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Early successional patterns of bacterial communities in soil microcosms reveal changes in bacterial community composition and network architecture, depending on the successional condition

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ABSTRACT

Soil ecosystem dynamics are influenced by the composition of bacterial communities and environmental conditions. A common approach to study bacterial successional dynamics is to survey the trajectories and patterns that follow bacterial community assemblages; however early successional stages have received little attention. To elucidate how soil type and chemical amendments influence both the trajectories that follow early compositional changes and the architecture of the community bacterial networks in soil bacterial succession, a time series experiment of soil microcosm experiments was performed. Soil bacterial communities were initially perturbed by dilution and subsequently subjected to three amendments: application of the pesticide 2,4-dichlorophenoxyacetic acid, as a pesticide-amended succession; application of cycloheximide, an inhibitor affecting primarily eukaryotic microorganisms, as a eukaryotic-inhibition bacterial succession; or application of sterile water as a non-perturbed control. Terminal restriction fragment length polymorphism (T-RFLP) analysis of the 16S rRNA gene isolated from soil microcosms was used to generate bacterial relative abundance datasets. Bray-Curtis similarity and beta diversity partition-based methods were applied to identify the trajectories that follow changes in bacterial community composition. Results demonstrated that bacterial communities exposed to these three conditions rapidly differentiated from the starting point (less than 12 h), followed different compositional change trajectories depending on the treatment, and quickly converged to a state similar to the initial community (48–72 h). Network inference analysis was applied using a generalized Lotka-Volterra model to provide an overview of bacterial OTU interactions and to follow the changes in bacterial community networks. This analysis revealed that antagonistic interactions increased when eukaryotes were inhibited, whereas cooperative interactions increased under pesticide influence. Moreover, central OTUs from soil bacterial community networks were also persistent OTUs, thus confirming the existence of a core bacterial community and that these same OTUs could plastically interact according to the perturbation type to quickly stabilize bacterial communities undergoing succession.

1. Introduction

Biological succession (BS), or more inclusively community dynamics, may be understood as the changes in the composition or architecture of a community assemblage at a specified location over time (Pickett and McDonnell, 1989; Meiners et al., 2015). Although BS theory has been mostly applied to study macro-organism communities, several microbiological studies have used the BS concept to explain the changes that occur in microbial communities over time (Schmidt et al., 2014; Zhou et al., 2014; Brannen-Donnelly and Engel, 2015) and over time and space (Bajerski and Wagner, 2013; Storey et al., 2015; Beam et al., 2016). However, few studies have described the changes in bacterial assemblages during early stages of bacterial succession, as in colonization of pristine soil or heavily perturbed substrates.

Perturbations are incredibly varied, as are their impacts on communities (Armesto and Pickett, 1985). As such, initiating factors may

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represent the dominant shaping force between perturbation and succession (Meiners et al., 2015). To measure perturbation effects, changes in community diversity and composition are usually analyzed (Shade et al., 2012; Itoh et al., 2014; Brannen-Donnelly and Engel, 2015). However, little is known about how perturbations affect the early compositional trajectories and community network architecture that a perturbation-induced succession produces. Conceptual models of successional dynamics of microbial communities assume that predictable changes in species composition occur during microbial BS (Nemergut et al., 2007; Redford and Fierer, 2009; Shade et al., 2012; Fukami, 2015). Species turnover processes, i.e. the replacement of species, has been shown to occur in a predictably manner in a variety of ecosystems (Schmidt et al., 2007; Redford and Fierer, 2009; Fierer et al., 2010). Nestedness, a measure that quantifies the overlap in species composition between high- and low-diversity times (Atmar and Patterson, 1993), is especially high in fragmented ecosystems (Ruhí et al., 2013) or during late BS stages of biofilm development (Jackson et al., 2001).

Advances in techniques to estimate changes in community composition have elucidated the processes of resistance and resilience to environmental disturbances. However, understanding changes in both community composition and the interactions between organisms in that community is necessary obtain sensitive indicators of the health status of an ecosystem (Burkhard et al., 2008; Fukami, 2015). While there are extensive data on interactions between populations in communities of macro-organisms, much less is known about the interactions between bacteria, mainly because these interactions are more difficult to observe and document. Advances in bioinformatics and statistics have provided a wealth of tools for the inference of community networks; however, these methods have not yet been applied to estimate the restoration potential of an ecosystem based on changes in microbial community networks. One of the methods used for network inference is based on the Lotka-Volterra (LV) model. Using this model, it is possible to hypothesize putative interactions though inference of both the sense of the interaction (who affects whom) and the type of interaction (positive or negative) between relevant actors in the community (Faust and Raes, 2012; Berry and Widder, 2014; Agler et al., 2016; van der Heijden and Hartmann, 2016).

Considering the importance of colonizing microorganisms in initiating succession, the bacterial community composition at the beginning of succession may play a central role in shaping soil ecosystem dynamics in response to perturbations, modulating land recovery, maintaining soil health (Berendsen et al., 2012; Chaparro et al., 2012; Itoh et al., 2014; Creamer et al., 2016), and promoting successful settlement and growth of plants (Chabrerie et al., 2003; Knelman et al., 2012). Studying the composition of soil bacterial communities and successional dynamics contributes not only to the understanding of bacterial community processes occurring in soil microbial ecosystems, but also to the development of strategies for sustainable land management, as an agro-resource.

This work describes how the successional conditions of soil bacterial communities affect the trajectories of compositional changes and how these changes are reflected in the structure of community bacterial networks. Using soil microcosms prepared with a non-irradiated/irradiated (1:19) soil mix, thus imitating colonization of a resource rich substrate, three soil successional conditions were tested: no perturbation, eukaryote-inhibition, and pesticide-amendment. The non-perturbed soil microcosms (i.e. controls) received only sterile water; the eukaryote-inhibition condition was amended with cycloheximide (CHX), a compound that strongly inhibits eukaryote development, by protein synthesis inhibition (Kota et al., 1999; Manzano et al., 2007; Holmes et al., 2014); and the pesticide condition was amended with the chloroaromatic pollutant herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), whose toxic effects are well-described (Kraiser et al., 2013). The choice of the compounds used (CHX and 2,4-D) obeys to the characteristics of both compounds. CHX can kill or inhibit a significant fraction of soil eukaryotes, diminishing the activity of bacterial predators and affecting the interaction between fungi and bacteria. However, the most remarkable use of CHX is as an antiprotozoal chemical. CHX has been used as a eukaryotic growth inhibitor both for bacterial isolation (Capozzi et al., 2012) and to test ecological hypotheses in bacterial predation experiments (Kota et al., 1999; Holmes et al., 2014). The pesticide 2,4-D, which has been used for decades for weed control, was chosen as an example of anthropic perturbation. This decision is supported by a wealth of studies available on the effects of 2,4-D on soil and particularly on microbial communities (Pérez-Pantoja et al., 2003; Vroumsia et al., 2005; Manzano et al., 2007; Gazitúa et al., 2010). Together, these treatments represent examples of disturbances produced by pesticides or by inhibition of eukaryotes.

Using varied statistical and computational data analysis strategies, we aimed to test the hypothesis that soil successional conditions determine both the trajectories that follow early compositional changes and the architecture of bacterial community networks, adding to our understanding of the ecological rules that dictate community stability.

