Supplementary information to

Current mixture toxicity assessments on soil organisms and applied risk assessments

Table S1 is provided as separate csv file.

1. Effect classification methods and strategies

A considerable number of the reviewed studies assessed mixtures for their null model deviations without the use of a formal risk assessment. Table S1 lists the major models for prediction, which are currently used to assess the effect of mixture toxicity on soil organisms. Most frequently, mixture models following the concept of independent action (IA) or concentration addition (CA), and the CA-derivative method of toxic unit (TU) summation, have been applied to compare predicted mixture effects to experimentally observed ones. The remaining three methods, namely the combination index(1, 2), the mixture dose-response analysis(3) and the additive index(4), base mixture effects on additivity or optionally on IA(3). This implies that the concepts do not differ fundamentally in their classification method to account for additivity. Some of the lesser applied methods comprise the isobologram method(5, 6), the median effect plot(7), the co-toxicity method(8, 9), the effect addition or concentration addition index(10, 11), the benchmark dose method(12-14) and the model deviation ratio(15, 16).

However, the threshold definitions that define whether an observed effect acts antagonistic, synergistic or is model conform vary considerably among the studies. For example, the classification can vary from as simple as judging the smallest deviation from the toxic unit concept (>1 TU or <1 TU) to be antagonistic or synergistic(17). Other classification systems consider some variation (combination index within factor 2 of the additivity prediction), such as the model deviation ratio(15) or statistical analyses(3). A particular finding is that the methods of the combination index and the co-toxicity coefficient tend to find more deviations from the null model, that is antagonism or synergism in experimental mixtures(6, 7, 9, 16, 18-20), than other commonly used methods such as the mixture dose-response analysis by Jonker et al. (2005)(3, 21-29). The claim of having detected synergism without an actual empirical analysis that confirms the synergism in the experimental data was the exemption(30-32).

Statistical testing has regularly been used to classify null model deviations and thus the methods and the corresponding results vary widely depending on the experimental design. Some studies relied on statistical descriptions of experimental effects using generalized linear models or generalized linear mixed models(33) to detect significant deviations via lowest sum of squares along with a Chi squared test (26, 29, 34). Others used ANOVA or similar tests for non-normal distributed tests to test for significantly different effects and set significance thresholds where effects would no longer be model conform and thus antagonistic or synergistic(35-37). Other straightforward methods are the calculation of confidence intervals of the mean effect(36, 38), where deviations that lie outside the confidence interval can be associated with effect classes (e.g.(18, 39)) or the application of the Student's t-test(35) or mean and standard deviation calculation with a comparison to a classification system(16). Some other studies directly compared calculated indices from experimental data and classified observed effects without statistical analysis for significant differences in relevant mixtures(5, 20, 30, 40-42).

The broad spectrum of applied methods to classify deviations from the null model reflect a current discussion on how to deal with statistical and biological significance of chemical mixtures but also multiple stressor exposures in experimental studies. Schäfer et al. (2023)(43) summarise that statistical significance of effect sizes from the null model is strongly dependent on the sample sizes of experiments and that detected significances not necessarily imply biological significance. For example, the frequent detection of synergistic interaction effects of mixtures in studies that applied the combination index (see above) may be caused by a limited sample size for the different tested mixtures and thus may be a statistical flaw that could be amended if the number of samples was increased to reflect experimental variation better. To avoid the shortcomings of the different approaches summarised above, it may be useful to apply standardized effect sizes like Cohen's d or Hedge's g(44) to classify null model deviations, as suggested by(43). The latter approaches are only effect size-dependent and do not rely on sample sizes.

Toxicokinetic-toxicodynamic studies have not been applied frequently for effect classification in studies with soil organisms. Toxicokinetic-toxicodynamic models add the time dependent development of

toxicity exposure as a result from single application of pesticides or their application in series(45, 46). The authors advance the General Unified Threshold model for survival (GUTS) to follow the concepts of CA or IA to enable the detection and prediction of antagonistic or synergistic effects. It provides the possibility to identify the pesticide(s) that may be responsible for the deviation from CA or IA. The proof of concept-studies demonstrate that these models are capable to predict mixture effects that deviate from CA and IA.

From this inconsistency in the definition of synergism and antagonism it appears that there is no clear convention for classification in currently applied methods for soil risk assessment of mixtures except for the agreement that deviations from the null model justify non-conformity. This observation has been noted elsewhere already(38, 47).

Table S2: Most frequently used descriptive methods for mixture effect classification and prediction in mixture experiments with soil organisms. The table lists methods that predict mixture effects to soil organisms and classify measured mixture toxicities as antagonistic, additive or synergistic. For the mathematical description of the methods, please refer to the referenced conceptual studies.

