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Soil bacteria show different tolerance ranges to an unprecedented disturbance

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Abstract Soil microbial communities have remarkable capacities to cope with ceaseless environmental changes, but little is known about their adaptation potential when facing an unprecedented disturbance. We tested the effect of incremental dose of microwaving on soil bacteria as a model of unprecedented stress. 16S rRNA gene qPCR at both the DNA and cDNA levels was used to characterize the total (DNA) and transcriptionally active (cDNA) fractions of the bacterial community. Amplicon sequencing of 16S rRNA gene transcripts was performed to decipher tolerance ranges within the community using the concept of functional response groups (FRGs). Increasing microwaving doses resulted in 90% loss in total and transcriptionally active bacterial communities after

6.8 and 4.7 min, respectively. Four distinct FRGs with peculiar phylogenetic signatures were identified, revealing a link between taxonomy and increasing stress doses. FRG1, the most sensitive group, was dominated by Actinobacteria. FRG2 and FRG3, with intermediate tolerance, displayed prevalence of Proteobacteria, while FRG4, the most resistant group, was driven by Firmicutes. While the most sensitive FRGs showed predictable responses linked to changes in temperature and soil water content associated with microwaving, more tolerant FRG4 members exhibited a stochastic response nested within the Firmicutes phylum, potentially revealing bet-hedging strategists. The concept of FRGs based on 16S rRNA gene transcripts stood as an efficient tool for unraveling

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bacterial survival strategies and tolerance ranges triggered by incremental doses of an unprecedented stress, with regard to phylogeny linkages.

Keywords Soil bacteria · Biodiversity · Disturbance · RNA · Functional response group · Bet-hedging

Introduction

Understanding how organisms adapt to environmental changes lies at the heart of ecology but is a relatively new pursuit in microbiology (Prosser 2012) and soil biology in general (Barot et al. 2007). Until recently, the typical high phylogenetic diversity and rapid succession rates of microbial communities have impaired in-depth studies of their dynamics, especially in response to disturbances (Prosser et al. 2007; Shade et al. 2012). The development of high-throughput DNA sequencing techniques has allowed systematic evaluation of phylogenetic relationships among microbiome members and has become instrumental in understanding community-wide patterns along successional environmental gradients (Dini-Andreote et al. 2015; Shade et al. 2013). In addition, adoption of trait-based approaches has proven to be a powerful tool in understanding the relationship between microbial community composition and ecosystem functioning (Krause et al. 2014). Trait-based approaches can explain, for example, a community's propensity to invasion (Mallon et al. 2015) as well as its diversity (Barnard et al. 2015; Bouskill et al. 2012; Salles et al. 2012). The integration of high-throughput DNA sequencing and trait-based approaches may serve to better understand microbial community responses to perturbations (Martiny et al. 2015) and how do they reorganize themselves following a known/unknown disturbance (Jurburg et al. 2017a, b). We define a disturbance as a transient event that either directly alters the community (e.g., application of an antibiotic), or alters the environment, thereby affecting the community (e.g., flooding; Rykiel 1985). While alterations may be seen and investigated at the ecosystem functional level, we focus here on the compositional changes occurring in microbial communities, both in terms of phylogeny, abundance, and diversity. The disturbance may have short-term effects on the community by changing its composition, but long-term effects may also be observed, as initial alterations may trigger feedbacks and further resulting shifts, or "legacy effects" (Nunes et al. 2016; Jacquiod et al. 2017a).

Understanding how a disturbance affects a microbial community relies often on the ability to classify the response of its members into functional response groups (FRGs, i.e., groups of organisms, which responds similarly to changes in their environment) and functional effect groups (FEGs, i.e., groups of organisms, which contribute similarly to ecosystem function, also known as "guilds") (Lavorel and Garnier 2002;

Lennon et al. 2012; Nunes et al. 2016; Jacquiod et al. 2017a, b). A FRG can be, for example, a group of soil organisms, which have similar pH tolerance ranges, while an FEG may correspond to the group of soil organisms, which are able to degrade lignin. FRGs and FEGs are neither exclusive nor inclusive of each other but can be strongly connected as groups of organisms with different tolerance to a stressor (FRGs) can contribute to the same function (FEG). Therefore, the disparity between members of FRGs and FEGs within a community results in the persistence of functions in a fluctuating environment. At the community level, the functional range is much broader than any of the respective individual tolerance ranges, and the system as a whole may be functionally resistant to disturbances despite genetic and member losses.

While in macroecology the study of functional response traits is more common than functional effect traits (Suding and Goldstein 2008), the opposite is observed for microbial systems where high diversity, rapid growth rates, and functional redundancy are often assumed to compensate for phylogenetic loss in response to environmental changes (Finlay et al. 1997). While a wealth of literature is available on the functional and structural responses of microbes to environmental change (reviewed in Griffiths and Philippot 2013), mechanistic insights into the relationship among environmental fluctuations, individual tolerance ranges, and the recovered community are lacking. Thus, while resolving FRGs of a microbial community is fundamental to understanding its responses to environmental change, FRGs are seldom quantified as it requires measuring community responses to a wide range of doses for the specific disturbance. Nevertheless, we propose the application of the FRG concept to describe soil microbes. FRG is an appropriate concept to study community-wide patterns instead of selected narrow trait-based approaches (FEG). Moreover, the phylogenetic coherence of microbial FRGs has recently been observed for a range of environmental parameters (Martiny et al. 2015), including long-term metal pollution of soil (Nunes et al. 2016) and sediments (Jacquiod et al. 2017a), as well as water quality affecting microbial trophic status (Jacquiod et al. 2017b).

