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Distinctions between similarly and dissimilarly acting mixture components unnecessarily complicate mixture risk assessments: Implications for assessing low dose mixture exposures

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Abstract

Distinguishing between mixtures of substances with similar and dissimilar modes of action is believed to have implications for judgements whether mixture risks might arise when all chemicals comply with their regulatory limits. However, differentiating between similar and dissimilar action unnecessarily complicates mixture risk assessments. Whether substances in a mixture have similar or dissimilar mechanisms is often difficult to decide. Only a few cases show the validity of dissimilar action; concepts based on similar action (dose addition) generally produce good approximations of observed mixture effects. Further, the quantitative differences of mixture effect predictions that follow from assumptions of similar or dissimilar action are rather small. To avoid underestimations of mixture risks, chemicals that produce common adverse outcomes should be assessed together, and this should not be restricted to chemicals with similar mechanisms. Assertions that compliance with Health-Based Guidance Values (HBGVs) protects against mixture risks can be de-constructed to reveal several false assumptions, among them that chemicals generally act according to dissimilar action and that HBGVs are equivalent to "zero-effect levels." The protection goals enshrined in HBGVs for single chemicals may not be realized when there is co-exposure to chemicals that produce the same effect, regardless of perceived modes of action of the mixture components.

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Abbreviations

DA, Dose addition; HBGV, Health-based Guidance Value; IA, Independent action; MOA, Mode of action; NOAEL, No-Observed Adverse Effect Level; TCDD, 2,3,7,8 Tetrachlor-dibenzo-dioxin; USEPA, United States Environmental Protection Agency.

Introduction

According to an often repeated and widely cited view [1,2], chemical mixture risk assessments must distinguish between mixtures composed of substances with similar modes of action (MOA) and those made up of chemicals with dissimilar MOA. The issue has implications for judgements of whether mixtures composed of large numbers of chemicals but present in low quantities—exposures often experienced by the general population—could pose risks to human health and wildlife.

Representative is the opinion of the EU Scientific Committees, who in 2011 proclaimed that "*for chemicals with different modes of action (independently acting), no robust evidence is available that exposure to a mixture of such substances is of health or environmental concern, if the chemicals are present at or below their "zero-effect levels"*" [1]. With the assumption that regulatory limits such as Health-based Guidance Values (HBGVs, including acceptable daily intakes, tolerable daily intakes, reference doses and similar) are equivalent to "zero-effect levels," they concluded that "*the effects of co-exposure to several substances all below the HBGV value should be assumed to be negligible if all substances have dissimilar modes of action.*" Continuous updating of HBGVs was offered as a policy for protection against mixture risks. In this view, additional risk assessment or management approaches are not generally required and should be reserved only for special cases. More recently, these assertions have reappeared in a critique by officials of the German Federal Institute for Risk Assessment of new European Commission suggestions for mixture risk management approaches [2]. In this critique, mixture risks are considered negligible, unless several conditions come together: Mixture components must have a common or interlinked MOA (how

this is to be understood is not further defined) and must produce hazards of high concern. Furthermore, the exposures to all components must be below regulatory limits and must be constant during windows of vulnerability.

The assertions in the studies by EU Scientific Committees and Herzler et al. [1,2] can be de-constructed to expose several assumptions which require scrutiny: The first is, that it is possible to clearly distinguish between similarly and dissimilarly acting mixture components. Second, that the immense diversity of chemicals that make up exposure scenarios in the real world implies that they generally act together by dissimilar modes of action. Third, that regulatory exposure limits set to protect human health are not associated with any toxicity ("zero-effect levels"). And finally, that estimates of "zero-effect levels" are available for the multitude of chemicals that constitute human exposures.

This article evaluates these assumptions by first tracing the distinctions between similarly and dissimilarly acting mixtures to the concepts of dose addition (DA) and independent action (IA, often also called response addition). This will be followed by a discussion of the conceptual and practical difficulties encountered while attempting to distinguish between similarly and dissimilarly acting mixtures. Next, the empirical evidence in support of dissimilarly acting mixtures and the validity of IA will be considered, followed by a summary of data of combined effects of multiple chemicals at low doses and a discussion of the implications for chemical risk assessment and management.

Dose addition, independent action, similar and dissimilar action: implications for mixture assessment groups

The theoretical importance of separating similar and dissimilar action derives from the assumptions underlying the concepts for predicting mixture effects from the toxicity of their components: dose addition (DA) and independent action (IA).