Method	Description	Example Studies	Conceptual reference
Concentration Addition (CA)	Mixture effects are predicted based on the sum of concentrations of each individual chemical in mixture, which are normalised to a concentration causing the equivalent effect if present singly (e.g. EC50). The toxic unit approach is a widely applied version of CA, while the additive index is a version of the toxic unit approach.	(6, 30) (14, 17, 18, 20, 25, 26, 28-31)	(48, 49)
Toxic unit (TU) summation	Mixture effects are predicted based on the sum of the concentrations of each individual chemical in a mixture, which are normalized to their respective equi-effective concentrations if present singly (e.g. EC50).	(5, 34, 39) (additive index)(40, 50) (additive index) (51)(additive index) (17)(simple classification for additivity deviation)(14, 18, 20, 22, 23, 52)	(53)
Independent Action (IA)	The fractional effects of each chemical concentration are summed up, assuming that each chemical in mixture is present at the equi-effective concentration as if present singly.	(21, 35) (11) (effect addition index used) (14, 18, 20, 22, 23, 25, 26, 28-31, 52)	(54)
Combination Index	A combination of the sum of single chemical concentrations provoking effect x in mixture, their fractional contribution to the observed mixture effect and the concentration that provokes effect x of each chemical, if present singly.	(7, 16, 18-20, 24, 25, 27, 28, 55)	(1, 2)
Mixture dose-response analysis	Specific parameters are added to the CA and IA models to account for dosel level dependent and dose ratio dependent deviations in experimental observations. Model conformity, antagonistic or synergistic deviations are classified according to fitted parameter sizes.	(26, 29, 52), (21-23, 34)	(3)
Additive Index	Additive index classifies biological activity (calculated from CA) as antagonistic, additive or synergistic.	(39, 41, 50, 51, 56, 57)	(4)

Table S3, S4 and S8 are provided as separate csv file.

2. Overview of experimental mixture studies in soils

Table S5: Frequencies of studied substances in mixture toxicity studies on soil organisms. Only studies are summarised here, where the mixtures were intentionally prepared and did not depend on field sampled soils and their contamination. The substances are grouped according to their frequencies in the studies.

Substance/Stressor	Frequency of being tested in mixtures
chlorpyrifos	17
cadmium	10
imidacloprid	10
atrazine, glyphosate	8
acetamiprid	6
abamectin, clothianidin, copper, flumioxazin, lambda-cyhalothrin, petroleum hydrocarbon	5
azoxystrobin, perfluorooctanoic acid, pyrene, s-metolachlor, temperature*, zinc	4
arsenic, benzo[a]pyrene, boscalid, butachlor, carbendazim, clethodim, cypermethrin, dicamba, drought*, fipronil, lead, lindane, phoxim, siduron	3
2-4-dichlorophenoxyacetic acid, acetochlor, deltamethrin, , difenoconazole, dimethoate, epoxiconazole, fenobucarb, fluoranthene, fluoxypyr, fomesafen, geothite, ibuprofen, microplastic, oxytetracycline, perfluorooctane sulfonate, phenanthrene, pirimicarb, prochloraz, sulfamethoxazole, tetracycline, toluene	2
2-4-6-trichlorophenol, 9-chloroanthracene, aldicarb, anthracene, arsenite, benzo[b]fluoranthene, bisphenol a, chlorantraniliprole, chromium, chrysene, ciprofloxacin, clofenotane, clopyralid, cyazofamid, cyhalothrin, cyproconazole, di-n-butylphthalate, di (2-ethylhexyl)phthalate, diazinon, dieldrin, diflufenican, dimoxystrobin, esfenvalerate, fenhexamid, fluroxypyr-meptyl, flutriafol, folpel,, halosulfuron, hexachlorobenzene, lauryl ether sulphate, lenacil, linear alkylbenzene sulfonate, linuron, mesotrione, methyl tert-butyl ether, metolachlor, metrafenone, metribuzin, metsulfuron-methyl, napropamide, nickel, nicosulfuron, nitrogen enrichment*, nonylphenol, norfloxacin, octylphenol, oxyfluorfen, paraquat, pendimethalin, pentachlorophenol, perfluorobutane sulfonamide, picoxystrobin, polyethylene, prosulfocarb, pyraclostrobin, salinity*, sulfamethazine, tebuconazole, tetraconazole, thiacloprid, trichloroethylene, triclosan, trifluralin, xylene, zinc-oxide, zinc oxide nanoparticles	1

*As part of abiotic factors, these stressors (drought, temperature, salinity etc.) were also used as part of toxic mixture experiments to assess chemical exposure together with the impact of abiotic stressors.

Table S6: Frequencies of tested soil organisms in mixture toxicity studies. Only those studies were considered where soil organisms were exposed to chemical mixtures or along with other environmental stressors such as soil moisture content or temperature. The organisms are grouped according to their frequencies in the studies.

