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Synergistic effects of chemical mixtures: how frequent is rare?

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Highlights

- Synergisms greater than 2-fold reported in roughly 5% of investigated mixtures.
- This frequency is representative of mixtures toxicology studies in the literature.
- Magnitude of synergistic deviations from additivity can be large (up to 100-fold).
- Further research to increase mechanistic understanding of synergisms is required.

Abstract

Chemical pollution is characterised by sequential and simultaneous exposure to unintentional complex mixtures. The almost infinite number of real-life mixtures poses major challenges for investigations of all possible exposure scenarios through whole mixture or component-based approaches. As a pragmatic approach in data-poor situations, the application of a Mixture Assessment Factor to single substances assessments under REACH was announced in the European Chemicals Strategy for Sustainability. Current proposals for this factor are based on the assumption that mixtures behave additively, assuming that synergistic interactions are rare. This assumption is based on eight reviews published in the last 30 years. Synergistic deviations from additivity greater than 2-fold were reported in roughly 5% of investigated mixtures. This was more, rather than less, frequent in the handful of suitable studies of low dose mammalian mixture toxicity. This frequency is representative of mixtures toxicology studies in the literature and should not be interpreted as the frequency of synergisms in real world exposures. Understanding the frequency and likelihood of synergisms would entail detailed understanding of the co-occurrence of groups of substances giving rise to such interactions in relevant environmental media. Assumptions that synergistic interactions in real-life mixtures are rare appear to be premature. While further research is required, potential synergisms should not be omitted from debates on the conservatism or otherwise of mixture allocation factor or other regulatory approaches to protect people and environment from mixture effects.

Keywords: chemical mixtures, synergism, mixture assessment factor, mixture allocation factor, MAF, interaction

Abbreviations:

CA: Concentration or dose addition

IF: Interaction Factor

MAF: Mixture Assessment Factor or Mixture Allocation Factor

MRA: Mixture Risk Assessment

POD: Point of departure

1. Context

All living systems including humans are sequentially and simultaneously exposed to complex mixtures of chemicals. Over 350 000 anthropogenic chemicals and mixtures of chemicals have been registered for production and use [1] and the myriad of by-products, metabolites and abiotically formed transformation products are not included in this figure. Chemical pollution is therefore a wicked problem characterised by exposure to unintentional complex mixtures found in air, water, soils, in food and household and consumer products. The "something from nothing" toxicological behaviour of chemicals, or observation of mixture effects when all individual chemicals are present at concentrations below their individual no-effect levels [2], has been demonstrated in a broad range of complex chemical mixtures [3].

The scientific understanding of mixture effects has indisputably advanced in recent years as has the development of methods to assess risks from combined exposures [4,5]. Nonetheless, the almost infinite number of real-life mixtures poses major challenges for investigations of all possible exposure scenarios through whole mixture or component-based approaches. Additionally, regulatory systems were designed to address single substances through different regulatory jurisdiction often aligned with specific uses, applications or processes rather than co-exposure to multiple chemicals regulated under different legislative silos [6]. The need for cross-cutting, intermediary, pragmatic approaches that can be implemented at relatively short notice to address potential mixture risks was recognised in the European Union Chemical Strategy for Sustainability [7]. The Strategy, published in 2020, announced the introduction of a Mixture Assessment Factor (MAF), sometimes referred to as a Mixture Allocation Factor, to single substance risk assessments under REACH [8].

The application of the MAF has been argued to differ from uncertainty factors applied in chemical hazard assessment to account for extrapolation of experimental data in animals to the real world [9]. It is driven by co-exposure considerations and therefore more akin to the risk cup/allocation factor concept applied in the context of cumulative exposure and risk assessments [10]. It has been defined as a factor by which the regulatory safety threshold for an individual compound needs to be divided to ensure the same level of protection against unintended mixture effect as the level of protection aimed for in single substance assessment [10] and is intended as a pragmatic default approach in data-poor situations.

