Ecological resilience of soil microbial communities

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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5 LEGACY EFFECTS ON RECOVERY OF SOIL MICROBIAL COMMUNITIES FROM PERTURBATION

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Manuscript in preparation for submission to Frontiers in Terrestrial Microbiology
ABSTRACT

The type and frequency of disturbances experienced by soil microbiota is expected to increase given predicted global climate change scenarios and intensified anthropogenic pressures on ecosystems. While the direct effect of multiple disturbances to soil microbes has been explored in terms of function, their effect on the recovery of microbial community composition remains unclear. Here, we explore the effect of multiple disturbances on the recovery of soil microbiota after identical or novel stresses. We set up soil microcosms and exposed them to a heat shock to create an initial effect, followed by another heat shock or a cold shock. We monitored the communities for 25 days following the treatments using 16S rRNA gene transcript amplicon sequencing. The application of a heat shock to soils with or without the initial heat shock resulted in similar successional dynamics, but these dynamics were faster in soils with a prior heat shock. The application of a cold shock had negligible effects on previously undisturbed soils but, in combination with an initial heat shock, caused the largest shift in the community composition. Our findings show that compounded perturbation affects bacterial community recovery by altering community structure and, thus, the community's response during succession.

INTRODUCTION

Ecosystems are expected to face increasing anthropogenic pressures and climatic oscillations (Hartmann et al., 2013; Millennium Ecosystem Assessment, 2005; Trenberth et al., 2014), but how these changes will affect the soil biota is poorly understood (Smith et al., 2015b). The contribution of the soil microbiota to terrestrial ecosystem services is critical, but their precise role in safeguarding the processes of the soil system under increased environmental constraints is largely unknown (Nemergut et al., 2014). Particularly, the influence of altered soil microbial community structures on the stability of soil functioning is poorly understood. The composition of microbial communities is often treated as a 'black box' (McGuire and Treseder, 2010; Nemergut et al., 2014). Microbial communities, both in the field and in micro/mesocosm experiments, often exhibit long-term changes in their structure following a disturbance (Allison and Martiny, 2008; Shade et al., 2012). These altered community compositions may be ecologically relevant if interactions between populations are ruptured or if the community's ability to resist invasion is affected, as has been recently shown (Fiegna, Moreno-Letelier, Bell, & Barraclough, 2015; Griffiths et al., 2007; Kuan et al., 2016; Mallon et al., 2015; van Elsas et al., 2012).

In particular, the increasing frequency of transient disturbances in soil ecosystems, resulting in compounded perturbation, represents a challenge for research. Compounded perturbation is defined as an ecosystem being stressed during the recovery process from a previous event (Paine et al., 1998). It has been suggested to have a '(negative) synergistic' effect on microbial communities (Paine et al., 1998), which is defined by the combined effect of both perturbations, being greater than the sum of their individual effects. In microbial systems we may distinguish between two cases of compounded perturbation, namely (1) mixed compounded perturbation, in which the first stress event differs substantially from the second, and (2) homogeneous compounded perturbation, in which the first and second disturbance events are of the same type. Previous experiments have often found a negative synergistic effect arising from mixed compounded perturbations (Kuan et al., 2006; Tobor-Kaplon et al., 2006). In these experiments, soils subjected to long-term disturbance, such as exposure to intensive agricultural practices or heavy metals, were exposed to an additional short-term stress, such as a temperature shock or...
an antibiotic (Kuan et al., 2006; Müller et al., 2002; Tobor-Kaplon et al., 2005, 2006). This compounded treatment often resulted in a slower (or null) recovery of function (i.e. substrate utilization rate) relative to soils without the prior disturbance. The opposite pattern has been observed in the case of homogeneous compounded perturbation: generally, the second disturbance exerted a lesser effect on the community or its functioning than the first. For example, soils previously exposed to extreme precipitation regimes were less functionally sensitive to further moisture pulses than unexposed controls (Evans and Wallenstein, 2012). Similarly, soils underlying an oak tree exhibited shifts in bacterial community composition in response to drying-rewetting regimes, while grassland soils in the same area, which experienced more radical natural fluctuations in moisture, exhibited no change (Fierer et al., 2003).