Organism	**Frequency of being used in mixture experiments**
Eisenia fetida	35
Folsomia candida	25
Microbial community	20
Eisenia andrei	8
Allium cepa	4
Aporrectodea caliginosa	4
Enchytraeus crypticus	4
Oppia nitens	4
Glycine max (varieties sculptor 000 and don mario 67i70 ipro)	3
Lumbricus rubellus	3
Raphanus sativus	3
Allolobophora chlorotica	2
Caenorhabditis elegans	2
Hypoaspis aculeifer	2
Lactuca sativa (one unspecfied variety and crispa)	2
Lumbricus terrestris	2
Sorghum bicolor	2
Arabidopsis thaliana, Brassica chinensis, Cantareus aspersus, Carum carvi, Centaurea jacea, Cucumis sativus, Dactylis glomerata, Earthworm (not specified), Earthworm community (not specified), Elymus lanceolatus, Enchylaena tomentosa, Enchytraeus albidus, Folsomia fimetaria, Galium mollugo, Iseilema membranaceum, Lolium multiflorum, Lycopersicum esculentum, Medicago sativa, Metaphire sieboldi, Medicago truncatula, Perionyx excavatus, Picea glauca, Pinus banksiana, Porcellionides pruinosus, Rice (not specified), Silene latifolia, Sinapis alba, Sorghum saccharatum, Zea mays	1

Table S7: Frequencies of measured toxicity endpoints in mixture toxicity studies. 46 relevant studies were found under the criterion that soil organisms were exposed to chemical mixtures or along with other environmental stressors such as soil moisture or temperature. The endpoints are grouped according to their frequencies in the studies.

Endpoint	Frequency of being quantified in mixture experiments
Mortality	53
Biomarker	41
Reproduction	37
Seed germination	25
Plant growth	21
Bioaccumulation	16
Growth, Bacteria abundance & diversity	14
Avoidance	12
Fungi abundance & diversity	8
Mycorrhization	6
Biomass	5
Plant health,	43
Microbial activity, Decomposition rate, Trophic Links, Archaea abundance & diversity	3
Neuronal functionality, Weight	2
Biodiversity, Protist abundance & diversity, Root elongation	1

3. Novel Prediction methods

Other studies experimented with multiple stressor exposure, that is chemical exposure was combined with an environmental stressor such as temperature or soil moisture/drought. These studies used effect assessment models, which were not limited to CA only. One mesocosm study examined the pesticide mixture effects of different application modes of up to ten pesticides on soil microbial ecosystem services (e.g. litter decomposition (tea bag index), soil aggregate stability and aggregate size) by the quantification of using multivariate analysis along with a range of null models to assess pesticide interactions.(58) Another study conducted a similar mesocosm test with effect analysis but included ten various stressors comprising temperature, nitrogen enrichment, drought, salinity, microplastics and exposure to an antibiotic, an insecticide, a herbicide, a fungicide and a heavy metal.(59) The null models applied in both studies were an additive model, a multiplicative model and a dominative model, which, like most other approaches discussed, relies on single chemical exposure data (bottom-up approach). In their assumptions the additive and the multiplicative model are the equivalents to CA and IA, respectively, while the dominative model only selects the strongest effect size within a stressor combination to predict combined effects.(60) By selecting multiple possibilities as null models the authors argued for detecting synergistic or antagonistic effects if none of the model-predicted effect sizes were outside of the calculated confidence intervals.(59)

The concept of stress addition is another approach capable to consider different types of stressors that go beyond chemical exposure.(61) It assumes that each individual has a stress capacity, which is "used up" to different degrees by different types of stressors, which are summed up to represent the overall stress impact. The approach assumes that all stressors on an organism can be normalised to identical units and act additively. However, the approach's caveat is that it requires full information about the stressor intensity and the effect in the organism from single exposures in order to be versatile and usable in multiple stressor studies. If these data are available, it is deemed that the model is capable for effect prediction equivalent to the CA and IA approaches.(62)

Overall, the inclusion of other environmental stressors together with chemical exposure in soil studies are not frequent.(21, 33, 58, 59, 62-64) This is likely caused by the strong link of the ecotoxicological discipline to chemical regulatory background, which currently considers exclusively chemical exposure as the only stressor to ecosystem health, ignoring all other non-chemical sources.(43) A more holistic ecological risk assessment approach including environmental stressors would be more relevant and likely more robust in terms of assessing the observed effects and the discussed models may provide a better foundation for predicting and mitigating risk to soil organisms from chemical mixtures. Yet, the effect quantification of environmental factors like humidity and temperature in different soil types on representative soil organisms are largely lacking. These data need to be generated to consider them in a holistic assessment together with chemical toxicity data, which would benefit the realism of a MRA. One new experimental indicator, E_{mix}, has been proposed to integrate multiple biomarker quantifications upon mixture exposure to metals and PPP residues in earthworms into one indicator along with multivariate analysis.(65) The use of E_{mix} as an indicator for exposure risk, however, is currently impractical, since a risk framework for the indicator is lacking as well as clear correlations of the indicator with exposure concentrations in the multivariate analysis. More investigations on the suitability of this indicator will give a clearer picture about its usability.

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