2. Materials and methods

2.1. Soil samples and microcosm design

Soil microcosms were prepared with a grassland soil from San Borja Park (SB) (33° 26′ S, 70° 39′ W) or with an agricultural soil from Calera de Tango (CT) (33° 36′ S; 70° 45′ W), both located near Santiago, Chile. Soil samples were taken to the laboratory, sieved through a 2 mm mesh and dried at 40 °C for 48 h. The homogenized material was subjected to physical and chemical characterization (Supplementary material, Table S1), using standard procedures (Sparks et al., 1996). One kilogram of each soil sample was irradiated with a 75-kGy dose of gamma radiation (a ^{60}Co source). Gamma irradiation was selected because it produces almost sterile soil (McNamara et al., 2003). The 75-kGy total dosages were applied as 25-kGy partial doses in three consecutive days. To check sterility, three non-amended soil microcosms (see below) containing only irradiated soil were incubated during the corresponding experimental period and metagenomic DNA was then extracted and analyzed. For microcosm experiments, irradiated soil was homogenized and mixed in a ratio of 19:1 with the respective CT or SB native soil. Microcosms were prepared in 50 ml glass beakers containing 10 g of soil mixture, covered with polyethylene foil, and incubated at 22 ± 2 °C. Three different treatments were tested: controls corresponding to the irradiated/non-irradiated soil mixture (19:1) plus 500 µl sterile water (water holding capacity 25%), as used in the next two treatments; eukaryotic-inhibition treatments amended with CHX to give a final concentration of 1 mg g^{-1} of soil; and pesticide treatments amended with the pesticide 2,4-D (400 mg kg^{-1} of soil).

2.2. Experimental settings

To investigate the patterns and trajectories that bacterial communities follow during different successional stages, two experimental setups were designed using different sampling schemes.

2.2.1. Comparing successional dynamics in two soils

To compare the response dynamics of two different soil bacterial communities, two sets of ten microcosms each were set up under the control conditions described above with CT or SB soils. Five sampling times were established (0, 12, 24, 48 and 72 h of incubation). In each sampling time, two microcosms of each set were sacrificed and 1-g homogenized soil samples from each microcosm were frozen immediately in liquid nitrogen and stored at −20 °C for no more than ten days before DNA extraction.

2.2.2. Comparing the effect of perturbations in very early successional dynamics

Using CT soil, control, eukaryotic-inhibition and pesticide-amended

conditions were tested in three sets of 24 soil microcosms each. Twelve sampling times were established (after 0.5, 1, 2, 4, 6, 10, 16, 24, 36, 48, 72 and 300 h). In each sampling time, two microcosms of each set were sacrificed and samples were removed, frozen, and stored as indicated above.

2.3. DNA extraction and T-RFLP analysis

The FastDNA spin kit for soil (MP Biomedicals, Santa Ana, California, USA) was used to obtain metagenomic DNA from 1 g soil sample following the manufacturer's instructions. Cellular lysis was performed using the Lysing Matrix E (a mixture of ceramic and silica beads), the lysis buffer MT and the FastPrep®-24 instrument (MP Biomedicals, Santa Ana, California, USA), at level 6 during 40 s. This protocol yielded DNA concentrations ranging from 0.02 to 0.9 μg μl^{-1} in a final volume of 50 μl, as quantified by spectrophotometry (Infinite® 200 PRO NanoQuant, Tecan Group Ltd., Männedorf, Switzerland). Metagenomic DNA was used as a template for PCR with primer pairs 8F (5′ AGA GTT TGA TCC TGG CTC AG 3′) (Lane, 1991), labeled with the fluorochrome 6-FAM at the 5′ end, and 1392R (5′ACG GGC GGT GTG TAC 3′) (Lane et al., 1985). Each PCR amplification contained 5 μl of 10× PCR buffer (200 mM Tris-HCl, pH 8, 500 mM KCl), 3 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM dNTP, 0.2 mg ml^{-1} bovine seroalbumin, 10–50 ng of soil DNA, and 1 U of Taq polymerase, in a total reaction volume of 50 μl. PCR reaction conditions were as follows: 94 °C for 5 min; 30 cycles at 94 °C for 45 s, 56 °C for 45 s, and 72 °C for 2 min; and a final extension at 72 °C for 7 min. PCR products were digested with 20 U *Msp*I or *Hae*III restriction enzymes in appropriate buffers for 3 h at 37 °C in a final volume of 20 ul. Each PCR product was digested separately with each enzyme to assess T-RFLP profiles consistency. As most profiles indicated the same trends in terms of the trajectories that follow compositional changes (Supplementary material, Fig. S1,), only the *Msp*I profiles are reported. After desalting, the DNA fragments were separated and detected through capillary electrophoresis (Macrogen, Korea) and analyzed using Peak Scanner software (v1.0, AB Applied Biosystems, USA). The fragment sizes were estimated using the internal standard LIZ 1200 as reference.

2.4. T-RFLP data processing and statistical analysis

Raw data were terminal restriction fragments (T-RFs) sizes, measured in base pairs, and individual peak areas measured in fluorescence units. Only T-RFs from 50 to 500 bp were included in the analysis. T-RFs representing less than 0.5% of the total area were not considered, and the data were standardized by calculating the area of each peak as a percentage of the total area (Morán et al., 2008). Relative abundances of bacterial phylotypes were determined from relative intensities of T-RF signals. The composition of each community was described in terms of operational taxonomic units (OTUs) represented by the T-RFs signals (Schütte et al., 2008). Beta diversity was primarily analyzed using Bray–Curtis distance matrices based on the square root-transformed abundance of each OTU. Non-metric multidimensional scaling (NMDS) analyses were used to group data according to their similarity (Clarke, 1993), and two-way crossed analysis of similarity (ANOSIM) was used to examine the statistical significance of grouping according to treatment factor (Clarke, 1993). The PERMANOVA test (Anderson, 2001) was employed to determine statistical differences between treatments and trough the time. Finally, the method proposed by Baselga (2010), which consists of partitioning the additive components of beta diversity, was used to analyze the contribution of "nestedness" and "turnover" processes during bacterial succession.

2.5. Beta diversity partition analysis

Beta diversity is a powerful method for studying changes in patterns of diversity at different spatial-temporal scales (Mousing et al., 2016; Florencio et al., 2016). As this report investigates changes in community composition in relation to temporal gradients under three distinct conditions, a similarity-driven approach was selected (Vellend, 2001; Baselga, 2010). To study the contribution of nestedness and turnover processes to the changes in beta diversity, pairwise differences in community composition were calculated between time lapses. This method facilitated the understanding of temporal patterns in the underlying community composition regardless of inter-temporal fluctuations in bacterial successions and revealed differences between the three tested conditions.

