

Co-occurrence networks depict common selection patterns, not interactions

Rong Li^{1,2}, Simone Weidner¹, Yannan Ou², Wu Xiong^{1,2}, Alexandre Jousset¹, Qirong Shen² and George A Kowalchuk^{1*}

¹ Ecology and Biodiversity Group, Department of Biology, Institute of Environmental Biology, Utrecht University, 3584 CH Utrecht, Netherlands

² Jiangsu Provincial Key Lab for Solid Organic Waste Utilization, National Engineering Research Center for Organic-based Fertilizers, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, Nanjing Agricultural University, Nanjing 210095, P.R. China

* Corresponding author, E-mail: g.a.kowalchuk@uu.nl

High-throughput interrogation of microbial communities has provided a quantum leap in our ability to characterize the phylogenetic composition of our microbial world. However, as ecologists, we aim to go beyond pure stamp collecting of who is present in a community. We seek to understand the drivers of community dynamics and the interactions that dictate community functioning. A range of tools has been developed to visualize co-occurrence patterns, generated for instance by High-throughput (HTP) tag sequencing of ribosomal RNA gene fragments, as networks of taxa that are positively or negatively correlated in their distributions (Barberán *et al.*, 2012). While such studies often reveal interesting changes in network topology as related to specific environmental gradients or experimental manipulations (Hilton *et al.*, 2021; Li *et al.*, 2021), interpretation of how such networks relate to actual interactions and community drivers has remained problematic and can even be misleading (Cardona *et al.*, 2016). Here, we use a set of manipulated soil-borne microbial communities to demonstrate that extra complex and tightly knit microbial co-occurrence networks can be generated by selection processes that have no link to actual ecological interactions. Thus, while co-occurrence network topology and complexity may indeed have ecological meaning, we purport that they are more related to common outcomes of population selection, as opposed to interactive activities.

As we continue to develop more advanced and thorough means to describe complex microbial communities, we obviously wish to move beyond cataloguing relative population densities toward detecting patterns of ecological significance. To drive such research forward, a number of approaches have been employed to examine evolutionary and ecological signatures within large microbial datasets (Horner-Devine and Bohannan, 2006; Stegen *et al.*, 2012; Dini-Andreote *et al.*, 2015), in order to examine how interspecific interactions, be they positive, negative or neutral, shape microbial community structure and assembly (Faust and Raes, 2012; Zelezniak *et al.*, 2015; Blasche *et al.*, 2021). The use of co-occurrence and correlation networks has increased significantly in recent years, as a means of organizing population distributions across a range of complex microbial communities from oceans to the human gut and for describing not only bacteria, but also fungi, protists and other organisms (Faust and Raes, 2012; Morriën *et al.*, 2017; Xiong *et al.*, 2018; Gao *et al.*, 2022). Indeed, a simple

search of the Web of Science (Fig. 1) demonstrates the widespread and growing use of such approaches.

Many different approaches (correlation networks (CoNet), local similarity analysis (LSA), maximal information coefficients (MIC), random matrix theory (RMT), sparse correlations for compositional data (SparCC), Pearson correlations, Spearman correlations, Bray–Curtis, and so on) and models (copula, null model, ecological, and lotka–volterra) have been explored for constructing co-occurrence networks, and were reported to exhibit different correlation technique usage (Weiss *et al.*, 2016). Resulting changes in network topology are then typically related to specific environmental gradients or experimental manipulations. While most researchers recognize that such networks do not demonstrate actual ecological interactions between species, the very term 'network' implies that such community portrayals yield interactive information (Morales-Castilla *et al.*, 2015; Cazelles *et al.*, 2016; Sander *et al.*, 2017). Real world interactions are far more complex than a mathematical linkage can convey. They involve specific physiologies, higher-order and indirect interactions, changing environmental conditions and spatially structured environments (Alexander *et al.*, 2015; Thakur and Geisen, 2019; Dal *et al.*, 2020; Vos *et al.*, 2013). Network analysis is clearly booming, but what insight does it lend to illuminating microbial interactions or drivers of community structuring?