Compounded perturbation of soil may thus have opposite effects on the soil bacterial community depending on whether the disturbances are mixed or homogeneous, but this is unclear, as they have not been rigorously compared. We hypothesized that these differing outcomes can be explained by mortality and the associated loss of microbial diversity, as well as the successional patterns that ensue and allow the colonization of the newly available niches (Figure 1). Systems with high species richness are expected to contain organisms with a broader array of environmental tolerance ranges, which should fare better across a wider range of environmental challenges or disturbances (Balser et al., 2001; Naeem and Li, 1997; Yachi and Loreau, 1999). In the case of homogeneous compounded perturbation, multiple similar disturbance events would have the strongest impacts on similar taxa and favor similar survivors, so the effect of the second event would be less perceptible (Figure 1, B). In the case of mixed compounded perturbation, different taxa may be impacted by a subsequent different disturbance, resulting in a further erosion of the community's diversity during recovery (Figure 1, D).

Successional dynamics further obscures the impact of compounded perturbations on microbial communities. Following a first perturbation, tolerant and resistant organisms will be favored. As succession proceeds, however, these populations might be outcompeted by rapidly-growing opportunists and, eventually, specialists, as easily digestible resources become scarce. Over time, resistant organisms are diluted out of the community by the arrival of new strategists (Placella et al., 2012), resulting in a community that is once again vulnerable to the disturbance (Figure 1, A). The effect of the second (same or different) disturbance is thus dependent on how far along a successional gradient the soil community is at the time of this perturbation. In soils exposed to mixed compounded perturbation, we expect the community to be most vulnerable immediately after the first perturbation, since its diversity will be affected to the greatest extent. Conversely, in homogeneous compounded perturbation, we expect the community to be the most resistant to the second perturbation immediately after the first, since it is dominated by tolerant organisms at this time.
Here, we explore the effect of disturbance legacy on the ability of the soil bacterial community to cope with a similar or a novel disturbance. To focus on the effect of the disturbances rather than environmental variability, we set up soil microcosms and exposed these to an initial, heat shock (along with un-exposed microcosms), allowing microbial communities to recover in order to create a legacy. Microcosms were subjected to one of six treatments: 1) heat shock-heat shock, 2) heat shock-cold shock, 3) heat shock-control, 4) control-heat-shock, 5) control-cold shock, 6) control-control; with 25 days between the two treatments. By monitoring the bacterial community composition in the aftermath of these extreme selective sweeps, we evaluated whether the presence and the type of a disturbance legacy affect the successional dynamics of the soil bacterial communities.

MATERIALS AND METHODS

MICROCOSMS

A total of 205 microcosms were prepared by adding 50 g of fresh soil to 200 mL glass jars covered with a loose aluminum foil cap. Microcosms were constructed using the top 15 cm of a loamy sand soil (soil-water pH 5.04) collected in April 2013 from a well-characterized agricultural field in Buinen, the Netherlands (52°55’N, 6°49’E), where seasonal variations in biochemical parameters have been previously characterized (Pereira e Silva et al., 2011, 2012b). Prior to the experiment, soils were homogenized by sieving through a 4-mm sieve, and soils were allowed to stabilize for one month at 4 °C. After the preparation of the microcosms, soils allowed to stabilize for two weeks. Soils were maintained at 21 °C, partially shielded from light in a temperature-controlled greenhouse, and at 65% water-holding capacity (adjusted with sterile water) for the duration of the experiment. Sampling was done destructively in quintuplicate.

