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Challenging the value of a pseudoreplicated study: A comment to Liu et al. (2024)

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A R T I C L E I N F O

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A B S T R A C T

The paper by Liu et al. (2024) presents measurements of herbivory and six plant traits considered potential antiherbivore defences in seven plant species across two study plots: one mined and one adjacent unmined plot. I argue that this experimental design is inadequate for testing the authors' hypothesis that differences in herbivory and plant traits between these two plots are due to pollution impact, as the study lacks replication of pollution levels. This issue, which could have been easily addressed when designing the study, reflects insufficient attention to experimental design and seriously compromises the validity of the research findings. I briefly reiterate what pseudoreplication is and which fundamental principle of statistical inference it violates.

1. Introduction

Liu et al. (2024) intended to test two hypotheses: (1) plant populations at a mine site experience lower levels of invertebrate herbivory compared to populations farther away from it, and (2) these plant populations are better defended against herbivores than adjacent populations. The introductory part of the cited paper indicates that its authors consider soil contamination as the immediate driver of projected effects on plant quality for herbivorous insects and, consequently, on herbivory levels.

The design of an unintentional observational experiment reported by Liu et al. (2024) defines the study plot as the experimental unit, while individual plants nested within species are evaluation units. Importantly, this design lacks replication regarding the treatment under consideration, involving only one contaminated (mined) and one non-contaminated (adjacent) plot. As a result, the data collected by Liu et al. (2024) do not allow for testing of the authors' proposed hypotheses. Instead, these authors tested hypotheses on the existence of differences between two particular plots, which are of little general interest.

Given that researchers often uncritically adopt the experimental designs of previously published studies, there is a risk that the publication by Liu et al. (2024) could lead to the emergence of other non-replicated studies. To mitigate this risk, I comment on the limitations of the conclusions made by Liu et al. (2024) resulting from non-replicated study design and suggest modifications in study design that could address these issues. I also reiterate what pseudoreplication is and which fundamental principle of statistical inference it violates. Finally, I discuss whether the significance of the data collected by Liu et al. (2024) justifies their publication despite a non-replicated study design. I hope my comments will contribute to the ongoing discussion on replication in field ecology (Filazzola and Cahill, 2021) and ultimately reduce research waste (as defined by Purgar et al., 2022) in ecology and environmental sciences.

2. Limitations of a non-replicated study

Effective experimental design is crucial for robust empirical research in natural sciences. However, many studies fall short by not replicating at the appropriate biological or organizational level, thus weakening the strength of causal inferences more than is commonly acknowledged (Marshall, 2024).

The comparison between one 'mined' and one 'adjacent' site is a classical example of a simple pseudoreplication, as defined by Hurlbert (1984). This design uses variations among individuals within each site to statistically evaluate differences between the sites. If the authors had limited the conclusions of their statistical analysis to the existence of between-site differences, no error would have been committed. However, interpreting these differences as effects of pollution constitutes pseudoreplication, since neither low nor high pollution levels were replicated.

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Pseudoreplication in ecology and environmental studies has been extensively discussed in numerous methodological papers, so a comprehensive presentation of this issue cannot be provided here. Therefore, I will limit my remarks to a few key points that may help readers understand the core of this error and its possible consequences, and I will provide a few additional examples of a pseudoreplicated design. For further details, readers are directed to Hurlbert (1984), Lazic (2010), Colegrave and Ruxton (2018), and other relevant publications.

First, unreplicated treatments are sometimes unavoidable and do not constitute an error in themselves. However, making strong claims of treatment effects based on statistical analyses where variation among multiple samples or evaluation units *within* experimental units is used as a surrogate for variation *among* experimental units is the essence of pseudoreplication (Kozlov and Hurlbert, 2006). Second, pseudoreplication occurs when observations are not statistically independent but are treated as if they are (Lazic, 2010). Third, correct identification of experimental units is crucial to ensure that the assumption of independence is not violated (Colegrave and Ruxton, 2018).

The following study designs have been previously classified as pseudoreplicated: (1) studies of pollutant toxicity involving one treatment and one control aquarium, each containing several fish; (2) studies of temperature effects on plant growth involving one chamber with multiple plants for each temperature level; and (3) studies of grazing effects on the growth of woody plant seedlings involving one fenced (exclosed) plot and one unfenced (control) plot. In all these designs, the experimenter cannot independently change the level of treatment applied to individual organisms within the aquarium, chamber, or exclosure. Consequently, the aquarium, chamber, and exclosure are the experimental units, and they should be replicated for each treatment level to properly test for the treatment effect (Hurlbert, 1984; Kozlov and Hurlbert, 2006).

Pseudoreplication often misleads authors into seeing patterns where none exist (Forstmeier et al., 2017). Consequently, pseudoreplicated studies create multiple, yet insufficiently recognized, problems for research synthesis, especially when researchers conducting this synthesis do not thoroughly examine the methodology of each selected study. First, pseudoreplicated studies often yield larger effect sizes than truly replicated studies (Zvereva and Kozlov, 2010, 2011), likely due to unconscious biases in selecting a single “most representative” experimental unit, e.g. study plot. Second, vote counting may classify a pseudoreplicated study as supporting or not supporting a hypothesis instead of excluding this study as providing inconclusive results. Third, the number of evaluation units (e.g., plant individuals) is usually larger than the number of experimental units (e.g., study plots), giving greater weights to effect sizes from pseudoreplicated studies in meta-analyses.