Here, we characterized a soil bacterial community according to the tolerance patterns of its members (FRG) when exposed to different doses of a stress to which they were never exposed before. Microwaving was selected, as it simultaneously affects a combination of environmental parameters including rapid temperature increase, water loss, and aggregate destructuration, whose entangled resulting consequences create an artificial and previously unseen impact on soil microbes. Not even soils under heat/drought legacies, which have been extensively studied, could have memory for such a stress, as it goes beyond the mere effects of water depletion and high temperature exposure. Indeed, while reported biological effects of microwaving are primarily due to direct

effect on water molecules (Trevors 1996; Islam and Weil 1998), changes in the physical and chemical characteristics of soils are also happening (Ferriss 1984; Wolf et al. 1987; Darbar and Lakzian 2007), creating a chaotic stress impacting microbial niches in unprecedented manners. Environmental analogies resembling this artificial stressor could be natural disturbances with low probability (e.g., sudden fires, volcanic eruptions) to extremely rare phenomena (e.g., meteorite impacts) which are all considered “stochastic” events from an evolutionary perspective, with little or no expected microbial resistance memory. We subjected a grassland soil with no prior history of heat/drought exposure to increasing pulses of microwaving in microcosms (resulting in up to 90 °C and 65% water loss) and analyzed the community composition by targeting the 16S rRNA gene (DNA/cDNA) in soil sampled shortly after the disturbance. Our hypotheses were (i) the applied disturbance dose will significantly affect bacterial community composition, as expected water/temperature changes will occur; (ii) the changes induced in the community will be incremental along the microwave exposure gradient; and (iii) as the selected stress is unprecedented, we expect little to no microbial resistance memory as the dose increases, with no connections between microbial responses and phylogeny. Small doses are expected to trigger the natural response of the community to cope with predictable shifts in temperature and soil water content (deterministic response), while larger doses will induce drastic physicochemical shifts in microhabitat conditions resulting in chaotic diversity loss in the active population, selecting for resistant individuals in a variable and unpredictable manner (stochastic response). Characterizing microbial FRGs provides the first steps toward a better understanding of selective mechanisms involved in soil microbial responses to unprecedented perturbations.

Material and methods

Soil characterization

Soil was collected from an acidic (pH $\approx$ 5.4) sandy loam agricultural field located in Buinen, the Netherlands (52° 55' 386" N, 006° 49' 217" E) (Dias et al. 2012; Pereira e Silva et al. 2012a, b, 2013). The field is used for potato cultivation, with crop rotation involving nonleguminous plants (i.e., barley). The soil has an organic matter content of $3.6 \pm 0.5\%$ of dry weight soil (Pereira e Silva et al. 2012a, b). The site is characterized by a temperate climate with an average temperature of 8.9 °C and average rainfall of 781 mm year⁻¹, which is evenly distributed throughout the year (<http://en.climate-data.org/location/106080/>). Mean monthly temperature extremes for the period of 1971–2012 were -0.8 and 23 °C (<http://worldweather.wmo.int/en/city.html?cityId=142>; <http://www.worldweatheronline.com/Nieuw-Buinen-weather-averages/Drenthe/NL.aspx>).

www.worldweatheronline.com/Nieuw-Buinen-weather-averages/Drenthe/NL.aspx).

Microcosm construction and disturbance treatments

For the construction of soil microcosms, 25 kg of bulk soil was obtained in April 2013 from the top 15 cm of four 2×2 m plots. In each plot, ten subsamples were obtained randomly using a spade. Soil was homogenized by sieving through a 4-mm mesh and stored for stabilization at 4 °C in partially open plastic bags for 2 months, during which soils were amended with 100 mL water and homogenized twice by hand. Twenty-eight wide-necked 200-mL glass bottles were prepared with 50 g of soil at 60% water holding capacity, covered with loosely attached aluminum caps and allowed to stabilize for 2 weeks. Four flasks were kept undisturbed as controls, while the remaining 24 flasks were exposed, in quadruplicates, to six doses of microwaving. The microwaving disturbance consisted of placing the microcosms without caps in an 800-W microwave oven (R201ww Sharp, Utrecht, the Netherlands) at high intensity, arranged in a circle near the edge of the circular tray. Exposure doses of 15 s, 30 s, 1 min, 2 min, 5 min, and 10 min were applied. Soil pH, temperature, and water loss were measured for all samples (detailed protocol in Supplementary data, S1). For practical reasons, a temperature stabilization period of 2 h was applied before sampling for DNA and RNA extraction.