Beta diversity partition analysis was performed in R (R Core Team, 2014), using the package "*Betapart*" (Baselga et al., 2013), which uses presence/absence data as input. Using the additive partitioning framework proposed by Baselga (2010) it is possible to decompose the total pair-wise dissimilarity of Sørensen (β_{sor}) into the turnover (β_{sim}) component and the nestedness (β_{sne}) component. The pair-wise dissimilarity of Simpson (β_{sim}) describes the differences due to turnover (McKnight et al., 2007) while βsne describes the differences due to nestedness. It is calculated as the difference between β_{sor} and β_{sim}. Then, β_{sne} represents the increasing dissimilarity between nested assemblages due to the increasing differences in species richness (Baselga, 2010). To evaluate if turnover or nestedness is the predominant process in bacterial community dynamics (Baselga et al., 2013), NMDS graphics was used. Dissimilarity matrices from binarized (presence/absence) T-RFLP data were calculated using the *betpart* package (Baselga et al., 2013). A dissimilarity matrix of total beta diversity (β_{sor}) and each beta diversity component (turnover and nestedness) from each condition were visualized. To improve the data interpretation, arrows showing the temporal trajectories were added to NMDS graphics.

2.6. Soil bacterial community networks inference and analysis

Standardized T-RFLP data were used to compare the effect of the pesticide amendment and eukaryotic inhibition through network construction. Using the mean of duplicate T-RFLP profiles to generate a data set that contained the average relative abundance of each OTU over time, the generalized LV equations (see below) were utilized as a dynamic model of a microbial community. The computed values of LV interaction coefficients (β_{ij}) were calculated in R (R Core Team, 2014) using the packages "doBy" and "prob". Based on T-RFLP relative abundance data, the parameters α_i and β_{ij} of an N-species LV model for each treatment were calculated. The dynamic of the OTU i (i = 1,…,n) was as follows: $\frac{dX_i}{dt} = X_i\left(\alpha_i + \sum_{j\neq i} X_j \cdot \beta_{ij}\right)$, where X_i represented the relative abundance of the OTU i, α_i represented the intrinsic growth rate of the OTU i, and β_{ij} corresponded to the influence of the average variation rate of the OTU i on the relative abundance of OTU j. This influence was positive or negative according to the sign of β_{ij} (Wangersky, 1978; Faust and Raes, 2012). Within this simplified system, the interaction coefficient β_{ij} was considered to be significant when the value of $P(\beta_{ij}) \neq 0$ was > 90%, with a 0.75 threshold value (Mounier et al., 2008). The output of this method was a square adjacency matrix (or interaction matrix) (Deng et al., 2012), which contained the value of interaction (β_{ij}) between every interacting OTU. In order to simplify the analysis, the value of β_{ij} was discretized, adopting values of −1, 0 or 1. The output of the inference networks corresponded to meta-community adjacency matrices (meta-community networks), representing all the possible interactions that occurred in a particular condition over time. To analyze the topological changes in bacterial community networks over time, sub-networks inferences were made from the meta-community network. Nodes and edges were subtracted from the network according to whether the respective interacting OTU was present or absent in the corresponding sample. Then, to detect conserved and persistent networks, meta-community networks were analyzed using a ranking of presence/absence of each OTU in time

at three persistence levels: 100, 91.7 and 86.3%, corresponding to OTUs that appeared in all 12, 11 and 10 samples, respectively. Only the connected components (i.e. more than two edges) of those sub-networks were considered.

Network analysis was performed entirely in Cytoscape (Assenov et al., 2008). First, to analyze the differences in the type of ecological interactions that occurred under different experimental conditions, the number of positive and negative edges between each condition was compared. Second, to analyze the differences in the topological changes of the sub-networks between the different conditions, a set of measures (network size, average clustering coefficient, average number of neighbors, and average path length) were calculated using network analysis tools (Creamer et al., 2016). Third, to identify the keystone OTUs of the inferred meta-community and sub-networks, the local measure of centrality, or "betweenness" (Estrada, 2007; González et al., 2010) was used. Briefly, betweenness calculates the degree of importance of each OTU i within the network by estimating how the network would look without the presence of a keystone (Berry and Widder, 2014; Agler et al., 2016; van der Heijden and Hartmann, 2016). Then, the size of each node in each network was scaled using this measure to improve the interpretative value of the networks visualization, such that the size of the node corresponded to the ranking of keystones (Creamer et al., 2016).

Statistics for the size of networks, average number of neighbors, average clustering coefficient, and average shortest path length were extracted from Cytoscape and plotted using the "gplots" (Warnes et al., 2009) and "plotrix" (Lemon, 2006) packages in R. Indices were calculated from average data of individual measurements on each node (OTU). Therefore, the number of measurements (except for Netwrok size index) equaled the number of OTUs comprising each network. In terms of bacterial community assembly, nodes represent OTUs, edges represent the interaction between OTUs, and neighbors are OTUs connected by an edge. As such, variations in network parameters represent changes in the richness of interacting OTUs (size); changes in the average connectivity of a node in the network (average number of neighbors); and changes in the average of the clustering coefficients for all nodes in the network (average clustering coefficient). These parameters have been used to describe hierarchical properties of networks through the extent of module structure in a network (Deng et al., 2012). In turn, the changes in the average shortest path represent the changes in the average expected distance between two connected nodes, which is biologically interpreted as the overall ability of the OTUs to influence their reciprocal activity or abundance for individual nodes (Scardoni and Laudanna, 2012). This parameter has also been interpreted as the speed of the network's response to perturbations (Zhou et al., 2010), where low values represent high speed. To test significant differences between treatments in average number of neighbors, clustering coefficient, and average shortest path parameters, a two-way ANOVA was used. Data corresponding to the index of "average shortest path length" did not comply with the homoscedasticity postulation and were square-root transformed for statistical analysis. A post-hoc Tukey test was also performed to test statistical significance between treatment pairs.

3. Results

3.1. Comparing early successional dynamics in two soils

To study early successional dynamics of bacterial communities, the 16S rRNA gene T-RFLP profiles from agricultural (CT) and grassland (SB) soil microcosms were compared. NMDS analysis of these T-RFLP profiles showed a clear grouping of samples according to soil type for all sampling times analyzed (Fig. 1). ANOSIM comparisons between soil type groups indicated that the observed groupings were statistically significant with a global R-value of 1 ($p = 0.001$). The R-value of 1 indicated that the observed differences in taxonomic composition were high, which is expected given the different soil origins (grassland versus agriculture) and their different physical and chemical characteristics (Supplementary material, Table S1). Despite the differences in the bacterial community structures of these soils, it appeared that the trajectories in both soils were similar (Fig. 1), supported by an R-value of 0.767 ($p = 0.001$) for the factor "time". Between 0 and 24 h, bacterial communities experienced structural changes diverging from the native, non-incubated soil (Fig. 1), but quickly returned to structures similar to the starting point. The differences in the trajectories between both soils primarily reflected the greater data dispersion exhibited by CT soil bacterial communities and the greater effect of dilution/wetting on the native community in CT soil compared with SB soil (native compared to 0 h time samples, Fig. 1).