Positive or negative links within co-occurrence networks have been shown to be poor predictors of actual interactions upon examination of one-to-one effects (Freilich *et al.*, 2018). However, such pairwise interactions should also be viewed within the context of other interactions within the community, as interactions may be indirect and other species in the network may impact one or both of the pairwise players. Species can also coexist and exhibit a correlation in their abundances through population selection by a third species (Holt and Bonsall, 2017) or unreported abiotic factors (Röttgers and Faust, 2018; Lv *et al.*, 2019). It has also been suggested that co-occurrence may be a result of dispersal limitation (Ulrich, 2004), or common selection due to specific environmental factors, without any actual direct or indirect interaction (Peres-Neto *et al.*, 2001; Freilich *et al.*, 2018).

We believe that this latter explanation is in many cases driving the topology of microbial co-occurrence networks. In order to examine this premise, we used a set of engineered soil-borne microbial communities that differed in the degree to

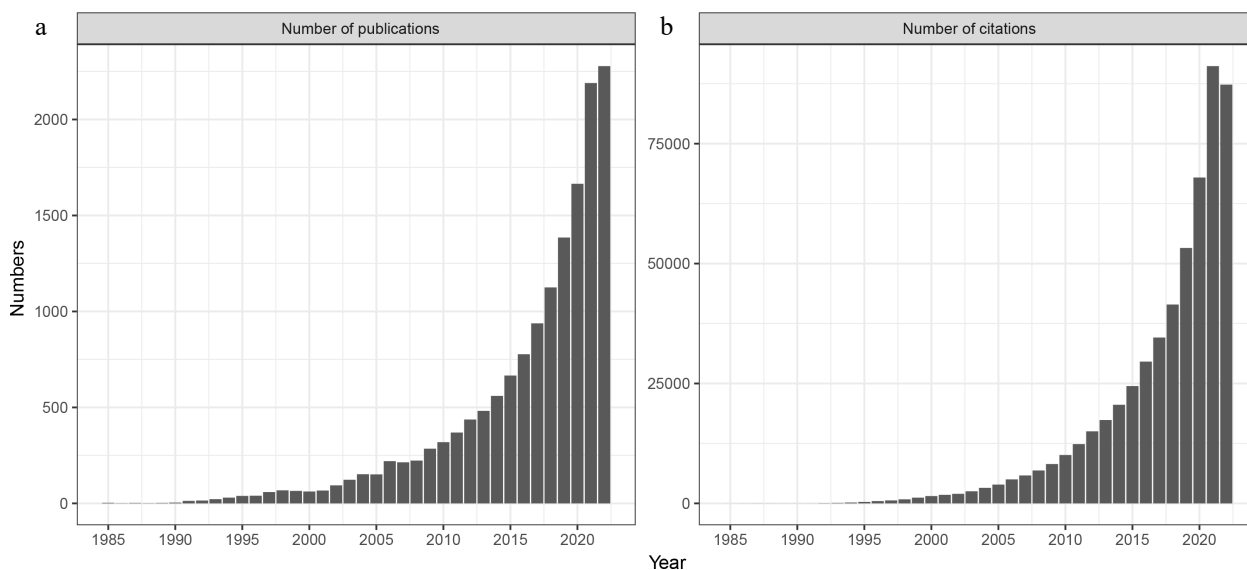


Fig. 1 (a) Number of publications and (b) citations referencing microbial network analysis (Web of Science from 1985 to Dec 20, 2022; search = network and microbiology or microbes or microbiome or microbiota or microflora as two separate subjects).