Microcosms were subjected to one of six treatments: an initial heat shock, followed by 25 days of recovery and 1) an additional heat shock, 2) a cold shock, or 3) control conditions for 25 additional days (treatments control-heat, control-cold, or control, respectively). The 25 interval between treatments was selected after initial microcosm experiments with identical soils and conditions revealed that bacterial communities were still recovering from a heat shock after 25 days. A detailed schematic of our experimental setup is provided in the Supplementary Information S1. The duration of the heat shock was selected after recording the effects of increasing durations of microwave heating (15 sec to 10 min) on the total copies of 16S rRNA transcripts, soil temperature, pH, and moisture loss, in order to generate a loss of between 33% and 57% of 16S rRNA transcripts (data available in Supplementary Information S2). During each heat shock, jars were uncovered, placed in an 800-watt microwave oven (R201ww Sharp, Utrecht, the Netherlands), subjected to 90 sec of heating at maximum intensity, adjusted for moisture loss, and covered immediately. The cold shock treatment consisted of placing jars in a -80 °C freezer for 6 h. Soils were sampled one day prior to disturbance (T0) as well as on days 1, 4, 11, 18, and 25 days after disturbance (T1-T25). Soils with an initial heat shock were sampled at these time intervals after both disturbance events.

DNA AND RNA EXTRACTION

DNA was extracted from 0.5 g soil using the MoBio PowerSoil DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions, with three additional 30-s rounds of bead-beating (mini-bead beater, BioSpec Products, Bartlesville, OK, U.S.A.). The concentration and band size of the extracted products were checked by electrophoresis using a 0.8% agarose gel with a SmartLadder (Eurogentec, Liege, Belgium).

For the RNA extraction, 2 g of soil were placed in 5 mL of LifeGuard Soil Preservation Solution (MoBio laboratories, Carlsbad, CA, U.S.A.) for ~24 hours at 4 °C, and then maintained in dry ice/-80 °C until extraction, which took place seven days after sampling. Extractions were performed with the RNA PowerSoil Total RNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions. Extracts were re-suspended in 1 mM sodium citrate, quantified using a Quant-iT™ RNA AssayKit (range 5-100 ng; Invitrogen, Molecular Approaches, Eugene, OR, USA) on a Qubit™ fluorometer (Invitrogen, by Life Technologies, Nærum Denmark). Samples
with total RNA concentrations < 20 ng μL⁻¹ were discarded. Products underwent an optimized DNase treatment from the DNA-free™ Kit (Ambion®, by Life Technologies™, Nærum, Denmark) protocol and were then subjected to reverse transcription using the Roche reverse transcription kit (Roche, Hvidovre, Denmark) with Random Hexamers (100 μM; TAG Copenhagen, Denmark). Further details are available in Supplementary Information S3.

16S rRNA GENE COPY NUMBER AND TRANSCRIPT QUANTIFICATION

Quantitative PCR of the 16S rRNA gene was run with reverse-transcribed RNA (cDNA) and DNA, respectively, using an ABI PRISM 7300 Cycler (Applied Biosystems, Darmstadt, Germany) targeting the 264-bp V5-V6 region using the primers 16SFP/16SRP (Bach et al., 2002). Reaction mixtures of 25 μL consisted of 12.5 μL SYBR Green PCR Master Mix (Applied Biosystems, California, U.S.A.), 0.5 μL of 20 mg mL⁻¹ bovine serum albumin (Roche Diagnostics GmbH, Mannheim, Germany), 2 μL of forward and reverse primers (10 mM), and 1 μL of template cDNA or DNA at a concentration of 10 ng μL⁻¹. Cycling conditions were as follows: 95 °C for 10 min, followed by 39 cycles of denaturation at 95 °C for 20 sec, annealing at 62 °C for 60 sec, and extension at 72 °C for 60 sec; fluorescence was detected after annealing. The specificity of the products was confirmed by melting curve analysis and checked on a 1.5% agarose gel. A standard curve was generated using linearized plasmids containing a fragment of the 16S rRNA gene cloned from *Burkholderia* sp. spanning six orders of magnitude (10²-10⁸). Amplification efficiency (E) was calculated according to the equation, E = (10⁻¹/Slope − 1). For all runs, 90% < E < 110%. The obtained data were log-transformed and are shown as the ratio of 16S rRNA transcripts to 16S rRNA gene copy number, which we use as a coarse estimate of average ribosomes per cell.

