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Towards unbiased interpretations of interactive effects in ecotoxicological studies

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ABSTRACT

Ecotoxicological research has increasingly focused on the interactive effects of chemical mixtures on biological models, emphasising additive, synergistic, or antagonistic interactions. However, these combination studies often test chemicals at unique concentrations (e.g. x:y), limiting our understanding of the effects across the full spectrum of possible combinations. Evidence from human toxicology suggests that interactive effects among chemicals can vary significantly with total concentration (e.g. x:y *vs.* 2x:2y), their ratio (e.g. x:2y *vs.* 2x:y), and the magnitude of the tested effect (e.g. LC_{10} *vs.* LC_{50}). Our non-exhaustive review of studies on binary mixtures in bee ecotoxicology reveals that such parameters are frequently neglected. Of the 60 studies we examined, only two utilised multiple total concentrations and ratios, thus exploring a broad range of possible combinations. In contrast, 26 studies tested only a single concentration of each chemical, resulting in incomplete interpretations of the potential interactive effects. Other studies utilised various concentrations and/or ratios but failed to capture a broad spectrum of possible combinations. We also discuss potential discrepancies in interactive effects based on different metrics and exposure designs. We advocate for future ecotoxicological studies to investigate a wider spectrum of chemical combinations, including various concentrations and ratios, and to address different levels of effects.

1. Introduction

The realisation that environmental contaminants are rarely encountered in isolation by organisms has sparked significant interest in the realms of environmental biology and ecotoxicology (e.g. Xiao et al., 2022). Similarly, within the medical domain, the attention towards mixtures has surged, as evidenced by studies highlighting the effectiveness of drug combinations in therapy compared to individual drugs alone, which also mitigate the risk of monotherapy resistance (e.g. Plana et al., 2022).

The complexity of interpreting the effects of chemical mixtures arises when post-exposure observed effects diverge from the expected outcomes, suggesting that the chemicals involved influence the effects of each other within the organism. Synergistic or antagonistic interactions are uncovered when observed mixture effects exceed or fail to meet the anticipated additive effects, respectively (Martin et al., 2021). Critically, interactive effects do not mean that the combined effects are only larger or smaller than the sum of individual effects, which would be defined as 'super-additive' or 'sub-additive' effects (Greco et al., 1995). Instead, genuine interactive effects are defined based on the assumption of non-interaction between the chemicals involved, using various null models such as Concentration Addition, Independent Action, Highest Single Agent and Zero Interaction Potency principles (Cedergreen, 2014; Vlot et al., 2019). Yet, because these null models rely on different formula and assumptions (e.g. identical toxicodynamic for each chemical), they possess advantages and shortcomings, and may confusingly yield different results (Vlot et al., 2019). Explicit detection and quantification of interactions can be done utilising both graphical techniques (i.e. response surface models), such as isobolograms, and quantitative indices, like the Chou-Talalay method (Altenburger et al., 2003; Roell et al., 2017; Twarog et al., 2021). For instance, the latter enables the computation of a Combination Index (CI), where $CI < 1$ implies synergism, $CI > 1$ implies antagonism, and $CI = 1$ implies additivity (Chou and Talalay, 1984). This index can be depicted along a gradient of Fraction Affected (FA) to result in effect-oriented FA-CI plots (Rodea-Palomares et al., 2015). Employing these methodologies, studies can qualitatively and quantitatively assess the presence or absence of interactive effects among chemicals.

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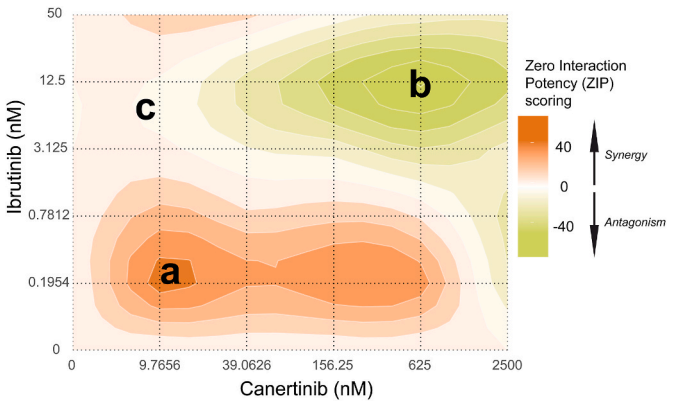

Fig. 1. Heatmap of drug combination (ibrutinib/canertinib) effects retrieved from data in Griner *et al.* (2014) showing Zero Interaction Potency (ZIP) scoring. **a.** Synergistic effects at low doses. **b.** Antagonistic effects at high doses. **c.** No interactive effects. ZIP models satisfy the assumption of both Concentration Addition and Independent Action models (Yadav et al., 2015).