3.2. Comparing the effect of a biotic and an abiotic perturbation at very early succession times in agricultural soil microcosms

NMDS analyses of the T-RFLP profiles showed that agricultural (CT) soil bacterial community structures changed immediately upon inoculation (0.5–2 h) with respect to the native bacterial community and that both amendments have low effects comparing with the wetting/dilution effect produced in control conditions (Fig. 2). Middle time period (10–36 h) T-RFLP profiles were more dissimilar between replicates but those from longer incubations (48–300 h) tended to cluster together and to be more similar to the initial community. Some outliers, i.e. T-RFLP profiles that have high dissimilarity with those of similar or the same incubation times, were observed but mainly at very early succession stages (Fig. 2). For example, control condition bacterial communities exhibited similarities between replicates of 11.2%, 55.3% and 63.5% at 1, 10, and 36 h, respectively; bacterial communities from the eukaryotic-inhibition treatment at 4 h showed 43.7% similarity between replicates; and bacterial communities from the pesticide-amended treatment showed 44.9% and 42.5% of similarity between replicates at 0.5 and 4 h, respectively. These outliers might be considered as alternative states of otherwise convergent trajectories. ANOSIM comparisons between treatments indicated that the observed grouping was statistically supported with a global R-value of 0.565 ($p = 0.001$) and pairwise R-values of 0.479 ($p = 0.001$) for the control/eukaryotic-inhibition pair, 0.625 ($p = 0.001$) for the control/pesticide-amended pair, and 0.688 ($p = 0.001$) for the eukaryotic-inhibition/pesticide-amended pair. To test for differences between treatments and time, a nested PERMANOVA test was performed and indicated that treatment accounted for a 15% of the variance in community structures (R2 = 0.158; $p = 0.001$). Time was the major factor explaining the differences found, accounting for more than a 40% of the variance (R2 = 0.407; $p = 0.001$), while the interaction of treatment-time was also statistically significant and accounted for almost a 21.5% of the variance (R2 = 0.215; $p = 0.001$) (Supplementary material, Table S2).

To compare the relative influence of turnover and nestedness during bacterial succession under the three experimental conditions, a beta-diversity partition analysis was performed. Pairwise analysis of total beta diversity (β_{sor}), turnover (β_{sim}), and nestedness (β_{sne}) components of species dissimilarity was visualized by NMDS (Fig. 3). β_{sor} and β_{sim} showed a similar distribution of samples, with a grouping pattern that resembled that obtained by multivariate analyses based on Bray-Curtis dissimilarity (Figs. 2 and 3). In both analyses, trajectories showed clear phases in community composition; with early (10–24 h) and late stages (48–300 h) differing, and very early stage (0–10 h) tending to be more similar with late stages. β_{sne} dissimilarity showed no clear grouping pattern associated with time (Fig. 3), which implies that nestedness has low influences in the changes observed during early succession of bacterial communities in soil microcosms.

3.3. Soil microcosm bacterial community network analyses

To test the effect of perturbations on the architecture of bacterial community networks, an inference network of OTU interactions was

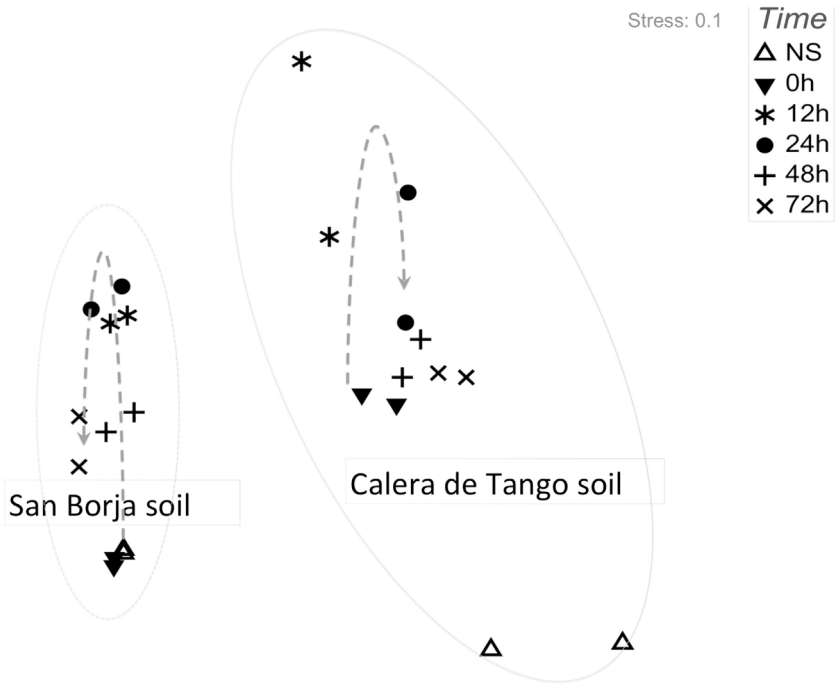

Fig. 1. Non-metric multidimensional scaling (NMDS) analysis of *Msp*I-terminal restriction fragment length polymorphism (T-RFLP) profiles for the 16S rRNA bacterial gene from Calera de Tango (CT) and San Borja (SB) soil microcosm metagenomic DNA, showing the trajectories and grouping of bacterial community structures during early successional stages (0–72 h). (△): native, non-incubated soil (NS); (▼):0 h; (✱) 12 h; (●) 24 h; (+) 48 h; and (×) 72 h. Each symbol corresponds to a single T-RFLP profile. Stress values for NMDS analysis are indicated at the upper right corner. Bray-Curtis dissimilarity matrices were calculated over square root transformed data.

constructed and interpreted. In general, control bacterial community networks demonstrated more negative than positive interactions, with a negative to positive interaction ratio of 3.3 (Supplementary material, Fig. S2, panel A). Eukaryotic inhibition strongly changed this ratio to 13.8, increasing the number of negative interactions (Supplementary material, Fig. S3, panel A). In contrast, pesticide application increased positive interactions, resulting in a ratio of 0.32 (Supplementary material, Fig. S4, panel A). These results suggest that antagonist interactions increase when eukaryotes are inhibited while cooperative interactions increase in the presence of a toxic pesticide.

Network analysis results also suggested that bacterial community assemblies reflected distinct patterns according to the perturbation (Fig. 4). Results from a two-way ANOVA (Supplementary material, Table 2) showed significant differences in the "average shortest path length" index ($F_{11,964} = 2.138$, $p = 0.015$) by time, while the response variables "average clustering coefficient" and "average neighborhood connectivity" showed significant interactions between treatment and time ($F_{22,964} = 3.754$, $p < 0.001$; $F_{22,964} = 69.644$, $p < 0.001$ for average clustering and average neighborhood connectivity, respectively). A post-hoc Tukey test showed significant differences between control/eukaryotic-inhibition and control/pesticide-amended treatments for the "neighborhood connectivity" index (see Supplementary material, Table S3.D). Together, these results indicated that control network dynamic differed to perturbed successions networks in connectivity, but not for both response speed and complexity.

Patterns observed for changes in network sizes (Fig. 4A) were quite similar to those for OTU richness (data not show), which is consistent with the fact that reconstruction of the sub networks was performed with presence/absence data. The oscillation of network index values observed under control conditions suggested that the networks of bacterial communities rapidly and frequently changed their complexity and thus their architecture. Moreover, both connectivity (average number of neighbors) and response speeds (average path length) followed almost the same trend observed with network size changes (Fig. 4B and D).