16S rRNA SEQUENCING AND ANALYSES

cDNA obtained from 10 ng of total RNA was used for 16S rRNA gene transcript amplicon sequencing, described in detail in Supplementary Information S3. Briefly, the primers 341F and 806R (Sigma-Aldrich, Brøndby, Denmark) flanking the V3 and V4 regions of the 16S rRNA gene were used to amplify a gene fragment of 460 bp (Berg et al., 2012; Yu et al., 2005). Sequencing of the 16S rRNA gene transcript amplicons was done using MiSeq reagent kit v2 (500cycles) and a MiSeq sequencer (Illumina Inc., San Diego, CA, U.S.A.).

Sequence analyses were prepared as follows: paired-end reads were mated and trimmed for primers using Biopieces (www.biopieces.org). Reads were quality-filtered with UPARSE (Edgar, 2013) with the following parameters: max expected error algorithm with –maxee 0.5. Dereplication was performed and singletons removed. OTUs were clustered at 97% using usearch_cluster otus and usearch_global. OTUs were chimeras-checked with UCHIME against Greengenes 2011 (DeSantis et al., 2006). Representative reads picked by usearch were classified using the Mothur Wang implementation against the RDP trainset PDS v9 (Schloss et al., 2009). Classifications were accepted at a threshold of 80% confidence at each taxonomic level. Qiime wrappers for PyNAST (Caporaso et al., 2010a), FastTree (Price et al., 2009), and filter_alignment.py (Caporaso et al., 2010c) were used to construct a phylogenetic tree. Alignments were built against the 2011 version of Greengenes (DeSantis et al., 2006) and filtered using—allowed_gap_frac 0.999999 and—threshold 3.0. Amplicon sequences were used as a measure of the composition of the microbial community.

STATISTICAL ANALYSES

All analyses were performed in the R environment (R Core Team, 2014b) using the Vegan (Oksanen et al., 2007), ade4 (Dray and Dufour, 2007), and Phyloseq (McMurdie and Holmes, 2013) packages. Prior to analyses, amplicon data were rarefied to 1474 reads per sample, using rarefy_even_depth from the Phyloseq package (seed.number = 266315).

Taxonomic richness was measured as the number of OTUs per sample, and evenness was measured as Pielou's evenness index. Significant differences between the control and all other treatments were compared for each time point using a two-tailed t-test (p < 0.05).

The rarefied data were also used to examine beta diversity through a Principal Coordinates Analysis (PCoA) of weighted Unifrac distances. The difference between treatments was evaluated with a PERMANOVA with 999 permutations.
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RESULTS

In previously undisturbed soils, the heat shock resulted in a significant, 8.3% average, decrease of normalized 16S rRNA gene transcripts compared to the controls on T4. This was followed by a rapid return to pre-disturbance levels (Figure 2A). A similar pattern was observed for the soils from the heat-heat treatment, which exhibited an 8.1% average decrease compared to control soils on T4. In previously undisturbed soils, the cold shock had no significant effect on the ratio at any point during the experiment (p > 0.12 for all comparisons between control-cold and control treatments). In contrast, in previously heat-treated soils, the cold shock led to a 10% average decrease compared to undisturbed controls on T1. This was followed by rapid recovery. Comparison of soils from the heat-heat and heat-cold treatments to soils from the heat-control treatment revealed similar patterns (Figure S5, A).

We also evaluated the effect of these treatments on α-diversity (total OTUs and Pielou’s J). The heat shock resulted in a significant decrease in these parameters in soils, regardless of prior disturbance. The effects in soils with prior disturbance were more severe, however, as on T1 soils from the control-heat treatment exhibited 30% decreases in richness and 12% decreases in evenness, on average. In contrast, soils from the heat-heat treatment exhibited average reductions of 42% and 23%, respectively (Figure 2, B and C).

