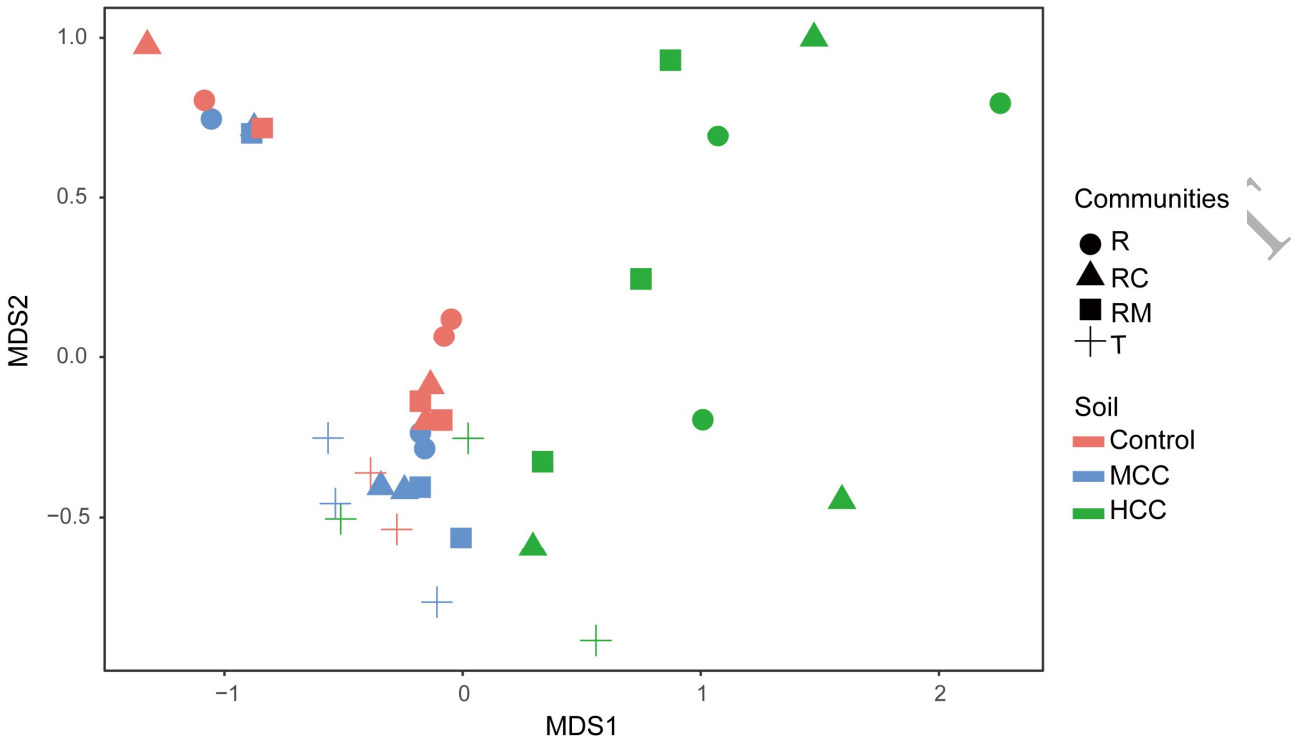

Figure 3. Impacts of long term soil Cu contamination on the taxonomic compositions of transconjugal pools and corresponding recipient communities as revealed by nonmetric 2-dimensional scaling analysis (NMDS). Abbreviations used: T, transconjugant pools; RM, recipient community filter mating with *E. coli* donor containing the pKJK5 plasmid; RC, recipient community from filter mating with *E. coli* donor without plasmid; R, recipient community from filter mating with only recipient community present; Control, low-Cu control soil; MCC, moderately contaminated soil; HCC, highly contaminated soil. Ordination is based on Weighted Unifrac distances.