For RNA extraction, 2 g of soil was placed in 5 mL of LifeGuard Soil Preservation Solution (MoBio Laboratories, Carlsbad, CA, USA), incubated for $\approx$ 24 h at 4 °C and shipped on dry ice to the University of Copenhagen, Denmark, where extractions were performed 7 days after sampling.

DNA and RNA extraction and reverse transcription

DNA was extracted from 0.5 g of soil using the MoBio PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions with three additional 30-s cycles of bead-beating (mini-bead beater, BioSpec Products, Bartlesville, OK, USA). The product was quantified and quality-checked by electrophoresing DNA extracts on a 1% agarose gel alongside a 200-bp molecular weight marker (SmartLadder, Eurogentec, Brussels, Belgium).

Total RNA was extracted using the RNA PowerSoil® Total RNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions and handled as in Nunes et al. (2016). Samples with total RNA concentrations < 20 ng/mL were discarded (Table S2). Products underwent an optimized DNase treatment using the DNA-free™ Kit (Ambion® RNA by Life Technologies™, Naerum, Denmark) protocol and then subjected to reverse transcription using the Roche reverse transcription kit (Roche, Hvidovre,

Denmark) with random hexamers (100 µM; TAG Copenhagen, Denmark) (detailed protocols can be found in the Online Resource 1). A 1:10 dilution of the obtained cDNA was used for PCR and qPCR amplifications in order to avoid inhibition.

16S rRNA gene copy number quantification and survival curves

The number of 16S rRNA gene and gene transcript copies were assessed in the controls and in the soils exposed to the different disturbance doses and used to quantify the responses of the transcriptionally active (cDNA) and whole (DNA) bacterial communities to the model disturbance. Copy number quantification was performed using a LightCycler96® (Roche) with the primers EUB338F (5'-ACTCCTACGGGAGGCAGCAG-3') and EUB518R (5'-ATTACCGCGGCTGCTGG-3') (Haugwitz et al. 2014) targeting the V3 region of the 16S rRNA gene allowing the amplification of a 215-bp fragment. Reaction mixtures of 20 µL consisted of 10 µL of 2× Brilliant III SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA), 1 µL of each primer (10 µM), 2 µL of template (either DNA or cDNA in a 1:10 dilution to avoid inhibition), and water. PCR cycling conditions were as follows: 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 56 °C for 10 s, and extension at 72 °C for 13 s, with a final melting cycle of 10 s at 95 °C, 60 °C for 60 s, and 97 °C for 1 s; fluorescence was detected after annealing. A standard curve was generated using a serial dilution of *Escherichia coli* DNA from 10² to 10⁶ copies/µL. Statistical analyses were performed using SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA, USA). One-way ANOVA tests were done ($\alpha = 0.05$) and Kruskal-Wallis one-way analyses of variance on ranks were performed when normality (Shapiro-Wilk test) was not achieved and/or variances were not equal. The number of gene copies per gram of soil was log-transformed and a linear regression fit was performed on the exponential phase of the curve describing gene copies as a function of microwaving time in order to calculate the decimal reduction (D_{10}) of the community using the equation $D_{10} = -\frac{1}{\text{slope}}$ (Crowther 1924). Spearman's rank correlations between the number of 16S rRNA gene copies per gram soil and sample temperature, water loss, and pH were performed using R 3.1.1 software (R Core Team 2014) (Shapiro-Wilk normality test $p \leq 0.05$; Online Resource 2).

Sequencing of 16S rRNA gene transcripts and analyses

Amplicon sequencing was done according to acknowledged best practices (Schöler et al. 2017; Vestergaard et al. 2017). cDNA, obtained from 10 ng of total RNA accordingly to the procedure described in S1, was used for 16S rRNA gene

sequencing. The primers 341F (5'-GTGCCAGC M G C C G C G G T A A - 3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Sigma-Aldrich, Brøndby, Denmark) flanking the V3 and V4 regions of the 16S rRNA gene were adapted from Yu et al. (2005) and used to amplify a gene fragment of 460 bp (Yu et al. 2005). A detailed protocol of the library construction is available in Online Resource 1. Paired-end sequencing of the 16S rRNA gene transcript amplicons was done using MiSeq reagent kit v2 (500 cycles) and a MiSeq sequencer (Illumina Inc., San Diego, CA, USA). Amplicon sequences were analyzed using qiime_pipe (https://github.com/maasha/qiime_pipe) with default settings, which performs sample demultiplexing, quality-based sequence trimming, primer removal, and paired-end reads assembly prior to a QIIME workflow (Caporaso et al. 2010). Settings for paired-end mating were overlap length of minimum 50, maximum mismatches of 15, and a minimum quality of 30. Briefly, criteria for sequence trimming were (1) reads shorter than 200 bp, (2) average quality scores lower than 25, and (3) six as maximum lengths of homopolymers. Chimera check was achieved with UCHIME (Edgar et al. 2011) and operational taxonomic units (OTUs) were picked at 97% sequence identity level. OTU representative sequences were selected by the highest abundance within the cluster and assigned to taxonomy using the RDP classifier, with a confidence threshold of 80%. The database used for annotation was produced using Biopieces (<https://code.google.com/p/biopieces/>) and consisted of a slice of the Greengenes Feb2011 release (DeSantis et al. 2006) database corresponding to the V4 region of the 16S rRNA gene. Annotation tables were generated at the genus level and rarefied to 2500 counts using the *rrarefy* function of the *vegan* package (Oksanen et al. 2013) in R 3.1.1 software (R Core Team 2014), providing sufficient community coverage as previously reported (Caporaso et al. 2011). Eliminated samples are listed in Online Resource 3.