DA, developed by Loewe and Muischneck [3], states that chemicals producing a common effect can be replaced with each other by equal fractions of equi-effective doses, without loss of combination effect. From this follows that multiple chemicals, when combined at fractions of their threshold dose, will produce a joint effect, but only if present in sufficient numbers and at sufficiently high levels [4]. These principles are fulfilled with combinations of chemicals that act through similar mechanisms or at the same site. For this reason, DA has been allied to "similarly acting" mixtures, even though the original article by Loewe and Muischneck [3] reveals nothing that links the idea to mechanisms or MOAs. As detailed below, the applicability of DA is not limited to mixtures of chemicals with similar MOAs.

Independent action (IA) [5] was originally conceived to deal with irreversible events such as mortality, where probabilistic principles apply. IA is commonly associated with toxicity through different mechanisms, and in more contemporary parlance could be defined as applying to mixtures composed of chemicals with no common molecular initiating events and few common key events. IA predicts that combination effects are not expected if all chemicals are present at quantities below their "zero-effect levels."

Conceptual and practical difficulties in distinguishing similar and dissimilar mixtures and implications for grouping decisions in mixture risk assessments

Separating similar from dissimilar action is not straightforward. In many cases, the mechanistic information needed to distinguish chemicals in terms of their MOA or mechanism of action is simply not available. It is also unclear how the terms "MOA" or "mechanism" should be applied to separate similar from dissimilar action in practice. For example, phthalates and 2,3,7,8 TCDD can reduce sperm numbers after exposure during gestation [6,7] (common MOA, therefore similar action?), but through different pathways and mechanisms not yet defined in every detail (dissimilar action?). Advocates of using strict mechanistic criteria of similar action would regard the grouping of phthalates and TCDD in common assessment groups as inappropriate. Yet, there is clear evidence that mixture effects from phthalates and 2,3,7,8 TCDD on sperm numbers occur [7].

In view of the enormous diversity of chemicals in "real world" exposure scenarios it is persuasive to assume dissimilarity of action by default [1]. What then is the empirical evidence that IA is generally valid for predicting the effects of most mixtures?

Scarce evidence for the general validity of IA as an assessment concept

Backhaus et al. [8], Walter et al. [9] and Faust et al. [10] went to great lengths to select mixtures of chemicals with strictly dissimilar MOAs for tests in luminescent bacteria and algae. With mixtures of up to 16 chemicals they demonstrated the superior performance of IA. DA overestimated the observed effects, but by a small margin. However, further examples proved difficult to find, especially with mammalian cells or multicellular organisms. To our knowledge, the only empirical example of the validity of IA with multi-component mixtures in higher organisms is in fish exposed to different hormonally active chemicals that disrupt reproduction in breeding experiments [11].

In contrast, there are numerous examples for the validity of DA with mixtures composed of chemicals showing a variety of different MOAs. Already in 1995, work with chemical mixtures in fish led van Leeuwen to conclude that "chemicals with different modes of … action can often almost behave according to concentration-addition" [12].

DA performed well in approximating the effects of chemicals that disrupt male sexual development by androgen receptor antagonism, suppression of foetal androgen synthesis or inhibition of steroid-converting enzymes [13–15], while IA often led to underestimations of effects (for a more detailed discussion see the study by Conley et al. [16]). In a very recent example, DA predicted accurately the effects of a mixture of eight chemicals that produce malformations in fish by a diversity of mechanisms [17].

The difficulties in finding additional reference cases for IA suggest that the theoretical principles of strict dissimilarity are confounded by the convergence of multiple effector chains or adverse outcome pathways on common downstream pathways better described by DA. Although downstream effects (e.g. smaller birth weight, poor semen quality, malformations etc) derive from a greater number of diverse MOAs, there seem to be biological limits to the number of strictly dissimilar MOAs for chemicals affecting the same adverse outcome. With rising numbers of mixture components therefore, combined effects approaching similar action better approximated by DA are increasingly likely.

Quantitative differences in mixture effect predictions derived from DA and IA

Are dissimilarity of action and IA therefore theoretically relevant, but of limited practical applicability? The available evidence suggests that this is indeed the case and supports the idea of default application of DA, even to mixtures that could be viewed as dissimilarly acting, as advocated by the European Food Safety Authority [18]. Examples where observed mixture effects exceed those anticipated by DA (synergisms) are relatively rare [19].