The R package 'SynergyFinder', purposely designed for analysing chemical combination data (Zheng et al., 2022), utilises a dataset sourced from Griner et al. (2014) to interpret a spectrum of potential chemical combinations. This package and associated dataset, increasingly used as a reference in the field of human toxicology (e.g. Kawale et al., 2024), but still underused in ecotoxicology, feature a high-throughput drug combination (ibrutinib/canertinib) wherein the viability of B-cell lymphoma was assessed using a 6 x 6 dose matrix design, i.e. 36 combinations. This study observed synergistic effects between the two drugs at low concentrations, but antagonistic effects at high concentrations, with sets of combinations exhibiting no interactive effects (Fig. 1). These findings underscore the necessity of considering the entire spectrum of potential chemical combinations to accurately characterise interactive effects among chemicals. In this paper, we emphasise the necessity of incorporating a broader spectrum of chemical combinations in future ecotoxicological studies, as interactive effects can be dependent on concentration, ratio, and magnitude. To illustrate the current gaps in accuracy within ecotoxicology literature, we focus on binary combinations of pesticides in bees, a critical and timely subject due to the alarming decline of these essential pollinators (Carnesecchi et al., 2019; Tosi et al., 2022). Additionally, we discuss further considerations for ecotoxicological studies, such as the importance of endpoint- and methodology-dependent interactive effects.

2. Concentration-, ratio- and magnitude-dependent interactive effects

Studies examining chemical mixtures often employ point-wise assessments, testing singular combinations such as one concentration *x* of pure chemical A mixed with one concentration *y* of pure chemical B (Favaro et al., 2023; Martins et al., 2022; Yao et al., 2018; Zhu et al., 2014). In this context, concluding that both chemicals show "synergistic effects" in response to an anecdotal synergism under a specific combination is a fundamentally biased extrapolation. Firstly, variations in the observed effects of mixed chemicals may occur along a gradient of total concentrations of these molecules. For instance, while a combination of the same concentration of chemical A and chemical B may exhibit synergistic effects, doubling the concentration of both chemicals could yield antagonistic effects (Fig. 2a). Secondly, variations may occur along a gradient of ratios. For example, for the same total concentration, a 1:2 ratio of A:B may result in synergistic effects, whereas a 2:1 ratio might lead to antagonistic effects (Fig. 2b). Thirdly, variations may occur along a gradient of magnitude of effects. For example, chemical A and chemical B may exhibit synergistic interactions at low level effects and antagonistic interactions at high level effects (Fig. 2c). It is therefore imperative to consider these total concentration-, ratio- and magnitude-dependent interactions when interpreting results from chemical mixtures on a given biological model (Jonker et al., 2005; Rodea-Palomares et al., 2015). In the aforementioned example, specifying the concentrations used for both chemicals is decisive for accurate interpretation: "a mixture containing a concentration *x* of chemical A and a concentration *y* of chemical B - and hence a *x/y* ratio - exhibits synergistic effects when considering the lethal concentration 50%" (Ritz et al., 2021). For future studies aiming at assessing interactive effects, optimal practice involves designing experiments that generate data over a wide spectrum of possible chemical combinations and over several possible magnitudes of effects.

3. Lack of accuracy in the bee literature

To stress the frequent oversight of total concentration-, ratio- and magnitude-dependent interactive effects within the existing literature, we conducted a review of studies investigating pesticide binary combinations in bees, building upon the analysis conducted by Tosi et al. (2022) (n = 33 studies). Bees are a crucial group of pollinators, highly threatened due to their frequent exposure to xenobiotics including pesticides, trace metals and plastic derivatives (Willcox et al., 2023). Their recurrent interactions with these chemical cocktails and the availability of domesticated species for agriculture and research purposes have made them powerful models to describe the impacts of

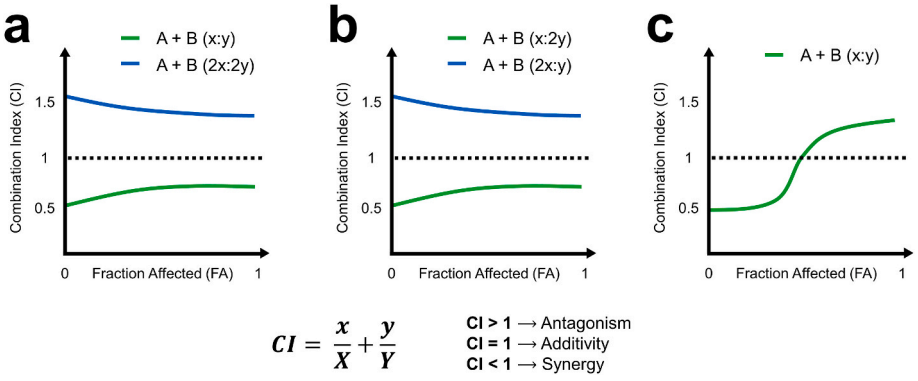

Fig. 2. Combination Index (CI) along a gradient of Fraction Affected (FA) for a binary mixture A + B. **a.** FA-CI plot showing concentration-dependent interactive effects. **b.** FA-CI plot showing ratio-dependent interactive effects. **c.** FA-CI plot showing magnitude-dependent interactive effects. Concentrations x and y are the concentrations of chemicals A and B within the combination of chemicals which together results in the FA. Concentrations X and Y are the concentrations of the chemicals A and B alone which result in the FA.