A large starting network size was observed for the pesticide-amended condition, with a rapid reduction until 24 h, followed by a sustained increase in size up to 300 h of incubation (Fig. 4A). When eukaryotes were inhibited, bacterial communities exhibited a similar behavior. Furthermore, changes in clustering parameters (Fig. 4C) indicated that community networks were highly dispersed (i.e. low levels of clustering) up to 48 h of incubation regardless of the starting conditions. While the average clustering coefficient values oscillated for control and pesticide-amended treatments, the eukaryotic inhibition maintained consistently minimal values for the first 72 h of incubation. Between 72 and 300 h, both control and eukaryotic-inhibition conditions exhibited a sharp increase the clustering coefficient (Fig. 4C), which correlated with the formation of an interconnected hub in the sub-networks from the 300 h sampling time (Supplementary material, Figs. S2 and S3, panels C). In contrast, amendment with 2,4-D produced a low level of clustering after 16 h of incubation with an increase after 32 h, a peak at 72 h, and a subsequent decrease (Fig. 4C) coinciding with the appearance and disappearance of peripheral networks (Supplementary material, Fig. S4, panels B and C). Such network disconnections affected the calculation of this coefficient.

3.4. Keystones and core networks

The relationship between structural changes in bacterial community networks and highly abundant, persistent OTUs was also addressed. The betweenness centrality of a node in a community network can indicate the extent to which a given OTU is structurally capable of holding together populations, i.e. two bacterial species could be interconnected through a keystone OTU (high betweenness centrality). Thus, a higher keystone ranking value corresponds to a higher relevance of the OTU in organizing community interactions. Although the richness of keystones was variable over time and among treatments (Supplementary material, Figs. S2–S4, panels B), some OTUs persisted with high values of centrality in almost all networks, concomitant with the keystones present in core networks that represent node groups that were persistent in the community (Fig. 5). OTUs were selected based on their observation in 100% of the instances (12 of 12 instances, extracted from the averages of the twelve times sampled) for each treatment, 91.7% of the instances (11 of 12), and 86.3% of the instances (10 of 12). Results indicate that several OTUs acted as network hubs, independent of the incubation conditions (Fig. 5). OTUs labeled with the numbers 88, 140 and 493 (corresponding to their fragment length sizes) occurred in 100% of samples (Fig. 5) and exhibited medium to high

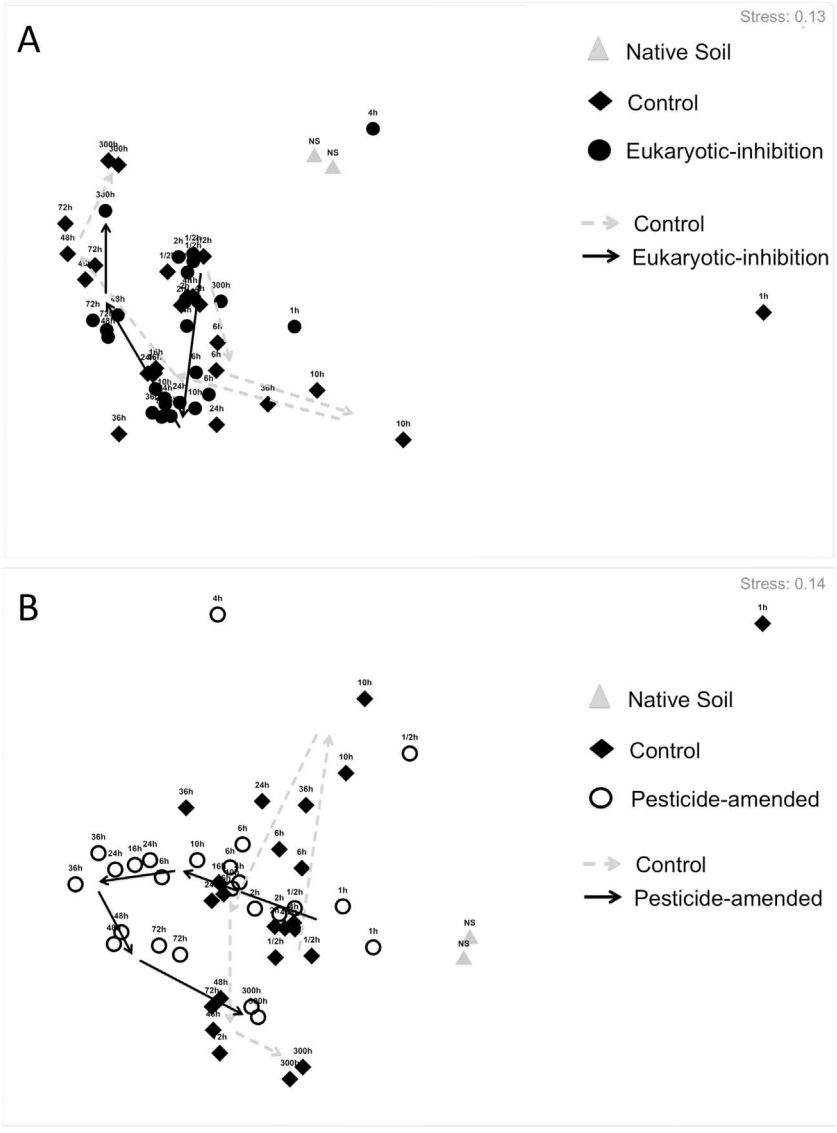

Fig. 2. Non-metric multidimensional scaling (NMDS) analysis of *Msp*I-terminal restriction fragment length polymorphisms (T-RFLP) profiles for the 16S rRNA bacterial gene, from Calera de Tango soil microcosm metagenomic DNA, showing the trajectories and grouping of bacterial communities during successional stages (0.5–300 h). (▲): native, non-incubated soil (◆): control condition, (●): eukaryotic inhibition condition, (○): pesticide amended condition. Each symbol corresponds to a single T-RFLP profile. Stress values for each NMDS analyses are indicated in the upper right corner. Bray-Curtis dissimilarity matrices were calculated over square root transformed data. R values for perturbed-control incubations were: A. control and eukaryotic inhibition, 0.479 (p = 0.003); B. control and pesticide-amended, 0.625 (p = 0.001). Note that the set of control T-RFLP profiles is the same in A and B.

betweenness centrality levels in both the meta-community networks and the sub-networks (Supplementary material, Figs. S2–S4). While these three OTUs had maximum persistence in bacterial communities all treatments, interactions among them were heavily dependent on the incubation condition. In control conditions, OTUs 88 and 493 influenced OTU 140 negatively and positively, respectively (Fig. 5A). When eukaryotes are inhibited, OTUs 88 and 493 negatively influenced each other, and OTU 140 benefited from OTU 88 (Fig. 5D). In the pesticide-amended condition, OTU 88 had a positive influence on both OTUs 140 and 493 (Fig. 5G). As expected, after decreasing the percentage of persistent OTUs, core networks increased in size (compare Fig. 5A with B and C; D with E and F, and G with H and I). In any case, it can be noted that most OTUs belonging to the conserved networks were redundant among treatments, indicating that these OTUs might be important in explaining the convergence levels detected through NMDS and multivariate analyses (Fig. 2).

Temporal changes in the average relative abundance of keystones between treatments were examined (Supplementary material, Fig. S5). Despite their generally fast and prolific propagation, OTUs 88 and 152 demonstrated consecutive peaks in abundance at very early (1 h) and early (between 16 and 36 h) stages, respectively, which may indicate that early bacterial succession of high abundance OTUs, follows turnover patterns similar to those reported for long term succession (van Breugel et al., 2007). The low abundance OTUs also exhibited a similar behavior (Supplementary material, Fig. 5S, panel B).