One of the greatest challenges to introducing a MAF and subject of much debate and contention is how to select an appropriate size for this factor [11,10]. Whilst the MAF has been said to target additional uncertainties encountered in chemical mixture risk assessment (MRA) including potential synergies, (eco)toxicological data gaps and lack of full composition information [12], the algorithms proposed to date to derive the size of the MAF for both environmental and health risk assessment are based on the explicit assumption that multi-component mixtures behave additively, adopting the principle of concentration or dose addition (CA) as a conservative default [11,10]. It is

therefore clear that the MAF, as currently discussed, would not account for the potential for synergistic interactions (more-than-additive). In earlier discussions on the size of a MAF, a separate Interaction Factor (IF), an additional MAF to specifically account for synergistic interactions, was discussed in the context of defined mixtures, such as biocide formulations [13]. A commonly encountered assumption is that synergisms occur rarely at concentrations close or below the point of departure (POD) concentrations of individual mixture components [11,14]. This assumption is typically justified by citing one or more of the several reviews that have attempted examine the frequency and/or magnitude of deviations from additivity [15] in the last 30 years. Implications of this assumption are that, without evidence of the contrary, the potential for synergistic effects can be considered negligible [14,16]. What is rare, frequent or negligible is however not strictly a scientific fact but represents a value judgment. The normative conclusions about the frequency of synergistic interactions of most of these reviews were written when the scientific and regulatory debates revolved around the feasibility and validity of component-based approaches to MRA. This context has now changed. While the factual evidence and numbers remain, their regulatory significance has shifted. In this commentary, the evidence base for the frequency and magnitude of synergistic interactions in chemical mixtures is briefly summarised before opening a debate around the interpretation of this evidence in the current regulatory context of the application of a MAF.

2. Experimental evidence on the frequency and magnitude of synergisms

2.1. Narcosis in aquatic organisms

Warne and Hawker [17] reviewed 104 equitoxic mixtures of a total of 182 chemicals with a predominantly unspecific, narcotic mode of action on aquatic organisms. Using a corrected Toxic Enhancement Index, they formulate their funnel hypothesis, i.e. the frequency of deviations from additivity decreases with the number of components in the mixture. These authors did not observe deviations that underestimated the predicted effect concentrations by more than a factor of 5. It is not possible to derive the frequency of synergisms from the data as presented.

2.2. Aquatic toxicity of pesticides

Deneer [18] reviewed the literature on joint effect of pesticides on aquatic organisms from 1972 to 1998, assembling data from 202 mixtures in 26 studies. Deviation from CA by a factor of more than two-fold was found in less than 10% of mixtures, and the frequency of synergisms and antagonisms were comparable. These proportions remained similar when excluding studies on algae, the frequency of more than two-fold deviation from CA was 6% (8/132 mixtures). The magnitude of synergistic deviation was as high as 20-fold in a mixture of deltamethrin and carbaryl. For 3 of these 8 mixtures, the underestimation of mixture effect doses by CA was greater than 5-fold.

2.3. Ecotoxicological endpoints

Belden et al. [19] reviewed 303 separate ecotoxicological mixture experiments with pesticides. About 5% of the 207 experiments that evaluated the CA model reported model deviation ratios greater than 2, most deviations from CA were less than 5-fold although a nearly 10-fold deviation was reported for a mixture of organophosphates in fish.

Vijver et al. [20] focused on 19 ecotoxicological studies (160 experiments) with organism exposed through water to mixtures of Cd, Cu or Zn, published between 1981 and 2009. Whilst these authors report that interactions were more frequent than additivity, their criteria to classify deviations from additivity were much stricter than in the other reviews summarised herein (0.1 or 0.2-fold as compared to 2-fold). Antagonisms appeared to be more frequent than synergisms. The largest synergistic deviation reported a 7.5-fold underestimation of the mixture effect dose by CA.