However, there are concerns that the default application of DA irrespective of the toxicants' MOA and mechanisms of action produces vastly over-protective mixture toxicity assessments, and is therefore poorly justified scientifically and in conflict with principles of proportionality in the regulatory management of chemicals risks.

Studies that have evaluated the performance of DA and IA side-by-side (e.g. the studies by Backhaus et al., Walter et al., Faust et al., Thrupp et al., Christiansen et al., Conley et al.,Conley et al., Ermler et al., Ermler et al., Orton et al., Villas et al. [8–11,13–15,20–23] all showed that the prediction differences between DA and IA, quantified as ratios of effect doses, are small, and normally do not exceed one order of magnitude.

DA-IA prediction differences are driven by four factors: the number of mixture components n, the slope of the components' dose response curves, the mixture ratio and the effect level under consideration [10,24,25]. With 100 mixture components, the ratio of mixture effect doses predicted by DA and IA cannot be larger than 100, but this can only occur when all components are present in equal fractions of equi-effective doses, a highly unlikely scenario. In any other situation, the ratio will always be smaller than n, the number of mixture components. Independent of the mixture ratio, the slopes of individual dose response curves of a given set of mixture components have a general limiting effect on the possible range of prediction ratios. Depending on the slope values and the effect level under consideration, DA may predict equal, higher or lower toxicities than IA as explained in extensive detail by Kortenkamp et al. [25]. Simulation studies with 100 chemicals that affect algal reproduction have shown that greater than 4.2-fold differences between the two predictions never occurred, a difference well within the "noise" introduced by experimental error [25]. Finally, under conditions of strict independence of action no mixture effects will arise below "zero-effect" levels of all single components, while DA predicts effects whose magnitude depends on the number of components in the mixture.

DA as a default, also for mixtures perceived as dissimilar: Implications for mixture risk assessments and decisions on cumulative assessment groups

Thus, if cases demonstrating the validity of IA are rare, if there are biological limits constraining the occurrence of strict independence of action and if the prediction differences between DA and IA are relatively small, then reflexions on presumed MOAs or mechanisms unnecessarily complicate decisions regarding the choice of DA or IA as the "correct" assessment concept.

All this argues for the general application of DA, irrespective of MOA or mechanisms, unless there are data to show that IA provides the better prediction of mixture effects, as proposed by the EU Scientific Committees [1], EFSA [18] and WHO-IPCS [26].

This has implications for building cumulative assessment groups in mixture risk assessments. Instead of a narrow focus on common mechanisms as practised in USEPA assessment groups for pesticides, and proposed recently for phthalates [27], broader criteria are needed. Chemicals with similar modes of action, based on *in silico*, *in vitro* and *in vivo* data should be grouped together, but

beyond that, the emphasis must be on common adverse outcomes, regardless of perceived mechanisms. Adverse outcome pathway networks may offer additional support in decisions about which chemicals to group together in mixture risk assessments [15,16,28]. Debates about similar or dissimilar action as such lead into a dead end.

Suggestions to restrict mixture risk assessments to chemicals with common or interlinked MOAs [2] are therefore of little use in risk assessment practice. At best, such proposals limit considerations of mixture risks to special cases in which only a sub-set of chemicals contributing to mixture risks are assessed together, introducing a bias towards underestimations of risks. At worst, they lead to largely fruitless debates about cumulative assessment group membership, thereby blocking mixture risk assessments entirely.

Observations of mixture effects at low doses and their implications

Experimental studies in the field of disruption of male sexual development have been particularly informative about mixture effects at low doses, often at fractions of the no-observed-adverse-effect levels (NOAELs) of individual mixture components and with chemicals that produce these effects through a variety of molecular initiating events and key event-relationships [7,13–15,29]. Malformations of the penis (hypospadias) were seen when each mixture component was present at doses that exceeded 25% of their individual NOAELs for this effect; other, less severe effects occurred at only 12% of individual NOAELs [15]. The data from genotoxicity studies [21] and from studies of developmental toxicity in fish [17] point in the same direction.

If experiments with even greater numbers of chemicals could be performed, they could demonstrate effects occurring at doses below HBGVs. If proof positive was required, this could no doubt be provided, but ethical and resource considerations prevent the realisation of such studies.