BACTERIAL Β-DIVERSITY AND COMMUNITY COMPOSITION

A PERMANOVA of the weighted-Unifrac distances between samples showed a significant effect of treatment (p < 0.001), time since disturbance (p < 0.001) and the combination of these two factors (p < 0.001) on community composition (Figure 3, Supplementary Information S4), however, soils from the control and heat-control treatments did not vary over time. In contrast, the heat-shocked soils (control-heat and heat-heat) exhibited large shifts in community composition following the second heat disturbance (Figure 3). Notably, these shifts in community composition occurred in two stages: in the first stage, samples from T1 and T4 clustered together and were the most different from the undisturbed, control samples, while in the second stage samples from T11-T25 clustered together. Soils

Figure 2. Effects of disturbance legacy of heat shock on the active community. Average ribosomes per cell (A), richness (B), and evenness (C) are shown as normalized ratios relative to the mean undisturbed control values for each respective time point. Average ribosomes per cell are measured as total 16S rRNA gene copies normalized by the number of 16S rRNA gene transcripts. Statistically significant differences between the undisturbed-control and each treatment along time are shown as hollow circles (two-tailed t-test, p < 0.05). Vertical black lines indicate the disturbance event. Normalizations of treatments with prior heat shocks relative to the heat-control treatment are available in Supplementary Information, S5.

using adonis from the vegan package. The recovery of community composition through time was evaluated through a Principal Response Curve (PRC, Van den Brink & Ter Braak, 1999). In order to select OTUs that responded to treatments or changed over time, multiple SIMPER analyses were performed, comparing differences between early (T1-T4) and late (T11-T25) recovery within treatments, as well as within recovery stages and between treatments. SIMPER analysis (Clarke, 1993) was used to select the OTUs accounting for 50% of the dissimilarity observed in pair-wise comparisons between all the conditions. This resulted in 46 OTUs, which were used for further comparative analysis. These were displayed clustered according to their temporal abundance patterns (vegan package, Euclidean distance, Ward’s clustering).
from the heat-heat treatment exhibited a temporal response pattern similar to that of the control-heat treatment, but with a faster rate of recovery: on T4, samples from the heat-heat treatment were more closely related to those of later time points than samples from the control-heat treatment (Figure 3, left panels).

We constructed a principal response curve (PRC, Figure 4) in order to compare the recovery trajectories of the bacterial communities exposed to the different treatments relative to the undisturbed control. For heat-shocked soils, the changes were not gradual: on T1, the microbial communities from the control-heat and heat-heat treatments exhibited compositions that were radically different from those of the heat-control and heat-cold treatments, but samples from these four treatments resembled each other by T4. Furthermore, the structure of the communities of soils from the heat-heat treatment was initially less affected than of those of soils from control-heat treatments, but the former increasingly diverged from the controls throughout the experiment. On the other hand, the communities in soils from the control-heat treatment exhibited a greater deviation from those of the control soils between T11 and T18; these showed signs of recovery by T25. A strikingly different pattern was observed for the cold-shocked soils: soils from the control-cold treatment showed no effect of the cold shock, while soils from the heat-cold treatment exhibited the largest deviations in bacterial community composition of all treatments, relative to the controls. This deviation increased over time, showing no signs of short-term recovery in community structure.

