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TECHNICAL COMMENT

Experimental tools for addressing effects of complex substance mixtures in soil

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Pollution is one of the most important factors responsible for reductions in biodiversity and nature's contribution to people (Díaz et al., 2019). Various environmental compartments, including soils, or individual target organism in the soil, are typically influenced not by single substances, but rather by a complex array of a wide range of compounds (Drakvik et al., 2020). These compounds, including an increasing number of manufactured chemicals, byproducts or compounds resulting from transformations in the environment, number in the hundreds of thousands. Addressing the impacts of such mixtures on soils and soil biota in experiments is important, but very challenging, as substances can occur in the environment in a virtually infinite number of different combinations.

A number of methods are available to estimate risk of exposure to substance mixes (Bopp et al., 2019). Such methods include computational work to predict effects from molecular similarities, conceptual tools, such as use of the Adverse Outcome Pathway (AOP) framework, prioritizing certain compounds or mixtures, and others. Nevertheless, experimental tools for addressing this issue are relatively limited, and often resort to the use of factorial experiments to study interactions among selected individual compounds.

A potentially useful approach that can be added to this toolbox is borrowed from a 'randomly sampling from a pool' design in biodiversity-ecosystem function research, a field in ecology that also has to deal with very high levels of diversity, in this case of species (Tilman et al., 1996). Recent experimental work has applied this idea to a set of global change factors (Brennan and Collins, 2015; Rillig et al., 2019), and we propose here that this may be useful for soil ecotoxicological studies interested in exploring the effects of many substances simultaneously.

How does this work? Imagine you have a pool of 100 substances that you wish to consider for their effects on soil biota. Obviously, a fully factorial design dealing with the presence/ absence of these substances fails already at far lower numbers of substances; for example, a fully factorial design including 10 substances (that is, factors) would already have 2^{10} = 1,024 treatment combinations - a prohibitively large number that cannot be realized for logistic and cost reasons. A potential way forward is to ask questions about the number of substances affecting a system, rather than about their identity and about the particular composition of compounds. One could thus assemble a gradient of influence (contamination) representing different numbers of compounds, for example zero (the control), 1, 5, 10, 50, and so on. Each of these levels will consist of replicates, for which compounds are drawn at random from the pool of 100 compounds. For example, replicates for level 2 would then be randomly determined combinations of two compounds, most likely with every replicate having a different combination of chemicals, and so forth (see Fig. 1). Ideally, one would also have the single factors separately replicated to estimate individual effect size. Given that the factor levels have a limited number of replicates (maybe n=10 for each level), this design results in a realistic number of experimental units and thus measurements overall.

Clearly, this design does not answer all questions (Manning, 2019). It cannot inform directly on the role of individual interactions (e.g. on particular 2- or 3-way combinations of certain factors); this is the price to pay for this design. However, combined with the effect size estimates for single factors, null models can be applied to test if effects are additive, or if certain substances have a disproportionately strong influence (the 'sampling effect',

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i.e. the probability of including a strongly acting substance in the random sample simply increases with the number of draws from the pool of substances). These can all be important pieces of information that may lead to follow-up experiments, which address more detailed questions, such as about particular factor interactions. This design would allow to test if there is a consistent pattern with an increasing number of substances, irrespective of what these chemicals