4. Discussion

Bacterial community structures in soil microcosms exposed to different conditions were compared through the culture independent molecular technique T-RFLP (Hartmann and Widmer, 2008; Schütte et al., 2008) and with a combination of multivariate and network based analyses. The results reported here indicate that soil bacterial communities exposed to a resource-rich substrate, as a result of extensive irradiation (i.e. almost sterile conditions) increased organic matter availability, and altered conductivity (Bank et al., 2008; Schaller et al., 2011; Francioli et al., 2016), undergo rapid changes in their structures at early stages. In addition, tend to recover the initial structure after longer incubations (Fig. 1), and quickly converge to similar structures between treatments (Fig. 2), despite differences in compositional trajectories according to experimental perturbation type.

To evaluate the effect of perturbations at early successional stages, two conditions were tested: eukaryotic inhibition and pesticide amendment. Eukaryotic inhibition was attained with the chemical CHX, which strongly decreases the activity of soil microbial eukaryotes (Ekelund and Rønn, 1994; Song et al., 2015) thus successfully inhibiting

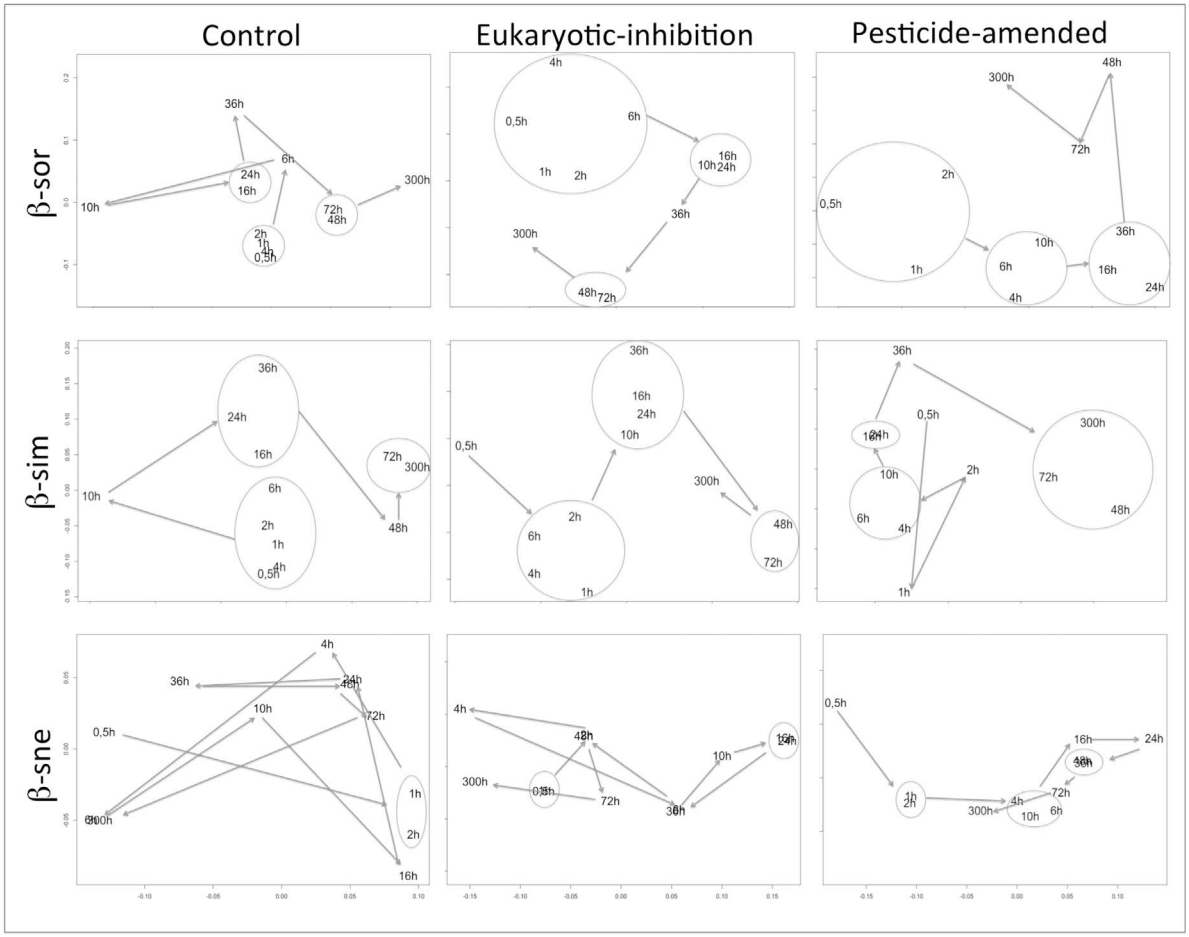

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination of the beta diversity partition proposed by Baselga et al., 2013, for soil microbial communities. All sampling times from control, eukaryotic-inhibition, and pesticide-amended treatments are shown in NMDS. Total beta diversity (β_{sor}) at top panel, turnover (β_{sim}) at medium panel and nestedness (β_{sne}) components at the bottom panel.

bacterivore protozoa in soil (Kota et al., 1999; Manzano et al., 2007; Badawi et al., 2012). However, it is quite possible that part of the restoration process of these bacterial communities is due to a gradual recovery in biomass and activity of microbial eukaryotes, as CHX is subjected to degradation during the incubation period (Badalucco et al., 1994) and CHX does not completely inhibit the entire soil eukaryotic microbiota (Badawi et al., 2012). Moreover, treatment of soil with CHX alters the abundance of fungi in soil and consequently affects the bacterial community, so it cannot be ruled out that the patterns of change observed under the influence of CHX are determined by the lack of interactions between both fungi and bacterial predatory protozoa. Also, the increase in negative interactions between OTUs under CHX treatment could be explained both by a decrease in mutualistic interactions between bacteria and fungi and by the increase in competition in the absence of bacterial predators.

The second condition tested was pesticide amendment. 2,4-D is a soil pesticide that has been used for decades in the field as an herbicide (Ahrens, 1994), and its effects on soil are well described (Fulthorpe et al., 1996; Kraiser et al., 2013). First, 2,4-D is toxic not only for plants, but also for bacteria because several of its biodegradation intermediates (2,4-dichlorophenol and 3,5-dichlorocatechol) strongly inhibit metabolism in bacteria (Schweigert et al., 2001; Pérez-Pantoja et al., 2003; Ledger et al., 2006). Secondly, 2,4-D may also serve as a carbon source for several bacterial and fungal taxa (Fulthorpe et al., 1996; Vroumsia et al., 2005). In contrast with the control and biotic perturbation, 2,4-D treatment elevated positive interactions in the bacterial community. This change may be explained by the "detoxification effect" (Ledger et al., 2006) that 2,4-D degrading bacterial populations exert on the 2,4-D sensitive bacterial groups. Microbial consortia degrade 2,4-D and some of its derivatives, establishing cooperative interactions (Haugland et al., 1990). Cooperative effects would predominate over the antagonist effects established among different 2,4-D-degrading bacterial taxa when this pesticide is used as an energy and carbon source. Although 2,4-D removal in soils is relatively fast (from hours to days, Manzano et al., 2007; Kraiser et al., 2013), and bacterial communities can restore over short time frames, this pesticide and its derivatives have been shown to cause significant changes in soil bacterial communities both at the taxonomic and functional levels (Gazitúa et al., 2010).