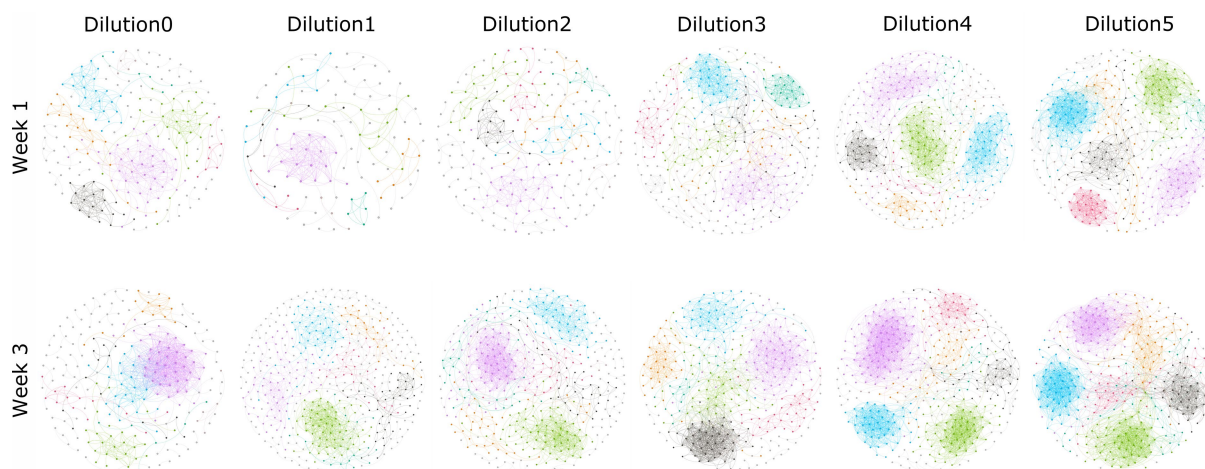


Fig. 2 Co-occurrence networks of rhizosphere bacteria microbiota from samples collected from week 1 and week 3 across the given soil suspension dilution treatments. Connections represent significant ($P < 0.01$) correlation as calculated by the Pearson method. Nodes represent different OTUs. Nodes and edges are colored by modularity class.

which populations were segregated across replicates. We then tracked rhizosphere community assembly and network topology, *via* a range of methods, over time. Briefly, we used a dilution series of a soil suspension re-inoculated back into its sterilized origin soil to create soil communities that had been subjected to different levels of population segregation (Supplemental Fig. S1). With such a dilution-to-extinction experiment setup, all starting communities derived from low dilutions have very similar species pools, as nearly all species remain present in the starting inoculum. On the other hand, replicates at higher dilutions receive more disparate species pools due to the dilution to extinction of different subsets of the initial community. As such, this imposed community 'drift' acts as a strong experimentally imposed segregation at high dilutions, but not at low dilutions. Soils were incubated for 61 weeks and then used to examine microbial community assembly on plant roots *via* 16S rRNA gene tag sequencing by primers 341F (5'-CCTACGGGNBGCASCAG-3') and 806R (5'-

GGACTACNVGGGTWTCTAAT-3') based on the Illumina MiSeq platform and network analyses in a replicated design (Supplemental Fig. S1). There are 12 sequencing samples in each dilution with three replicates per treatment which was derived from one origin soil (in a total of four origin soils) and with 446-814 OTUs in each sample, depending on the dilution and the sampling time, used to compute one network.

Co-occurrence network analyses of rhizosphere communities showed distinct patterns with respect to the dilution of the starting communities, as well as the age of the plant (Fig. 2), with denser and stronger networks with increased dilution and plant age. In addition, the number of links per network also showed increased trends with dilution, independent of the co-occurrence model used (see Supplemental Fig. S2; e.g. CoNet, Pearson or Spearman). The Spearman correlations between node (or link) number with dilutions in each network were calculated, and significant correlations can be observed in some networks (see Supplemental Table S1). In our manipu-

Co-occurrence networks depict common selection patterns

lated communities, we have no reason to assume that communities at higher dilutions are more 'connected' or contain more microbial interactions, which can be supported by the decreased number of OTUs involved in the network (see [Supplemental Fig. S2](#)). Rather, the imposed segregation of populations, which is greater at higher dilutions, resulted in a greater preponderance of positive and negative co-occurrence patterns and an increased number of nodes derived from multiple replicates (see [Supplemental Fig. S3](#)). Similarly, network strength is increased with the continued growth of the plant ([Fig. 2](#)). We believe that this result is due to the increased selective action of the plant, which increases with plant size. Indeed, it has previously been observed that co-occurrence network complexity increases with plant growth stage ([Shi et al., 2016](#); [Ceja-Navarro et al., 2021](#)).