**OTUs EXPLAINING THE VARIATION**

We selected the OTUs which explained 50% of the differences between the communities with respect to treatments and sampling time. These 46 OTUs clustered according to four response patterns, denoted as cluster a, b, c, and d (Figure 5). The OTUs in cluster a consisted of phyla that encompass many typically opportunistic taxa (α-, β-, and γ-Proteobacteria, Fierer et al., 2007). These taxa decreased in relative abundance immediately following the heat shock (i.e. were not heat-resistant) and then gradually increased during the second successional stage (T11-T25). This second-phase increase was greater, or occurred earlier, in the heat-heat treatment than in the control-heat treatment. For example, the average relative abundance of a conspicuous OTU classified as a Phenyllobacterium sp, increased from 0.01% one day after heat shock to 3.6% on T25 of the control-heat treatment, but achieved a relative abundance of 5.12% by T4 of the heat-heat treatment. Cluster b contained OTUs assigned
Acidobacteria (one OTU), but showed no clear patterns. Cluster c contained only rare (less than 1% on average) members of the Proteobacteria (i.e., Porphyrobacter sp., Rhodanobacter sp.) and Bacteroidetes (three Chitinophagaceae OTUs), which were most abundant in soils exposed to cold shocks and decreased permanently in all soils exposed to heat, and maintained average relative abundances below 1%.

**DISCUSSION**

Understanding how disturbances or selective sweeps shape communities and their response to further perturbation is fundamental to our knowledge of the dynamics of the soil biota over time. Disturbances can trigger successional dynamics in soil microbiomes, which is analogous to secondary succession in macroecology (Placella et al., 2012). We have previously shown that microbial community recovery is a deterministic, directional process, and that successional dynamics gradually leads the community away from the post-disturbance dominance of tolerant taxa in the case of transient disturbances (Jurburg et al., 2016b). The use of extreme disturbances was designed to evaluate community assembly during secondary succession rather than the implications for natural environments. By monitoring the soil bacterial community after either an extreme heat shock or a cold shock and in the presence or absence of a prior heat shock, we examined how such a compounded perturbation affects secondary successions, and whether the identity of the legacy prior to perturbations (i.e. heat-heat vs. heat-cold) affects the outcomes.

In control soils, the heat shock had a stronger and more significant effect on the bacterial community than the cold shock. This was apparent from the heat-heat treatment at this time. Several taxa exhibited pronounced peaks in relative abundance following the treatment in the control-heat treatment: an OTU assigned to the Planococcaceae increased in average relative abundance from 1.4% of the community in controls to 11.7% and 6.8% on T1 in the control-heat and heat-heat soils, respectively, and then decreased. Other taxa exhibited peaks in soils from the heat-heat treatment, but experienced rapid decreases thereafter regardless of prior heat shocks: an OTU assigned to Paenisporosarcina increased to 5.1% and 10% of the community in the control-heat and heat-heat treatments respectively, but decreased to less than 1% of the community thereafter. Finally, cluster d contained only rare (less than 1% on average) members of the Proteobacteria (i.e., Porphyrobacter sp., Rhodanobacter sp.) and Bacteroidetes (three Chitinophagaceae OTUs), which were most abundant in soils exposed to cold shocks and decreased permanently in all soils exposed to heat, and maintained average relative abundances below 1%.
analysis of the normalized 16S rRNA data, as well as the α- and β- diversity values. Furthermore, the bacterial community composition recovered from the heat disturbance in two distinct stages, exhibiting radical changes between T4 and T11 (Figure 3). We exposed soils to a second perturbation after 25 days of recovery to increase the possibility that the communities were still undergoing successional dynamics during the second perturbation. Indeed, the effect of the initial heat shock was sustained over time: communities exposed to heat shock maintained lowered levels of α-diversity throughout the experiment, probably due to the permanent removal of members of the community which were not heat-resistant (i.e., Figure 5, cluster d). The speed of recovery following the disturbance was also dependent on the metrics used to assess it: the number of potentially active bacteria returned to pre-disturbance levels by T25 in all soils, while the community composition in soils from the control-heat treatment remained different and showed no indication of recovery (Figure 2). This highlights the complexity of bacterial communities relative to their growth rate, and the need to assess soil microbial recovery with more complex metrics that account for successional patterns.