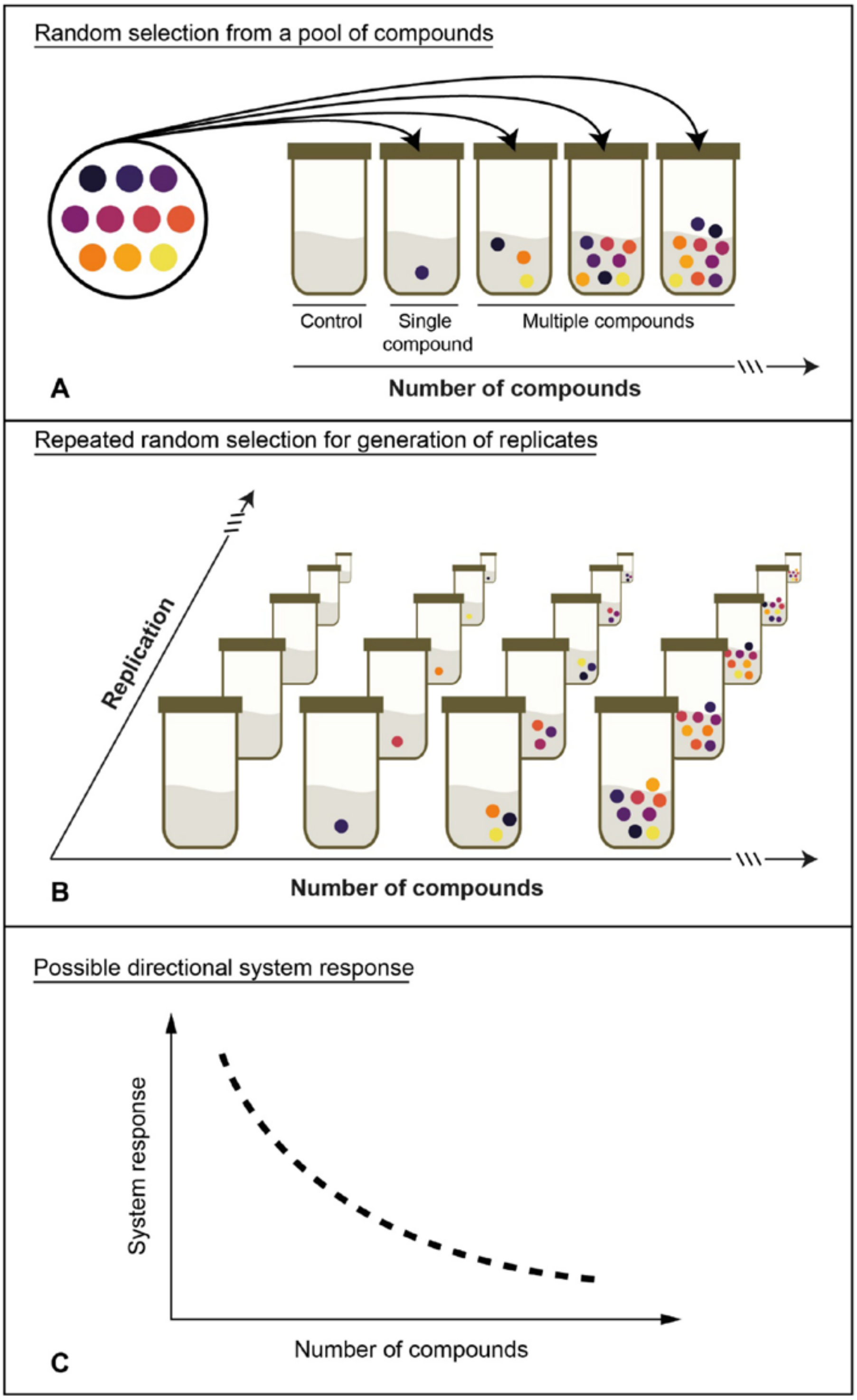

Figure 1. Sketch of an experimental design involving random draws from a given pool of items (in this case chemicals of concern) to create a gradient in the number of chemicals acting on a target system.

are and how they affect the target organism or soil property. Such a clear directional response along the gradient of factor number has in fact been observed for green algae (Brennan and Collins, 2015) and global change factors affecting soil processes and biodiversity (Rillig et al., 2019). It is possible, however, that different responses appear in purely chemical-based experiments, perhaps because of the potential for direct chemical interactions (even though many of the global change factors used in the two studies mentioned are, in fact, chemicals, such as pollutants or nutrients).

Another important consideration in dealing with cocktails of chemicals is the sequential exposure problem (Ashauer et al., 2017; Bopp et al., 2019); that is, not all chemicals may arrive and act at the same time. This is somewhat more difficult to integrate into the proposed experimental design, but could be approached by conducting multiple runs in which the order of substances, not just which ones, is selected at random. This could help estimate the importance of order of substance additions to soil along this gradient of number of substances. This approach is a direct parallel to the study of priority effects in community ecology.

This "random sampling" experimental design can help achieve progress in the study of complex substance mixtures by discovering if knowing the number of substances alone has predictive power. Such a finding could have consequences for regulatory processes and the development of soil protection policies. Clearly, adopting such an approach to complement existing ones would require a shift in the mindset from considering individual substances and particular interactions towards a coarser level of resolution. There is an ever-increasing number of substances and substance classes that may affect soils, including microplastics (Machado et al., 2018), poly- and perfluoroalkyl substances (Nakayama et al., 2019), or lithium (Robinson et al., 2018). Therefore, adopting such an approach may be what is required to get a handle on all the chemical pollutants currently affecting our soils and its biodiversity, and those that will in the future.

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