It has been suggested that co-occurrence networks can be misleading if other factors, such as habitat filtering, result in non-random patterns in the abundance of multiple taxa ([Berry and Widder, 2014](#)). In other words, if one seeks to zoom in on one particular parameter driving co-occurrence network structure, it is important to keep all other factors that may influence this structure constant. Here, the numbers of intra- and inter-treatment links both showed increased trends in co-occurrence networks of rhizosphere microbiota with the level of dilution (see [Supplemental Fig. S4](#)). We demonstrate that our imposed segregation of populations, which can be seen as a random selection, resulted in more tightly knit network topologies – a result that most likely has nothing to do with increases in actual interactions. The distribution of microbial species in the environment is clearly not independent, and we suggest that estimations of microbial responses to environmental filtering need to be considered, for instance *via* generalized joint attribute modeling ([Clark et al., 2017](#)). Our example provides an empirical warning regarding the ecological interpretation of co-occurrence networks. We show that co-occurrence network structure and complexity can be principally driven by common patterns of imposed selection, thereby providing a strong cautionary message to the interpretation of functional interactions from such approaches.

ACKNOWLEDGMENTS

This work was supported by the Key project at central government level: The ability establishment of sustainable use for valuable Chinese medicine resources (2060302); Natural Science Foundation of Jiangsu Province, China (BK20200544); the Priority Academic Program Development of the Jiangsu Higher Education Institutions (PAPD), the National Natural Science Foundation of China (42107141), the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (ALW.870.15.050) and the Koninklijke Nederlandse Akademie van Wetenschappen (530-5CDP18).

Conflict of interest

Li R, Shen Q and Kowalchuk GA are the Editorial Board members of *Journal Soil Science and Environment*. They were blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer-review handled independently of these Editorial Board members and their research groups.

Supplementary Information accompanies this paper at (<https://www.maxapress.com/article/doi/10.48130/SSE-2023-0001>)

Dates

Received 31 December 2022; Accepted 8 March 2023;
Published online 28 March 2023

REFERENCES

- Alexander JM, Diez JM, Levine JM. 2015. Novel competitors shape species' responses to climate change. *Nature* 525:515–18
- Barberán A, Bates ST, Casamayor EO, Fierer, N. 2012. Using network analysis to explore co-occurrence patterns in soil microbial communities. *The ISME Journal* 6:343–51
- Berry D, Widder S. 2014. Deciphering microbial interactions and detecting keystone species with co-occurrence networks. *Frontiers in Microbiology* 5:219
- Blasche S, Kim Y, Mars RAT, Machado D, Maansson M, et al. 2021. Metabolic cooperation and spatiotemporal niche partitioning in a kefir microbial community. *Nature Microbiology* 6(2):196–206
- Cardona C, Weisenhorn P, Henry C, Gilbert JA. 2016. Network-based metabolic analysis and microbial community modeling. *Current Opinion in Microbiology* 31:124–31
- Cazelles K, Araújo MB, Mouquet N, Gravel D. 2016. A theory for species co-occurrence in interaction networks. *Theoretical Ecology* 9:39–48
- Ceja-Navarro JA, Wang Y, Ning D, Arellano A, Ramanculova L, et al. 2021. Protist diversity and community complexity in the rhizosphere of switchgrass are dynamic as plants develop. *Microbiome* 9:96
- Clark JS, Nemergut D, Seyednasrollah B, Turner PJ, Zhang S. 2017. Generalized joint attribute modeling for biodiversity analysis: median-zero, multivariate, multifarious data. *Ecological Monographs* 87:34–56
- Dal Co A, van Vliet S, Kiviet DJ, Schlegel S, Ackermann M. 2020. Short-range interactions govern the dynamics and functions of microbial communities. *Nature Ecology & Evolution* 4(3):366–75
- Dini-Andreote F, Stegen JC, van Elsas JD, Salles JF. 2015. Disentangling mechanisms that mediate the balance between stochastic and deterministic processes in microbial succession. *Proceedings of the National Academy of Sciences of the United States of America* 112:E1326–E1332
- Faust K, Raes J. 2012. Microbial interactions: from networks to models. *Nature Reviews Microbiology* 10:538–50
- Freilich MA, Wieters E, Broitman BR, Marquet PA, Navarrete SA. 2018. Species co-occurrence networks: Can they reveal trophic and non-trophic interactions in ecological communities? *Ecology* 99:690–99
- Gao C, Xu L, Montoya L, Madera M, Hollingsworth J, et al. 2022. Co-occurrence networks reveal more complexity than community composition in resistance and resilience of microbial communities. *Nature Communications* 13:3867
- Hilton S, Picot E, Schreiter S, Bass D, Norman K, et al. 2021. Identification of microbial signatures linked to oilseed rape yield decline at the landscape scale. *Microbiome* 9:19
- Holt RD, Bonsall MB. 2017. Apparent competition. *Annual Review of Ecology, Evolution, and Systematics* 48:447–71
- Horner-Devine MC, Bohannon BJM. 2006. Phylogenetic clustering and overdispersion in bacterial communities. *Ecology* 87:S100–S108
- Li B, Roley SS, Duncan DS, Guo J, Quensen JF, et al. 2021. Long-term excess nitrogen fertilizer increases sensitivity of soil microbial community to seasonal change revealed by ecological network and metagenome analyses. *Soil Biology and Biochemistry* 160:108349