COMPOUNDED PERTURBATION AND RECOVERY

Our results support the notion that compounded perturbation of soil yields so-called “ecological surprises” (Paine et al., 1998). Our heat and cold shock disturbances targeted different portions of the bacterial communities in soil. Furthermore, the cold shock, which had weak effects on the community on its own, had a drastic effect on soils with a prior heat shock, suggesting a synergistic effect of the two disturbances. In fact, the samples from the mixed compounded perturbation treatment (heat-cold) exhibited a significantly lower number of normalized 16S rRNA copies one day after the cold shock than those from any other treatment, at any other time in the experiment, and community structures in soils from this treatment increasingly deviated from all other treatments over time (Figure 4). The lowered evenness in the communities from the heat-cold treatment relative to those in the heat-control treatment suggests that the cold disturbance, in combination with the prior heat shock, disproportionately altered the dominance patterns. This aligns with an earlier finding that suggested that community evenness is crucial in favoring functional stability under stress in communities of denitrifying bacteria (Wittebolle et al., 2009). Our results further show that decreased evenness results in more profound changes in bacterial community structure from further perturbation. Most of the taxa which became abundant following a cold shock in previously undisturbed conditions were suppressed by the heat disturbance (Figure 5, cluster d), and were thus already suppressed in soils from the heat-cold treatment by the time the cold shock was applied, possibly explaining the stronger effect of the cold shock on previously heat-shocked soils. This aligns with our initial conceptual framework (Figure 1, D). Clearly, the chronology and type of disturbance events in soil is important to determine the outcome of additional perturbations. Further research is necessary to quantify the synergistic effects of mixed compounded perturbation on microbial community recovery.

The overwhelming diversity and variability found in soil bacterial communities and the heterogeneity of the environment which surrounds them are common obstacles for the detection of clear, replicable patterns of community assembly. The selection of extreme disturbance treatments in this experiment allowed us to clearly detect different successional dynamics depending on the combination of disturbances. However, the highly controlled microcosm environment and the extreme nature of disturbances prevent us from applying our findings to natural systems, in which dispersal is much greater, disturbances are more subtle, and other organisms (i.e. mesofauna and plants) may play a role in modulating the observed dynamics. Thus, further research is also needed to determine the applicability of our results under more natural scenarios.

During community recovery from the heat shock, we observed two distinct successional stages, regardless of whether the soils had been pre-exposed to a heat shock. The first stage (T1-T4) was characterized by a community, which differed from those in both controls and the rest of the recovery trajectory (Figure 4), likely explained by the rapid increase in relative abundance of the Firmicutes, particularly Sporosarcina and Paenisporosarcina (Figure 5, cluster c). Many members of the Firmicutes can form heat-resistant endospores, and may be stimulated to germinate by elevated temperatures (Galperin, 2013), which allows the hypothesis that they would be favored by the heat shock.
In particular, several species of *Sporosarcina* have been documented to tolerate temperatures of 80 °C for over 10 minutes, and to produce abundant spores within 3-4 days (Pregerson, 1973). In soils without prior exposure to a heat shock, many Firmicutes persisted at higher relative abundances than in the controls for the rest of the experiment, but they were quickly depressed to near-control abundances in soils, which had been previously exposed to a heat shock. Furthermore, OTUs representing other Firmicutes in this group (i.e. *Planococcaceae incertae sedis* and *Paenisporosarcina*) increased only transiently in both cases, but exhibited a higher relative abundance during this “peak” in soils, which had been previously exposed to a heat shock. This may be due to the lower diversity in these soils, whereby these survivors made up a larger proportion of the community.