Diversity and statistical analysis

Rarefaction curves (Online Resource 4) and diversity indices (richness, Shannon's diversity, Shannon's evenness, and Chao-1) were calculated using the PAST software ver. 2.17 (Hammer et al. 2001). Differences in diversity indices were determined using one-factor ANOVA (SigmaPlot 12.5) associated with individual Z scores applied to lone values against the whole sample distribution when the number of replicates was less than 3 (for 5 min of microwaving). Analysis of beta-diversity was performed by multivariate analysis using the packages *vegan* (Oksanen et al. 2013), *ade4* (Dray and Dufour 2007), and *made4* (Culhane et al. 2005). Singleton taxa were removed from the datasets before further analyses as they may be the result of sequencing errors. Briefly, the rarefied and trimmed dataset was normalized by center and

scaling, and a principal component analysis (PCA) was performed. A pattern search was then applied to the PCA by grouping the samples accordingly to disturbance dose, using the between-group analysis method (BGA; Culhane et al. 2002). The significance of the selected grouping factor was tested with a Monte Carlo simulation (10,000 permutations). A paired group cluster analysis, using Bray-Curtis dissimilarity index (PAST ver. 2.17), was plotted alongside the BGA.

ANOVA with a Benjamini-Hochberg FDR correction (STAMP v.2.0.5, Parks et al. 2014) was performed on each taxon in order to select the taxa exhibiting changes in abundance with increasing microwaving dose. As only two replicates were preserved for the 5-min dose, a Z test comparing each of these replicates to all the other doses was performed. Here, taxa were only selected when the two-tailed p value < 0.01 , and the value (μ_0) presented, at least by one of the 5-min replicates, was lower than the minimum or higher than the maximum found in the other doses. The selected taxa were plotted in a heatmap of centered and scaled counts created using *gplots* (Warnes et al. 2015), *vegan* and *rioja* (Juggins 2015), and *RcolorBrewer* (Neuwirth 2011), and SIMPER analysis was performed (similarity percentage) using Bray-Curtis dissimilarity index in order to determine how much dissimilarity (in percentage) was explained by these taxa (PAST ver. 2.17).

Functional response groups, corresponding to groups of microorganisms which respond similarly to the increasing disturbance dose, were identified as described in a previous work (Nunes et al. 2016). Briefly, cluster analysis was applied (complete agglomeration method and bootstrapping 10,000 times) on the centered and scaled counts of the selected taxa, using Euclidean distance and a cutoff of 7.5 (*pvcust* package; Suzuki and Shimodaira 2006). Average behavior of each FRG with increasing disturbance dose was presented in a scatterplot.

To determine whether taxa within each FRG exhibited a phylogenetic signal, a phylogenetic tree was created using QIIME's *make_phylogeny.py* (FastTree) and pruned using the package *ape* (Paradis et al. 2004). Phylogenetic distances between and within groups (MPD—mean pairwise distance and MNTD—mean nearest taxon distance) were calculated using *picante* (Kembel et al. 2010). Random groups of the same size of each FRG were included in order to confirm the results. The significance of phylogenetic signals was tested with 1000 within-FRG permutations and presented in Online Resource 5.

Results

Physicochemical properties and qPCR results

Exposure to increasing microwave doses led to a temperature rise and a loss of soil moisture content (Kruskal-Wallis, $p < 0.001$; Fig. 1). Soil pH was, in most cases, not affected

by the treatment (Kruskal-Wallis, $p = 0.06$) but was significantly higher after a 10-min exposure (t test and Mann-Whitney, $p < 0.05$). In order to assess the effects of such an unexpected and transient stress on both the total and the transcriptionally active soil bacteria, quantitative PCR of the 16S rRNA gene was performed using both DNA and cDNA templates (Fig. 2). Following a small increase in 16S rRNA gene copy numbers at low microwaving doses, a decrease was observed for longer exposures (Fig. 2). Associated survival curves revealed convex patterns, with a decimal decrease of $1\text{-log}(D_{10})$ in the number of gene copies after exposure to 6.75 (DNA) and 4.66 min (cDNA) of microwaving, respectively (red dots in Fig. 2). Extractable RNA was below the level of detection for two of the four samples exposed to 5 min of disturbance and for all the samples exposed to the highest disturbance dose (10 min). Both temperature and water loss were negatively correlated with total and transcriptionally active bacterial communities (Online Resource 2).