- Lv X, Zhao K, Xue R, Liu Y, Xu J, et al. 2019. Strengthening insights in microbial ecological networks from theory to applications. *mSystems* 4:e00124-19
- Morales-Castilla I, Matias MG, Gravel D, Araújo MB. 2015. Inferring biotic interactions from proxies. *Trends in Ecology & Evolution* 30:347–56
- Morriën E, Hannula SE, Snoek LB, Helmsing NR, Zweepers H, et al. 2017. Soil networks become more connected and take up more carbon as nature restoration progresses. *Nature Communications* 8:14349
- Peres-Neto PR, Olden JD, Jackson DA. 2001. Environmentally constrained null models: Site suitability as occupancy criterion. *Oikos* 93:110–20
- Röttgers L, Faust K. 2018. From hairballs to hypotheses—biological insights from microbial networks. *FEMS Microbiology Reviews* 42:761–80
- Sander EL, Wootton JT, Allesina S. 2017. Ecological network inference from long-term presence-absence data. *Scientific Reports* 7:7154
- Shi S, Nuccio EE, Shi ZJ, He Z, Zhou J, et al. 2016. The interconnected rhizosphere: High network complexity dominates rhizosphere assemblages. *Ecology Letters* 19:926–36
- Stegen JC, Lin X, Konopka AE, Fredrickson JK. 2012. Stochastic and deterministic assembly processes in subsurface microbial communities. *The ISME Journal* 6:1653–64
- Thakur M P, Geisen S. 2019. Trophic regulations of the soil microbiome. *Trends in Microbiology* 27(9):771–80
- Ulrich W. 2004. Species co-occurrences and neutral models: reassessing JM Diamond's assembly rules. *Oikos* 107:603–9
- Vos M, Wolf AB, Jennings SJ, Kowalchuk GA. 2013. Micro-scale determinants of bacterial diversity in soil. *FEMS Microbiology Reviews* 37:936–54
- Weiss S, van Treuren W, Lozupone C, Faust K, Friedman J, et al. 2016. Correlation detection strategies in microbial data sets vary widely in sensitivity and precision. *The ISME Journal* 10:1669–81
- Xiong W, Jousset A, Guo S, Karlsson I, Zhao Q, et al. 2018. Soil protist communities form a dynamic hub in the soil microbiome. *The ISME Journal* 12:634–38
- Zelezniak A, Andrejev S, Ponomarova O, Mende DR, Bork P, et al. 2015. Metabolic dependencies drive species co-occurrence in diverse microbial communities. *Proceedings of the National Academy of Sciences of the United States of America* 112:6449–54

Co-occurrence networks depict common selection patterns



Copyright: © 2023 by the author(s). Published by Maximum Academic Press, Fayetteville, GA. This article is an open access article distributed under Creative Commons Attribution License (CC BY 4.0), visit <https://creativecommons.org/licenses/by/4.0/>.