The second stage (T11-T25) was characterized by a gradual increase in the relative abundance of predominantly Proteobacteria. The copiotrophic nature of many Proteobacteria is well known (Fierer *et al.*, 2007). It is likely that the relative abundance of such opportunistic taxa increased as the survivor advantage of heat-resistant Firmicutes faded, as we have previously shown (Jurberg *et al.*, 2016b). Alternatively, some slow-growing, oligotrophic Proteobacteria may have benefitted from the release of complex compounds resulting from the disturbance. For example, a lowly abundant (0.001%) *Phenylobacterium* sp. increased in relative abundance more rapidly in soils exposed to the heat-heat treatment than in the control-heat treatment. *Phenylobacterium* strains are able to degrade phenolic compounds (Reznicek *et al.*, 2015), which were likely released following the heat shock, and were probably more abundant after the second shock. In soils that were pre-exposed to a heat shock, the onset of the increase in the relative abundance of presumed proteobacterial copiotrophs occurred earlier, likely because they already occurred at higher abundances in the recovering communities. While our experiment was not designed to tease out individual interactions, this accelerated increase in the relative abundance of opportunists may have resulted in the earlier displacement of Firmicutes, and a more rapid shift to the second successional stage in soils that were pre-exposed to a heat shock.

### IMPLICATIONS

The findings of this study largely match the theoretical framework as shown in Figure 1. As expected, the effect of compounded perturbation was largely dependent on the type of disturbance disturbances. An initial heat shock did not affect the vulnerability of the bacterial communities to a second heat shock, but resulted in an accelerated shift to the second successional stage. This is likely due to a higher percentage of opportunists in the recovering community, which resulted from the first perturbation. The increased number of taxa, which were characteristic of the second successional stage may have caused a faster shift towards the second successional stage upon re-exposure.

In successional gradients driven primarily by competition, an increased biodiversity, and the resulting competitive pressure, may slow down the successional dynamics (Drury and Nisbet, 1973). In our system, the lowered community diversity in soils pre-exposed to a heat shock relative to that in the undisturbed soil may have resulted in lowered numbers of competitors for the resources available after the heat shock, and thus a faster transition towards the opportunists that are characteristic of the second successional stage. In this way, soils with a disturbance legacy have become ‘specialized’ in recovering from a specific perturbation. Our results from the mixed compounded perturbation treatment show that this specialization comes at a cost, however. Soils that had been pre-exposed to a heat disturbance exhibited disproportionately larger shifts in community composition in response to a weaker (cold) disturbance than soils without this prior heat shock. In this case, the order of perturbation may be crucial. We did not test the effect of a heat disturbance on soils pre-exposed to a cold shock; however, it is plausible that the effects of this treatment would have been weaker, as the community quickly recovered from a cold shock.

Our findings show that disturbances affect soil bacterial communities, likely removing vulnerable individuals and altering dominance patterns. This affects the community’s resilience to further perturbation. Previous theoretical work has concluded that a soil microbial community’s resilience is largely determined by the soil’s exposure to disturbances in the past (Hawkes and Keitt, 2015). Our findings are consistent with this notion. We here suggest that a soil’s disturbance legacy must be considered in a “legacy budget”, as there
may be a critical threshold of disturbance intensity and frequency, at which the soil community loses its compositional—and even functional—integrity. We only tested one disturbance frequency, but we suspect that, given the successional patterns observed in the heat-shocked bacterial communities, the responses observed are highly dependent on disturbance frequency. For example, Kim et al. showed that soil bacterial communities subjected to an increasing frequency (every 7, 14, 28, and 56 days) of dilution into sterile soil collapsed when the dilutions were weekly, resulting in highly ‘erratic’ community compositions (Kim et al., 2013). As in our experimental setup, their disturbance (90% dilution) was designed to evaluate community assembly during secondary succession rather than the implications for natural environments. Further research is needed to disentangle the relative influences of disturbance intensity and frequency on the response of soil microbial communities, as well as the role of abiotic factors such as soil type, in buffering the microbiota from environmental changes. This is particularly important in a world in which environmental fluctuations are expected to intensify, and soil microbial communities will need to be able to withstand a wide range of fluctuations in order to maintain their ecological integrity.

ACKNOWLEDGEMENTS

We would like to thank Nick de Vetten and Paul Heeres for providing us with soil samples, Esther Chang for her helpful comments, Britt Danhoff for help with lab work and S. Tem for help with analyses. This research was supported by the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7 2007/2013 under REA Agreement n° 289949 (TRAINBIODIVERSE). The authors declare no conflict of interests.