Figure 4

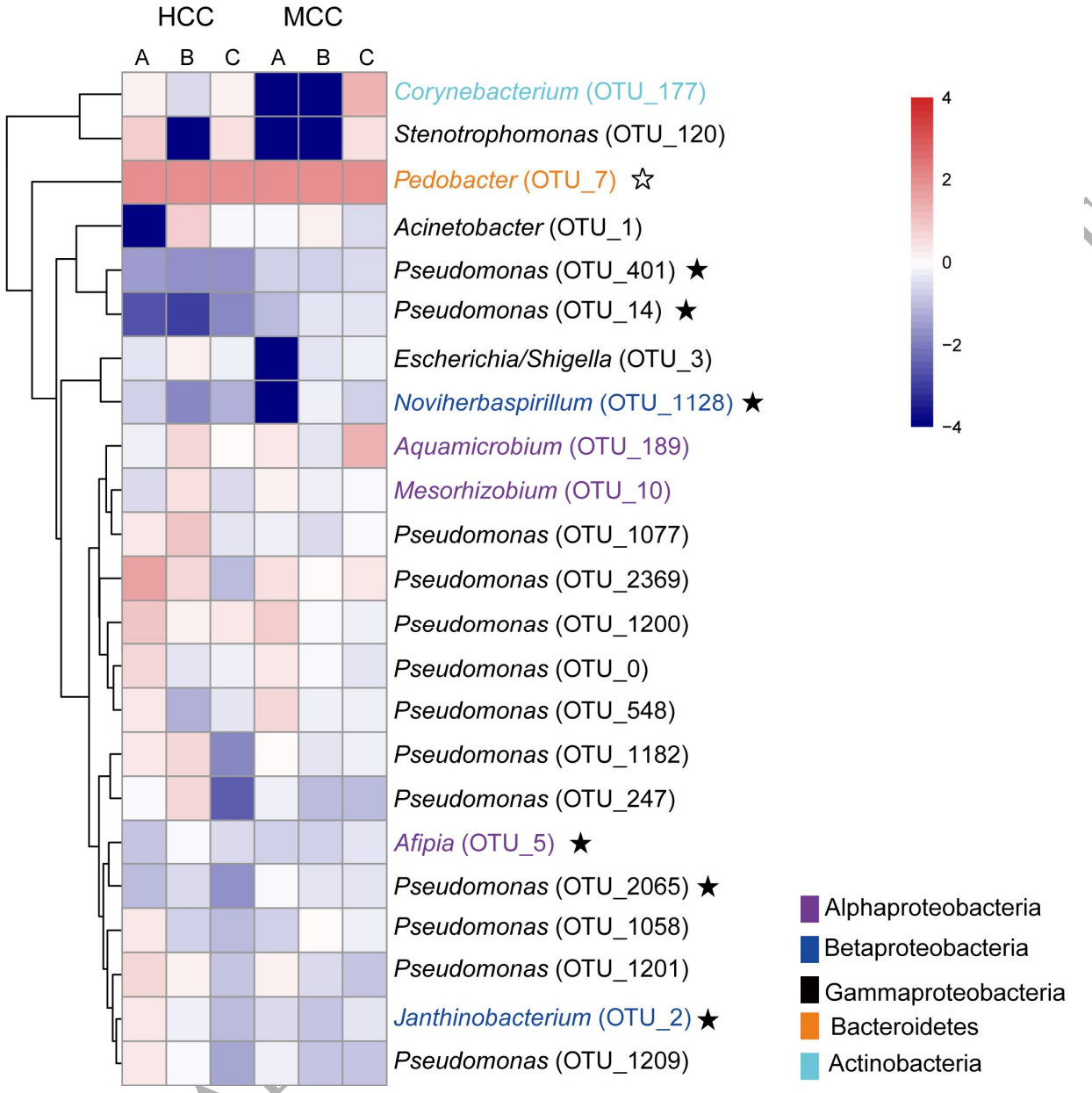

Figure 4. Heatmap clustering of the 23 operational taxonomic units (OTUs) shared between all recipient communities with an average relative abundance above 0.01% across all transconjugant pools. Clustering was performed based on similarity in how their individual permissiveness (log δ value) was altered across the copper gradient using the Bray-Curtis similarity metric. Increased permissiveness relative to low-Cu control soil is shown in red and decreased permissiveness is

shown in blue. The 23 OTUs belonged to 5 different phyla as indicated by color coding. MCC, moderately Cu contaminated soil; HCC, highly Cu contaminated soil. A, B, and C are biological replicates. Solid stars indicate OTUs with more than 2-fold decreased individual permissiveness in either MCC or HCC soils. The empty star indicates that OTU 7 was only permissive in HCC and MCC soils and the log δ value for OTU 7 was consequently set to the highest possible value.