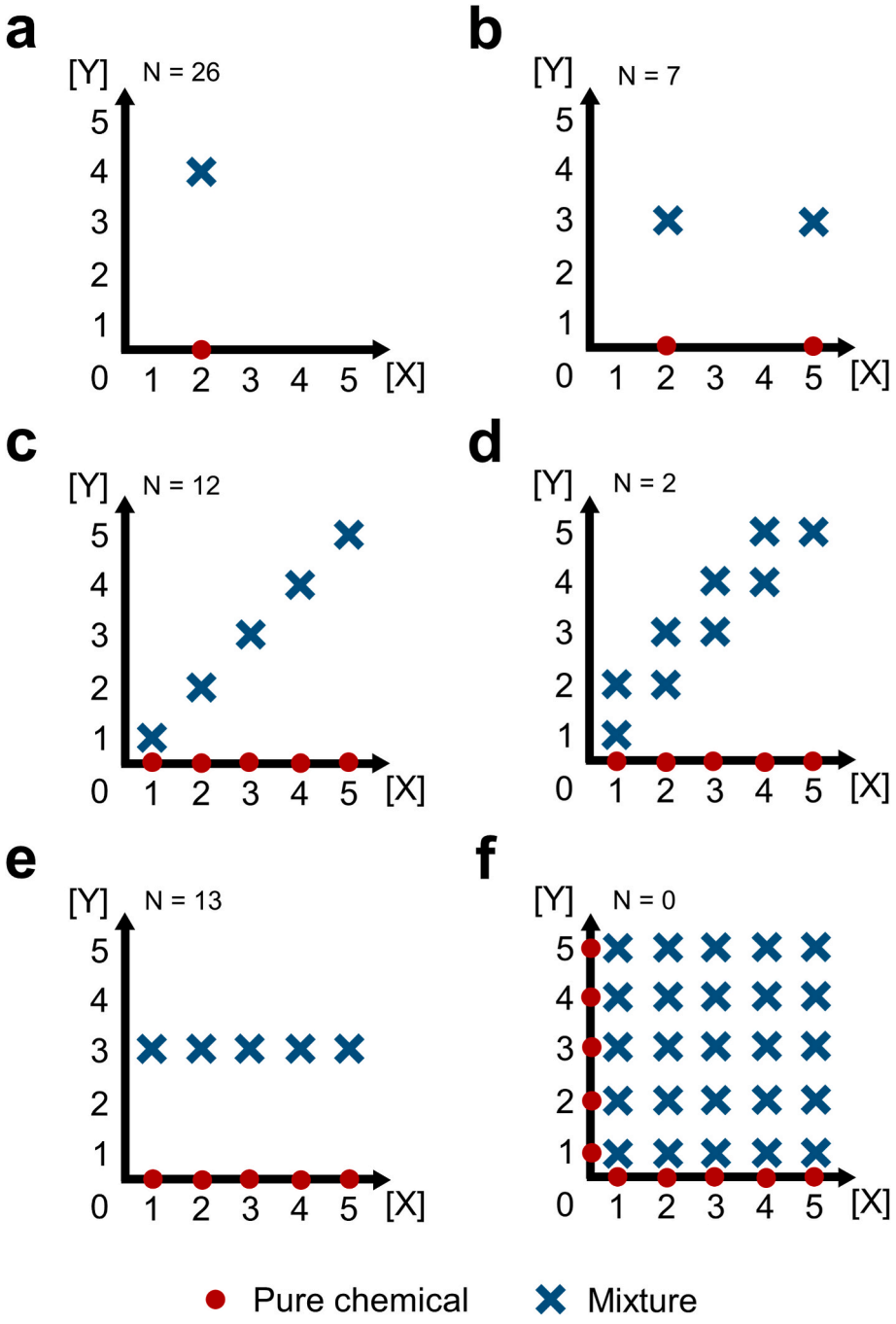

Fig. 3. Illustrations of design strategies for testing the effects of a binary mixture of chemicals at various concentrations. **a.** Unique total concentration and unique ratio of the mixture, compared to the equivalent concentration of one pure chemical. **b.** Several total concentrations of the mixture with a unique ratio each, compared to the equivalent concentrations of one pure chemical. **c.** Several total concentrations with a constant ratio, compared to the equivalent concentrations of one pure chemical. **d.** Several total concentrations with several constant ratios, compared to the equivalent concentrations of one pure chemical. **e.** Dose-response curve for one chemical with or without a given concentration of another chemical. **f.** Full design, all possible concentrations and ratios, with dose-response curves. The number of studies from the bee literature is reported for each design strategy (N total = 60).