Both perturbations produced, at early stages, a more restricted set of community structures, as judged by lower data dispersion and fewer outliers compared to the control condition (Figs. 1–3). However, despite the differences in both the successional conditions and the trajectories that follow the changes in the community composition, community structures converge in later stages (48–300 h). Such a convergence could be related to the persistence of keystone bacterial assemblages, regardless of the successional condition. While the persistence of keystone OTUs is subject to possible variation in the abundance of the taxa that constitute each OTU, it is possible that changes in the relative abundances of OTUs have biological relevance, by failing to ensure that a single taxon is represented in each OTU (Blackwood et al., 2007). It should therefore be understood that a given OTU keystone might correspond to conglomerates of non-phylogenetically related taxonomic groups. To this end, our definition of keystone does not require that a network node (OTU) be comprised of a single taxa, but instead as a "betweenness" OTU in the relation of the rest of the OTUs (Agler et al., 2016). Then, the relationships between the keystones

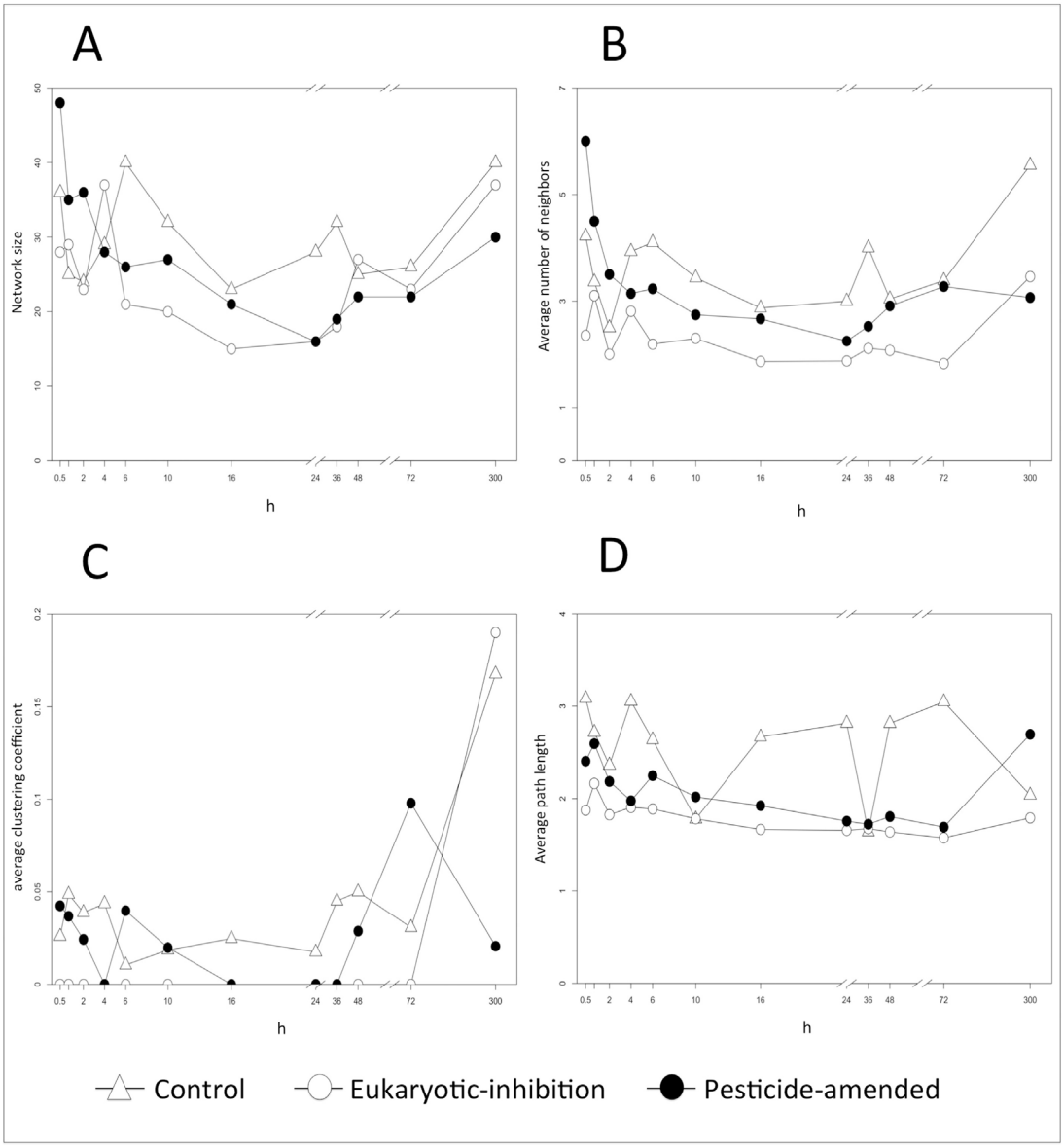

Fig. 4. Line plots showing changes in network parameters for the time series experiment performed with Calera de Tango soil microcosms. A. Network size (number of nodes), B. Average number of neighbors, C. Average clustering coefficient, and D. Average path length. Scale breaks were added at 24 and 72 h.

OTUs depend exclusively on the mathematical relationships between the changes in relative abundances of such OTUs. Using this framework, our results indicate that the presence of pesticides and the absence of eukaryotes modulate the way keystones interact. Therefore, plasticity in keystone interactions could represent a resilient property of bacterial communities, which allows them to respond to disturbances. In this way, interaction plasticity would increase the robustness of community networks (Ramos-Jiliberto et al., 2012), a hypothesis that parallels the topological plasticity found in mutualistic networks in response to external perturbations in macro-organism communities.

Reports suggest that soil bacterial communities are resistant and resilient (Girvan et al., 2005; Shade et al., 2012) when faced with a short-term pulse disturbance such as those described here. Community restoration begins after a decline in community metrics (i.e. diversity metrics) proportional to the perturbation effect (Shade et al., 2012). The resiliency response restores these metrics to pre-disturbance levels, thus recovering the initial community structure. Results reported here show that this process occurs very rapidly (less than 24 h). However, we find that in response to perturbation, bacterial communities feature states of high and low richness (data not show) and present strong variations in the community composition, from which a stochastic dynamic is inferred during the first stages of succession. Therefore, the non-linear dynamics that drive the compositional changes in early bacterial community succession reported here also indicate that stochastic processes are relevant to prime the community for restoration. This early stochasticity could be explained by the high availability of carbon at the beginning of the succession (Francioli et al., 2016). Moreover, similar early stochastic microbial dynamics have been recently proposed in bacterial community succession in pinewood decomposition (Kielak et al., 2016) and grassland microcosms (Hao et al., 2015).