16S rRNA gene transcript analysis

In order to estimate the tolerance ranges of transcriptionally active community members, 16S rRNA gene amplicon sequencing was performed on cDNA. A total of 407,871 reads were assembled, and quality-checked reads per sample varied from 2888 to 47,633 (Online Resource 3). Samples excluded or lost during experimental procedures are listed in Online Resource 3. No significant differences in diversity indices were detected between the control, 15-s, 30-s, 1-min, and 2-min doses (Fig. 3, ANOVA, $p > 0.05$); however, a significant decrease in Shannon diversity and evenness was detected after 5 min exposure (Fig. 3, Z scores, $p < 0.001$). Moreover, the microwave doses had a strong effect on the soil bacterial community structure, with different community compositions resulting from increasing exposure (Fig. 4). The BGA showed that the first axis (43.3%) separated the control and all other doses from the 5-min dose, while the second axis (20.1%) showed a gradual effect of increasing microwave doses on the community composition (Fig. 4a; BGA inertia ratio = 0.34; Monte Carlo simulation $p < 0.001$). This gradual change in composition with increasing doses, followed by a drastic change after 5 min of exposure, was also consistent in the cluster analysis and in the analysis of abundance at high taxonomical ranks (Fig. 4b). The relative abundance of Actinobacteria dropped with increasing microwaving dose, while the relative abundance of Firmicutes decreased with the lower doses, followed by an increase at the highest doses (Fig. 4b; ANOVA, $p < 0.05$). Proteobacteria and Alphaproteobacteria presented the opposite pattern of Firmicutes, their relative potential activity increased with lower doses and decreased at the highest microwaving dose (Fig. 4b; ANOVA, $p < 0.05$).

Fig. 1 Effects of microwaving doses on soil temperature (diamonds), water loss (squares), and pH (triangles). Error bars represent standard error of average values ($n = 4$)

Functional response groups analysis

The relative potential activity of 76 taxa (genus level)—representing 86.6% of the total community—changed significantly with increasing microwaving doses (ANOVA, $p < 0.05$ and Z score, $p < 0.01$). These 76 taxa were responsible for 83.9% of the dissimilarity between the doses (SIMPER, Bray-Curtis dissimilarity index; Fig. 5) and were classified into four FRGs, according to their tolerance ranges, showing a progressive shift from an Actinobacteria- (FRG1) to a Proteobacteria- (FRG2 and FRG3) and finally Firmicutes-dominated community (FRG4) (Figs. 5 and 6). Most FRGs displayed significantly lower mean pairwise distance (MPD) indices than the null model simulations ($p = 0.001$; Online Resource 5), indicating significant phylogenetic relatedness of constituting members within each group. The exception was FRG2 (MPD; $p = 0.969$; Online Resource 5) which, despite the fact of having a phylogenetic signal significantly lower than the null model simulation when assessed by mean nearest taxon distance (MNTD; $p = 0.001$), presented a MNTD value (0.049) closer to the null model simulation (0.051) than the other FRGs, indicating lack of relatedness or higher phylogenetic diversity within the group (Online Resource 5). These results support the diversity analyses, showing conservation of

response traits at the phylum level, especially for FRG1, FRG3, and FRG4 (Fig. 5).

FRG1 (25.9% of the total abundance), which contained genera such as *Arthrobacter*, *Mycobacterium*, *Nitrospira*, and *Solirubrobacter*, constituted the most sensitive group, showing a progressive decline in the relative abundance of 16S rRNA transcripts with increasing exposure doses (Fig. 6). FRG2 and FRG3 (representing 40.9 and 4.4% of the total abundance, respectively) contained individuals with intermediate sensitivity to microwaving, exhibiting the highest potential activity at lower doses and decreasing after prolonged exposure (Fig. 6).

FRG2, which included, e.g., *Niastella*, *Nitrosospira*, *Rhodanobacter*, and *Rhodoferrax*, showed an enhanced sensitivity to the disturbance, while members of FRG3 exhibited a broader tolerance range, with a decrease in 16S rRNA transcription only after 2-min exposure. FRG4 (representing 15.4% of the total taxa abundance) corresponded to the most resistant organisms, showing relatively high 16S rRNA production at the largest dose (Fig. 6), and included genera such as *Bacillus*, *Clostridium*, *Rhizobium*, and *Ruminococcus*. However, while the microbial response observed in FRG4 was clearly associated with a strong phylogenetic signal with many Firmicutes, the responses in the two replicates exposed

Fig. 2 Survival curves of the total (a) and the transcriptionally active (b) soil bacterial communities after exposure to microwaving. The log-transformed number of gene and gene transcript copies per gram of dry soil are plotted against the increasing microwaving dose. D_{10} is represented by red dots in the charts and was calculated for doses from 15 s to 5 min. Error bars correspond to standard error of average values ($4 \leq n \leq 8$)

Fig. 3 Richness (a), Shannon (b), evenness (c), and Chao-1 (d) indices calculated from 16S rRNA gene transcript sequence data for the different microwaving doses. Error bars represent standard error of average values ($2 \leq n \leq 4$)

to a 5-min dose were very variable with different Firmicutes OTUs responding in each of them (Fig. 5).