environmental pollutants, alone or in combination, in wild animals. We further conducted a search of the Scopus database for recent experimental studies between 2022 and 2024 (n = 27 studies), identifying a total of 60 relevant studies (Supplementary Material 1, see 'README' sheet for search string, output filtering and classification). Our analysis revealed that approximately 40% (26 of 60) of these studies examined only a single total concentration and ratio of the binary mixture, comparing it solely to an equivalent concentration of one of the individual chemicals (Fig. 3a). Furthermore, approximately 10% (7 of 60) of the studies evaluated various concentrations of the binary mixture, each with its distinct ratio, again compared solely to the equivalent concentrations of individual chemicals (Fig. 3b). Similarly, another 20% (12 of 60) of the studies investigated multiple concentrations of the binary mixture with a fixed ratio, compared to equivalent concentrations of individual chemicals (Fig. 3c). Only two studies adopted an almost comprehensive approach by examining multiple concentrations and ratios of binary mixtures in comparison to individual chemicals (Fig. 3d). Notably, around 20% (13 of 60) of the studies employed shifts in dose-response curves, particularly the lethal concentration 50% (LD_{50}), as a means to delineate interactions. These shifts were

determined by maintaining a constant concentration of the second chemical (i.e. anchored approach; Fig. 3e). As only the LD_{50} was investigated in these studies, they failed to capture magnitude-dependent interactive effects. Strikingly, none of the studies employed a fully comprehensive combination design (Fig. 3f), i.e. an experimental design that would test all potential concentrations and ratios as depicted in Fig. 1. These findings demonstrate that a significant proportion of studies fail to account for total concentration-, ratio- and magnitude-dependent interactive effects, thus highlighting both biases and substantial gaps in our understanding of the impact of chemical mixtures on these highly threatened animals.

4. Further considerations in combination studies

The potential variance in outcomes across the diverse metrics is another important consideration in the realm of ecotoxicological studies. A combination of two chemicals, each administered at specific concentrations, may manifest distinct interactive effects across different endpoints (e.g. the activity levels of disparate enzymes). Moreover, variations in these interactive effects may arise due to differences in experimental methodologies, encompassing factors such as acute *vs.* chronic exposures, as well as oral *vs.* topical administration routes (Bjergager et al., 2017; Zou et al., 2012). Consequently, we urge forthcoming investigations to fully acknowledge biases in their interpretations of interactive effects. Building upon the aforementioned example, optimal practice would imply describing interactions as follows: "a mixture comprising concentration *x* of chemical A and concentration *y* of chemical B - and hence a *x/y* ratio - elicits synergistic effects on mortality (LD_{50}) following acute topical exposure." Although the present comment solely addresses the combination of xenobiotic stressors, equivalent practices are required for experiments exploring the interplay between xenobiotic and non-xenobiotic stressors, such as the combination of pesticides with poor-quality diets. For instance, in bumble bees, exposure to a sulfoximine insecticide along with a sugar-deficient diet had additive impacts on the likelihood of laying eggs, but synergistic impacts on their abundance if laid (Linguadoca et al., 2021). Hence, it is also imperative to refine the characterisation of interactive effects and enhance the design of associated experimental frameworks applicable to such investigations.

5. Conclusion

Studies dealing with chemical combinations must ensure transparency in the interpretation of their results, particularly by detailing the concentrations, ratios and effects investigated, including in their abstract. Considering concentration-, ratio- and magnitude-dependent interactive effects in ecotoxicological studies is critical to correctly interpret and quantify the impacts of chemical mixtures. To reach confident conclusions regarding the nature of interactions involved, studies should explore a much wider spectrum of possible chemical combinations, encompassing various concentrations and ratios, and addressing various levels of effects. Such practices are indispensable for enhancing our understanding of chemical interactions and facilitating comprehensive data aggregation in reviews. A higher degree of transparency and rigour will ultimately advance our knowledge of the intricate dynamics governing the post-exposure effects of chemical mixtures on biological systems.

CRediT authorship contribution statement

Antoine Gekière: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Guillaume Ghisbain:** Writing – review & editing, Supervision. **Maxence Gérard:** Writing – review & editing. **Denis Michez:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data are in supplementary.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2024.119572.

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