Cedergreen [21] completed the Belden et al. [19] and the Vijver et al. [20] datasets with additional searches on mixtures of antifoulants and an update with papers published up to 2013. A total of 67 studies could be included in a quantitative analysis of the frequency of synergistic interactions for pesticides, metals and anti-foulants, respectively. Synergy occurred in 7%, 3% and 26% of the 194, 21 and 136 binary pesticides, metals and antifoulants mixtures, respectively. The magnitude of the deviation from CA was generally less but could exceed 10-fold. For pesticides, 95% of the synergistic mixtures contained combinations including cholinesterase inhibitors or azole fungicides, two groups of pesticides known to interfere with metabolic degradation of other xenobiotics.

2.4. Ecotoxicological and mammalian mixture studies

We conducted a systematic review of mixture experiments published between 2007 and 2017 covering all chemicals and toxicity outcomes [22]. Our searches resulted in an inventory of 1220 mixture experiments from 761 eligible studies, of which about a quarter reported synergisms. Approximately two thirds of these experiments were conducted with binary mixtures, and the funnel hypothesis [17] could neither be confirmed nor refuted. Most experiments relied on low-cost assays with readily quantifiable endpoints and outcomes of relevant for human risk assessment (e.g. carcinogenicity, genotoxicity, reproductive toxicity, immunotoxicity, neurotoxicity) were rarely addressed. About half of the 1220 entries were rated as “definitely” or “probably” low risk of bias.

Of the 557 experiments that reported synergistic or antagonistic interactions, 388 reported sufficient information to allow a quantitative reappraisal of their claims. Only twenty percent of those (N = 78) reported synergisms more than two-fold higher or lower than the predicted additive doses (Fig. 1). Strong synergisms (4 to nearly 100-fold in an *in vitro* androgen receptor reporter gene assays in Chinese hamster ovary

cell line) were observed in 9 *in vitro* and one *in vivo* mammalian study and 32 ecotoxicological studies (deviations between 4 to 50-fold).

Previous concerns about the synergistic potential of combinations of triazine, azole and pyrethroid pesticides at environmentally relevant doses were confirmed, while evidence of synergisms with endocrine disrupting chemicals, particularly anti-androgens, emerged.

2.5. Synergisms at low doses in human/mammalian toxicology

Boobis et al. [23] retrieved 43 studies published between 1990 and 2008 reporting synergisms in mammalian test systems at doses close to the PODs for individual chemicals. The focus of the literature search does not allow one to comment on the frequency of synergisms at low doses. The magnitude of synergisms was included in 11 papers, and this includes studies where deviations from effect doses predicted for additivity that were less than 2-fold. None of these synergistic deviations exceeded a factor of 4. Synergisms at low doses were observed for mixture of organophosphate pesticides, thyroid axis disrupting chemicals and carcinogenic solvents.

Elcombe et al. [24] conducted a review of the peer reviewed studies published between 2000 and 2020 relating to low-dose mixtures of chemicals (defined as those in which all components were at or below their POD) in mammalian *in vivo* systems. Of the 30 eligible mixture studies that used component-based methods, only 7 employed experimental designs which allowed for comparison to additivity predictions. Nonetheless, nearly half (3 of 7), all mixtures of endocrine disrupters, reported responses significantly greater than additivity, suggesting synergy.

3. **Discussion**

In reviews that allowed estimation of the frequency of synergistic deviations from additivity greater than 2-fold, synergisms were reported in roughly 5% of investigated mixtures. This was more, rather than less, frequent in the handful of suitable studies of low dose mammalian mixture toxicity [24]. It should also be noted that these figures do not account for potentiation, where the combination of one active and one inactive component leads to exacerbations of effects. This frequency is representative of mixtures toxicology studies in the literature and should not be interpreted as the frequency of synergisms in real world exposures. Understanding the frequency and likelihood of synergisms would entail detailed understanding of the co-occurrence of groups of substances giving rise to such interactions in relevant environmental media. Socianu et al. [11] derived a generic chemical mixture that approximates a mixture co-exposure profile of the EU general population from the HBM4EU-Aggregated dataset which included phthalates, known endocrine disrupters, and pyrethroid pesticides. Exposure to some of the culprits identified to be involved in synergistic interactions is therefore not a rare occurrence. Both classes of compounds have been associated with synergistic effects in experimental studies. Notwithstanding that CA has been demonstrated to be a useful default assumption for MRA, on the basis of currently

available evidence, assuming that synergisms are rare enough to be negligible is premature.