Discussion

Survival curves and DNA/cDNA resolution

Microwaving had a pronounced effect both on the bacterial population size and composition, leading to 90% loss among the transcriptionally active community after 4.66 min of exposure, confirming its status of harsh unprecedented stressor. These results are in accordance with the literature, which reports a general decrease in the number of microorganisms with increasing microwaving doses (Vela and Wu 1979; Wainwright et al. 1980; Speir et al. 1986; de Boer et al. 2003; Velikonja et al. 2014), but also some degree of resistance at up to 6 min of exposure (1000 W microwave oven; Wainwright et al. 1980). The convex shape of the survival curves indicated a typical inactivation behavior of the soil bacterial community (Online Resource 6; Bazin and Prosser 1988), revealing different levels of sensitivity within the same community. The temperature range applied here was insufficient to fully destroy DNA molecules (Marguet and Forterre 1994; Thiel et al. 2014). As a consequence, a larger D_{10} value was estimated from DNA compared to cDNA, likely reflecting the higher stability of DNA molecules (Lindahl 1993; Berg et al. 2002) and also the probable accumulation of environmental DNA progressively released from soil particles (Pietramellara et al. 2009), dead cells, and extracellular

DNA movements in soil associated to changes in water status (Ceccherini et al. 2007). Thus, cDNA-based qPCR proved to be more sensitive in detecting the response patterns along increasing microwaving doses compared to DNA-based qPCR. Indeed, the bacterial response seen after 15-s exposure was more pronounced and variable at the cDNA level. The use of microbial inactivation approaches (Botelho et al. 2007; Parmegiani et al. 2010; Nunes et al. 2012, 2013) in combination with qPCR was a powerful and successful tool, which can be easily applied to other stresses in controlled experiments.

Phylogenetically driven response at low microwaving doses

As microwaving was significantly changing the soil bacterial community, and as cDNA allowed better response estimation than DNA, 16S rRNA gene amplicon sequencing at the cDNA level was applied to identify responding taxa. Dissimilarity analysis revealed enhanced variability with increasing dose, indicating a shift from predictable to heterogeneous responses (Figs. 4 and 5). The four identified FRGs correspond to transient successional changes in the transcriptionally active bacterial community with increasing exposure, from FRG1, showing early activity, to FRG2–3 at intermediate doses, and FRG4 at the highest exposure. Moreover, FRG1, 3, and 4 were characterized by significant phylogenetic signals. While FRG1 was dominated by Actinobacteria, which are known to increase their relative abundance in microcosm setups due to moisture (Jacquiod et al. 2013), FRG3 had a clear predominance of Alphaproteobacteria which are

Fig. 4 Composition of the transcriptionally active bacterial communities. 2D representation of between-group analyses (BGA) obtained from principal component analysis of centered and scaled data using the different doses of microwaving as grouping factor (**a**). BGA ratio and respective p values were determined by Monte Carlo simulation using 10,000 permutations. Constrained cluster analysis of 16S rRNA gene transcript sequence data obtained before and after exposure to microwaving, using the Bray-Curtis dissimilarity index (**b**). Stacked horizontal bars depict the

abundance of taxa present in each sample. Separation between samples is the result of differences at genus level in the relative abundances of the taxa present. Colors in the stacked horizontal bars correspond to the phylum level classification of each taxon, with exception of Proteobacteria which were classified to class level. Stars represent the significance of the differences found in the phylum abundance: (*) $0.05 \leq p$ values < 0.01; (**) $0.01 \leq p$ values < 0.001; (***) p values ≤ 0.001

often considered typical soil opportunists/copiotrophs (Philippot et al. 2010), and FRG4 was driven by Firmicutes, also known for their resistance/tolerance to heat (Vos et al. 2009). FRG2 did not display significant signs of relatedness, as it was constituted by members from wide phylogenetic origins, which could be interpreted as a response of generalist strategists from broad taxonomical origins. Most FRG1 members (e.g., *Arthrobacter*, *Solirubrobacter*, *Acidisoma*, *Nitrospira*, and unclassified Acetobacteraceae) are nonspore formers with low resistance to high temperatures (Watson

et al. 1986; Funke et al. 1996; Singleton et al. 2003; Jones and Keddie 2006; Belova et al. 2009). While FRG2 included mesophilic genera such as *Niastella* (Weon et al. 2006), *Nitrosospira* (Head et al. 1993), *Phenylobacterium* (Eberspächer and Lingens 2006), *Rhodoferrax* (Hiraishi et al. 1991), and *Rhodanobacter* (Nalin et al. 1999), whose reported optimal growth temperature range was reached within the first 30 s of exposure (~ 30 – 35 °C), FRG3 had an apparent broader heat tolerance range, with members surviving a 2-min exposure (~ 66 °C). This broader temperature range is in

Fig. 5 Heatmap plotting the relative abundances of 76 taxa based on 16S rRNA transcripts differing significantly between microwaving doses. *p* values were determined using ANOVA ($\alpha = 0.05$; Benjamini-Hochberg correction) associated with a *Z* test ($\alpha = 0.01$) and data are centered and scaled to the average of each taxon abundance. Microwaving dose increases from the left (control—green) to the right (5 min microwaving—dark red) and each vertical bar corresponds to a sample. Sample clustering (constrained average agglomeration method) and SIMPER analysis were based on Bray-Curtis dissimilarity index, while centered and scaled taxa clustering were based on Euclidean distance (complete agglomeration method; bootstrap = 10,000). Functional response groups (FRG) were identified using a distance cutoff of 7.5 and are represented in the lateral dendrogram by colorful branches. All tests were based on rarefied data (2500 sequences)

accordance with the thermotolerant Alphaproteobacterial taxa found in FRG3 (e.g., some Sphingomonadaceae, *Brevundimonas*, and *Caulobacteraceae*), as well as with the genus *Deinococcus*, known for having species with very effective DNA repair systems conferring high resistance to temperature, ionizing radiation, and desiccation (Slade and Radman 2011).