Despite the need for proper replication being recognized and appreciated decades ago, a substantial part of research data (more than half in some disciplines!) still originates from pseudoreplicated studies (Zvereva and Kozlov, 2010; Ramage et al., 2013; Lazic et al., 2020; Hubert et al., 2024). Although some scientists view pseudoreplication as a pseudoissue (Oksanen, 2001; Schank and Koehnle, 2009), a non-replicated study design had never been shown to have advantages over a replicated design, and addressing pseudoreplication has always been challenging (Davies and Gray, 2015). Was pseudoreplication unavoidable in the study by Liu et al. (2024)?

3. The study by Liu et al. (2024) could have been replicated with minimal additional effort

When a researcher records the outcomes of an unintentional pollution treatment that has already occurred, both common sense and the fundamental statistical requirement for independence of observations clearly dictate that the sampling plot should be considered the experimental unit. All objects of interest within a plot (e.g., individual plants) serve as evaluation units and are much more interdependent than experimental units located at different distances or directions from the pollution source (Vorobeichik and Kozlov, 2012).

Statistical analysis comparing two groups of objects evaluates between-group differences relative to within-group variation. Accurately assessing within-group variation is only possible when each group consists of more than one object. Therefore, to explore the impact of environmental disturbances caused by pollution, data must be collected from at least two polluted and two unpolluted plots (Kozlov and Hurlbert, 2006).

It seems feasible that Liu et al. (2024) could have randomly selected at least two spatially distinct study plots from suitable locations within both polluted and unpolluted areas. This would involve (1) defining the spatial boundaries of both areas, (2) selecting at least four plots of a certain size (e.g., 50 × 50 m) within each area, and (3) randomly (in a true statistical sense) selecting two plots within each of the compared areas. Given that Liu et al. (2024) chose their control site 1.1 km from the polluted site, I think that a 100-meter distance between the polluted plots would be sufficient to consider these as true replicates. For the control plots, I would recommend positioning them in opposite directions from the polluted ones.

Implementing a replicated design would not necessarily require more work. The number of conspecific plant individuals sampled from each plot could have been reduced from 10–25 to 5–10 without any loss of statistical power.

4. Is the publication of a pseudoreplicated study justified by the value of its data?

Davies and Gray (2015) proposed that a strict application of the concept of pseudoreplication can limit our ability to extract valuable insights from natural experiments and monitoring data. Are the data reported by Liu et al. (2024) valuable enough to justify publication despite the non-replicated study design?

Liu et al. (2024, p. 165) claimed that their manuscript addresses “the gaps in knowledge on enemy attack and plant defences in metal-polluted sites.” However, I do not agree that this topic is so understudied that it constitutes a research gap. Already by 2009, 17 datasets reported changes in leaf/needle damage by insects along pollution gradients (Zvereva and Kozlov, 2010), although only one of these datasets was collected near a copper-nickel smelter. Kozlov et al. (2009) reported 61 datasets on insect herbivory, each based on 10 study sites representing entire pollution gradients. Among these, 31 datasets were collected from 14 plant species in the impact zones of eight non-ferrous (copper or nickel-copper) smelters located in boreal and temperate forests across the Northern Hemisphere. Significant among-site variation in herbivory was observed in 24 of the 31 datasets; nevertheless, there was no overall effect of trace metal contamination on insect herbivory (Kozlov et al., 2009). Similarly, measurements of insect herbivory in mountain birch (*Betula pubescens* ssp. *pumila*) from 21 sites around the Monchegorsk copper-nickel smelter in northwest Russia from 1999 to 2016 revealed significant among-site variation, which was not associated with the level of pollution (Kozlov et al., 2017). Multiyear data revealed a slight decrease in insect herbivory in aspen (*Populus tremula*) and no pollution effects on herbivory in birch (*B. pubescens*) growing near the Middle Ural copper smelter relative to unpolluted sites (Belskaya and Vorobeichik, 2013, 2015; Belskaya and Zamshina, 2024). Compared to these previous studies, the data published by Liu et al. (2024) provide no conceptually novel insights into plant-herbivore interactions in metal-polluted sites.

I refrain from discussing the importance of the data on putative plant defences, as Liu et al. (2024) did not investigate whether these data (or metal concentrations in plants, which were not measured) explain between-site differences in insect herbivory. Moreover, a current meta-analysis (Zvereva et al., 2024) found no overall correlation between herbivory and either concentrations of plant secondary metabolites or values of physical leaf traits, which are routinely considered antiherbivore defences. This conclusion calls for a re-evaluation of the relationships between insect herbivory and plant functional traits on both ecological and evolutionary timescales.

5. Conclusion

The failure by Liu et al. (2024) to replicate the levels of pollution (and related environmental disturbance) shifted the focus of their study from testing the proposed hypotheses on the impact of pollution and mining activities to merely exploring differences in herbivory and plant defences between two study plots. This issue, which could have been easily addressed during the planning stage, stemmed from insufficient attention of Liu et al. (2024) to study design and significantly undermined the value of their research findings.

Declaration of competing interest

The author has no relevant financial or non-financial interests to disclose.

CRediT authorship contribution statement

Mikhail V. Kozlov: Writing – original draft, Investigation, Conceptualization.

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