Selecting the size for a MAF accounting for additive interactions is already contentious. Such decisions can be informed by scientific evidence, e.g. the recent analysis by the Swedish Chemical Agency KEMI found that MAF values of 10, 20 and 50 seemed sufficient for over 70%, 95% and all the mixtures analysed, respectively [10]. The desired level of protection is a value-laden societal choice attempting to balance the consequences of over- or under-conservatism under uncertainty. Some concerns regarding over-conservatism stem from the view that the application of uncertainty factors during individual substances' hazard assessment may already result in undue overprotection, particularly for human health [25]. Beyond muddying the transparency of uncertainty analyses, such assumptions do not necessarily hold true [26,27]. For example, chemical-specific inter-individual toxicodynamic variability can exceed the default assumption of $10^{1/2}$ [28].

In this context, increasing a MAF based on assumption of additivity, e.g. by an additional IF, to account for potential synergisms is likely to be met with some resistance, even before discussing the potential size of such a factor. The latter should be informed not only by the frequency but also by the magnitude of deviations from additivity. Whilst the largest deviations from additivity have been reported in *in vitro* systems (up to 100-fold), the magnitude of synergistic deviations from additivity can be large; up to around 30-fold in *in vivo* ecotoxicological studies, whilst available data on *in vivo* mammalian study does currently allow any conclusion to be drawn [22]. An alternative would be to apply an IF to groups of chemicals known or suspected to give rise to synergisms on the basis of currently available evidence. This may however discourage the generation of new knowledge and understanding of synergistic interactions in chemical mixtures.

When discussing the conservatism of assessment or allocation factors, it should be stressed that default factors are typically a pragmatic response to lack of data. Such regulatory solutions should be conservative enough to encourage and reward the generation of data and mechanistic understanding by allowing such factors to be refined if adequate evidence is provided. Scientific evidence of synergistic or antagonistic interactions could help progress towards priorities for further research, namely; building a FAIR open evidence database of toxicokinetic or toxicodynamic interactions comparable to those available in pharmacology, increasing mechanistic understanding particularly in realistic, i.e. unbalanced (as opposed to equipotent) environmental mixtures, as well as test the funnel hypothesis.

Finally, debates around conservatism should also account for the uncertainty related to factors beyond the scope of chemical risk assessments. Living organisms during most of their lifetime often must cope with environmental stress deviating from the optimal environmental conditions used in (eco)toxicological experimental settings. A review of more than 150 studies covering stressors including heat, cold, desiccation, oxygen depletion, pathogens and immunomodulatory factors combined with a variety of environmental pollutants revealed that synergistic interactions between the effects of various natural stressors and toxicants were reported in more than 50% of the

245 available studies [29]. In a context of rapid global environmental change, we may have
246 to accept that the space in the risk cup that can be allocated to chemical pollution
247 before it overflows is shrinking.

248 4. **Conclusions**

249 Based on evidence available to date, assumptions that synergistic interactions in real-
250 life mixtures are rare and their likelihood therefore negligible appear to be premature.

251 Further research aiming to increasing mechanistic understanding of the likelihood of
252 synergisms and the frequency of co-occurrence of groups of chemicals giving rise to
253 such synergisms is required.

254 In the meantime, potential synergisms should not be omitted from debates on the
255 conservatism or otherwise of mixture allocation factor or other regulatory approaches
256 to protect people and environment from mixture effects.

257

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Declaration of interests

☐ 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.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Olwenn V Martin reports a relationship with European Chemicals Agency that includes: board membership. Olwenn V Martin reports a relationship with Food Packaging Forum that includes: board membership and consulting or advisory. Olwenn V Martin reports a relationship with European Commission Joint Research Centre Ispra that includes: funding grants. Olwenn V Martin reports a relationship with European Commission that includes: funding grants. Olwenn V Martin reports a relationship with European Environment Agency that includes: funding grants. Olwenn V Martin reports a relationship with UK Food Standards Agency that includes: paid expert testimony. None