Soil microbial communities are known to react rapidly to sudden environmental changes, e.g., through the production of heat-shock proteins or stabilizing solutes upon an increase in temperature (Holden et al. 1999). Moreover and as an example, it has been shown that microbes respond within less than half an hour to soil rewetting (Iovieno and Bååth 2008; Meisner et al. 2013, 2015) and that subtle temperature increases (e.g., ~1 °C) alters microbial composition in macroaggregates (Fang et al. 2016). Thus, in the 2 h after the

microwaving shock, it is likely that members of FRG2 and FRG3 also benefitted from nutrients released from the lysis of sensitive cells that perished when exposed to the lower doses (easily degradable; Darbar and Lakzian 2007), and/or from nutrients released from disrupted soil aggregates (with variable complexity; Jablonowski et al. 2012; Wang et al. 2014). Indeed, FRG2 and FRG3 also contained taxa with particular nutrient spectra. For instance, *Phenyllobacterium*, *Niastella*, and *Rhodanobacter* (FRG2) harbor representatives with the ability to hydrolyze complex compounds, such as phenyl compounds, chitin, carboxymethyl cellulose, or lindane (pesticide) (Lingens et al. 1985; Weon et al. 2006; Zhang et al. 2010). Taxa belonging to the family Sphingomonadaceae (*Sphingomonas*; FRG3) are capable of degrading xenobiotic substances (Ederer et al. 1997). Individuals from the *Hyphomicrobium* genus (FRG3) are

Fig. 6 Effects of microwaving doses on the tolerance ranges of the four identified functional response groups (FRGs). Dashed color lines show the average behavior of 76 taxa significantly affected by microwaving doses (ANOVA with Benjamini-Hochberg correction, $\alpha = 0.05$; Z test, $\alpha = 0.01$), grouped in four FRGs accordingly to a cutoff of 7.5 in the cluster analysis of the centered and scaled number of counts (complete agglomeration method; bootstrap = 10,000). FRGs represent the three main strategies adopted by the soil bacterial communities when exposed to microwaving: perish (FRG1—green); be enhanced by low/middle intensity doses and perish with high (FRG2 and FRG3—respectively light blue and blue); and resistant communities relatively enhanced only by high doses (FRG4—red). Values > 0 correspond to an amount of 16S rRNA gene transcripts higher than the average obtained for that FRG, while values < 0 correspond to an amount of 16S rRNA gene transcripts lower than that same average. Error bars represent standard error of average values ($2 < n < 4$)

methylophils capable of using 1-C compounds (Urakami et al. 1995) and *Lysobacter* spp. (FRG3) are capable of degrading complex polysaccharides like chitin (Jacquiod et al. 2013) or carboxymethyl cellulose. These genetic capacities may provide a significant fitness boost, as a wide nutritional spectrum would allow members of FRG2 and 3 to take advantage of resources being released and solubilized by disruption of soil aggregates with warmed water and cell content from dead FRG1 members. These observations are also coherent with reported copiotrophic lifestyles of Alpha-, Beta-, and Gammaproteobacteria, which mainly constitute FRG2 and 3 (Philippot et al. 2010). This highlights the presence of a certain degree of predictability in the recorded phylogenetic-driven bacterial responses to lower microwaving doses, which is in accordance with our original hypothesis.

Phylogenetically driven heterogeneous response at high microwaving doses

At higher temperatures, it is likely that specialized adaptive features, such as DNA repair mechanisms and spore formation, are necessary for survival. Indeed, FRG4, which is mainly representing the 10% potentially active taxa at 4.66 min of exposure, contained the most heat-resistant taxa, especially known thermophilic members from Firmicutes. Among them, the Gram-positive, endospore-forming *Bacillus* and *Clostridium* genera are known for their resistance to both heat and desiccation (Vos et al. 2009). The Gram-negative,