Stochastic variations in species composition during early successional stages could be related to stochastic processes of species turnover (Francioli et al., 2016; Socolar et al., 2016). However, changes in community composition reported here, which are attributable to turnover processes, clearly distinguish three different phases of early bacterial succession based on patterns of species turnover resemblance (see Fig. 3, β_{sim} graphs). Therefore, some non-stochastic processes must be operating during early bacterial succession in order to explain the compositional changes and convergence observed (Fig. 2). A recently

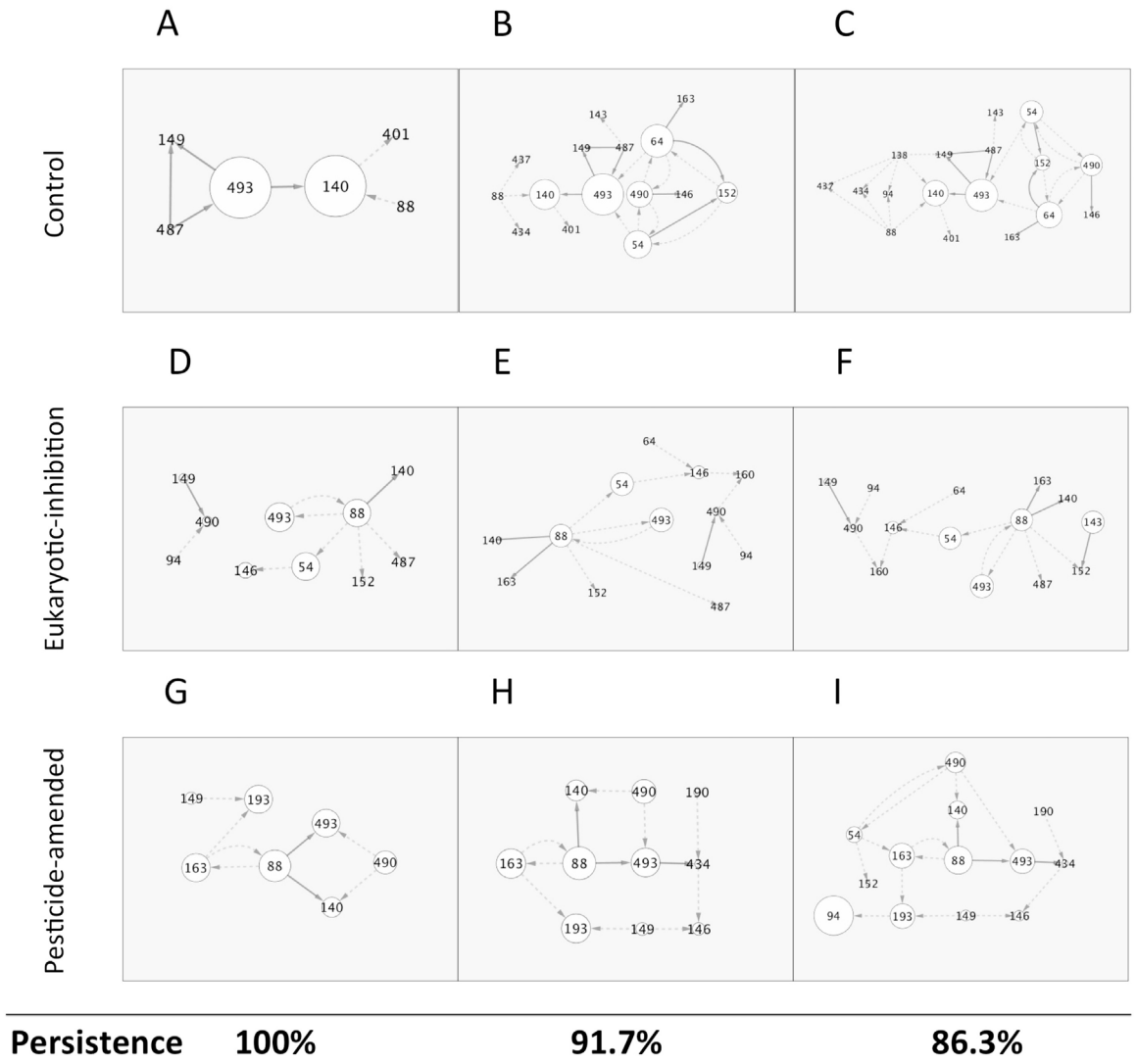

Fig. 5. Network diagrams showing core-networks of interactions based on significant positive LV interaction parameters. The nodes size is relational to the betweenness centrality value obtained from meta-networks (Keystone level). The threshold for positive interaction was > 0.75 (p = 0.01). Only connected components are shown. Black lines indicate positive influence, and dotted grey lines indicate negative influence. Panels present bacterial succession core-networks at three different levels of persistence percentage (100%: A, D and G; 91.7%: B, E and H; 86.3%: C, F and I). Control, eukaryotic-inhibition, and pesticide-amended (as indicated in the legend of Fig. 2) successions are shown at top (A–C), middle (D–F) and bottom G–I), respectively.

published study shows similar results (Francioli et al., 2016). In both this case and our work, variations in species composition were related to both stochastic (turnover) and non-stochastic (niche factors) processes. (Fig. 3). Moreover, the present work shows that perturbations do not affect species turnover processes during succession. Thus, the chemical amendments at the beginning of bacterial succession in soil microcosms affect community composition but not the turnover process that follow bacterial community succession. Since compositional change processes operating on bacterial communities do not depend on the successional condition, interactions between populations represent more valuable indicators of soil health. In this way, interactions can be used to explain how the changes in trajectories that follow disturbed and non-disturbed communities are related to ecological processes and to the resilience and stability of these communities.

In large-ecosystems, persistent low abundance populations are considered keystone species while high abundance population are considered dominant species, but both groups may be ecosystem population drivers (Power et al., 1996). However, in the present study, both low and high abundance OTUs may be keystones or drivers, since they were identified using the betweenness centrality measure, which is useful to determine the importance of each interacting OTU in the bacterial community (Berry and Widler, 2014; Agler et al., 2016). Under this framework, removal of a keystone species, regardless of its relative abundance, results in loss of community network stability. Results present here support the notion that interactions between both high and low abundance populations are important for maintaining ecosystem stability (Berlow 1999; McCann, 2000). Therefore, diversity dynamics, which involve changes in composition and abundance, could also affect the antagonistic and synergistic trophic interactions between different members of the resident community (Thébault and Fontaine, 2010). Recently, it has been demonstrated in root-associated bacterial communities that network variables explain resistance to pathogen invasion and/or plant health by the underlying interaction network architecture (Wei et al., 2015). Taken together, these studies show that network-based models of the dynamics of bacterial communities have consistently better explanatory power compared with classical diversity based models such as alpha diversity indices or Bray-Curtis distances.

In summary, our investigation represents the first to consider sampling in the early hours of bacterial community succession in soil. Our work indicates that the trajectories that follow early changes in bacterial community composition and the architecture of the bacterial community networks are affected by eukaryotic inhibition (i.e. CHX)

and pesticide amendment (i.e. 2,4-D). While these particular disturbances may be representative of those produced by human industrial activities, we acknowledge that additional experimental conditions (including longer exposures and different concentrations) must be tested in order to make generalizations about these compounds. Our data demonstrate that investigations into bacterial succession should consider the high degree of temporal variability in their sampling design and capitalize on the power of studying community networks to better understand the mechanisms by which communities respond to the environmental conditions. For example by increasing the antagonistic interactions between OTUs in the absence of eukaryotes, while cooperative interactions increase in the presence of the pesticide. Finally, studying bacterial community dynamics with a wealth of statistical tools improves the resolution of ecological phenomena that operate during BS, and, in turn, can serve to gauge ecosystem stability.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2017.07.015.

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