nonspore-forming, nitrogen-fixing *Rhizobium* genus was also found within FRG4, confirming the previously attested capacity to survive prolonged microwaving (Nelson 1996). However, despite the Firmicutes-driven phylogenetic signal, the response of FRG4 members was highly variable, as seen on the abundance patterns of the two remaining samples that yielded RNA after 5 min exposure, with different Firmicutes OTUs responding in each replicate (Fig. 5). Moreover, no RNA was extracted at the highest disturbance dose (10 min), suggesting the collapse of the active community fraction. These observations (the gradual shift of the community concomitantly to the variable response after exposure to the 5-min dose) are coherent with the underlying assumption of a stochastic response within the community, probably dependent on the physiological state/phenotype of its individual members when exposed to high disturbance doses, and perhaps also pure chance due to soil heterogeneity/safe spots. Nevertheless, a significant phylogenetic signal was overlaying this stochastic response, as the selected taxa were mostly belonging to Firmicutes. This suggests that conserved genetic traits involved in this stochastic response were phylogenetically selected and maintained within communities, supporting the idea of bet-hedging microbial strategists in soils (Cohen 1966; Beaumont et al. 2009; Rajon et al. 2014). According to the bet-hedging theory, the existence of different phenotypes within a community living in a given environment increases the survival odds of its members in case of a novel disturbance, resulting in variable responses (Cohen 1966; Beaumont et al. 2009). It is likely that the most intense microwaving doses resulted in higher selective pressures toward few taxa, whose tolerance depended on their physiological state as previously suggested (Rajon et al. 2014).

A good candidate genetic trait potentially involved in this stochastic response could be sporulation. Indeed, dormancy is an important aspect of soil bacterial adaptation, which is known to be associated with prior 16S rRNA accumulation (as reviewed by Blazewicz et al. 2013). Endospore formation seems to be important following long exposure to microwaving, especially within the FRG4. Moreover, germination of endospores by microwaving was previously reported for *Bacillus*, *Clostridium*, and other genera within Firmicutes (Vaid and Bishop 1998; Pellegrino et al. 2002; Aslan et al. 2008; Ammann et al. 2011). Another important and archetypal feature of Firmicutes is that some members are prone to acquire novel beneficial genetic traits increasing their fitness via horizontal gene transfer (Lanza et al. 2015). This is particularly true for representatives from *Bacillus* and *Streptococcus*, which display notable natural competence (Johnsen et al. 2009; Evans and Rozen 2013; Mell and Redfield 2014). As it can either be advantageous, neutral and deleterious to incorporate environmental DNA from unknown sources, being prone to horizontal gene transfer typically fits the spirit of being a bet-hedging strategist. Therefore,

the detected increase in the relative abundance of 16S rRNA gene transcripts assigned to Firmicutes in FRG4 may reflect the transcriptional activity of growing bet-hedging survivors, benefiting from nutrient profusion, environmental DNA “up for grab,” and available niches left behind by sensitive members from other FRGs.

Functional redundancy may maintain ecosystem function upon disturbance

While reaching the limits of what can be said based on 16S rRNA gene amplicon sequencing, our results provide a first glimpse of the immediate responses of soil bacterial communities to unexpected disturbances, as well as insight into how functional redundancy may contribute for maintaining ecosystem functions in case of environmental change. For example, among the four FRGs identified in this study, three contained nitrogen-fixing bacteria. The heat-sensitive FRG1 was dominated by Actinobacteria and included *Arthrobacter* spp., which contains members that are potential nitrogen-fixing bacteria displaying nutritional versatility (Funke et al. 1996; Jones and Keddie 2006). Within FRG2, the genus *Bradyrhizobium* is known to be capable of fixing atmospheric nitrogen. Finally, FRG4 by far the most heat-resistant group of bacteria contained the genera *Clostridium* and *Rhizobium* both of which have members capable of fixing atmospheric nitrogen (Vos et al. 2009). This would indicate that, even after severe member loss due to temperatures above 60 °C, key genetic traits like nitrogen fixation are likely maintained.

The nitrogen fixation function seemed to prevail thanks to substituting taxa taking over empty niches, indicating that functional redundancy in soil bacteria might be a crucial feature for disturbance recovery. Some nitrogen-fixing genera perished with low and intermediate microwaving doses (*Arthrobacter*, FRG1; *Bradyrhizobium*, FRG2), while other genera take over or simply are not affected and may perform the same function after exposure to high microwaving doses (*Rhizobium*, FRG4). Nitrogen fixation is quite common among soil bacteria (van Elsas et al. 2006), unlike other specialized functions performed by few groups like nitrification (Nannipieri et al. 2003). Nitrifiers such as *Nitrospira* or *Nitrosospora* were found only in FRG1 or FRG2, respectively, suggesting that ability to convert ammonia to nitrite and further on to nitrate would be compromised even at the lowest doses. In this way, the immediate effect of a disturbance on soil community function is indeed highly dependent on the type of genetic trait considered (Schimel 1995; Girvan et al. 2005).

Conclusion

By focusing on the immediate responses of the community to an unprecedented disturbance, we provided an in-depth

analysis of microbial community tolerance ranges. The concept of functional response group was efficiently applied to resolve tolerance ranges, microbial successions and generalist/specialist strategies at lower doses, as well as a pronounced phylogenetically driven microbial stochastic response associated to higher doses. Moreover, we have shown that stress tolerance-related traits are conserved at high taxonomic level and demonstrated that PCR amplicon sequencing of cDNA from 16S rRNA gene is a powerful experimental approach to define functional response groups.

Research questions related with recovery, successional stages, and disturbance memory were addressed in two follow-up studies where a 1.5-min dose was applied (Jurburg et al. 2017a, b). Nevertheless, future research should be performed in order to better understand how do the microbial communities reorganize and recover over time, and to elucidate the exact mechanisms behind this.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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