Mixture risks, regulatory limits and "zero-effect levels"

Proper application of IA for judging the health risks from combined exposures in the low dose range requires clear distinctions between zero effects and small effects: Before an exposure scenario involving multiple chemicals can be declared safe under IA, certainty is required that the individual effects of all components are indeed zero.

While toxicologists have defined thresholds and "zero-effect levels" into existence, it has fallen to biometricians and statisticians to emphasise the difficulties involved in determining "zero-effect levels" by experiment or by statistical means. Due to the limited resolving power of toxicological assays, in practice this task cannot be realised [30,31].

Considerations of the mathematics underpinning IA highlight the scale of the problem: IA predicts that the combined effect of 100 chemicals with a 1% effect each will be 63% of a possible maximal effect. With an effect of only 0.1% from each component, the expected combined response will still be 9.5%. However, effect magnitudes of 1% and smaller are beyond the discriminatory power of most toxicological tests and will almost certainly be overlooked. With the usual number of animals per dose group (20–50) in guideline toxicity tests, the minimum detectable effect magnitudes range from 10% to 30% of a possible maximal effect [32]. With 50 animals per dose group, long-term bioassays for carcinogenicity struggle to resolve a 10% cancer incidence. In their landmark low-dose study of dibenzo-al-pyrene carcinogenesis in trout, Bailey et al. [33] had to use no fewer than 4535 fish per dose to demonstrate tumour incidences of 0.1%. The measurement of incidences of only 0.02% required 6429 animals per dose group; the entire experiment consumed over 40,000 fish. Similar numbers of animals had to be used to detect cancer incidences of 1% in rodents [34]. Ruling out combination effects with multi-component mixtures according to IA would require the detection of very small effects of the individual components, especially with large numbers of chemicals in the mixture, to a level of refinement and sophistication that is currently unachievable. Similar challenges exist with *in vitro* assays where the detection of small effects is limited by the sensitivity of the measurement methods applied [31].

Not only according to DA, but also under the assumption of strict independence of action, small effects that can no longer be demonstrated directly in single chemical toxicity testing, may reveal themselves as combination effects, provided chemicals are combined in sufficient numbers. With a mixture of 16 toxicants shown to comply with IA, Faust et al. [10] detected a 18% inhibition of algal reproduction when all chemicals were present at concentrations estimated to produce 1% inhibition (EC01). This can only mean that the estimated EC01 were not "zero-effect levels."

The issue is well recognised. The EU Scientific Committees [1] emphasised that NOAELs derived from experimental studies do not always equate with "zero-effect levels," and that "*exposures to these levels may also contribute to mixture effects of dissimilarly acting substances.*"

Accordingly, they redefined the key issue: "*The question, therefore, is not if exposures to mixtures of substances at the NOAEL or NOAEC for each component represent a potential risk, but if exposures to mixtures well below these levels, and in particular at the level assumed to be safe for each component*

(TDI, DNEL, PNEC or equivalent) may produce adverse effects" [1].

What then of the idea that HBGVs are "zero-effect levels"? The EU Scientific Committees [1] write: "*The HBGVs are hence **expected** to represent a value at which no effects are produced; thus for threshold substances, the assumption is that this value is equal to or lower than the no effect level; thus an E(Ci) = 0 should be **assumed** for exposures at the HBGV level. Consequently, the effects of co-exposure to several substances all below the HBGV value should be **assumed** to be **negligible if all substances have dissimilar modes of action*** (emphases added)."

However, there are well-recognised uncertainties in setting HBGVs. What was judged as "safe" only a few years ago, is evaluated today as associated with human health risks. As knowledge about the toxicity of chemicals grew with the accumulation of further data, HBGVs have tended to decrease. The most striking recent example is the new exposure limit for bisphenol A proposed by the European Food Safety Authority [35], 20,000-fold lower than the previous value. Perfluorinated chemicals, PCDDs and lead, to name a few, are similar cases. To our knowledge, there is no example where a HBGV for a single chemical has been corrected upwards.

Continuous refinement of HBGVs is therefore essential. But the Committees offer this as a policy to also protect humans from mixture risks. Accordingly, if HBGVs turn out not to be "zero-effect levels," … *the conclusion should be that the HBGV should be recalculated for offering a proper level of protection*" [1].

Re-assessment of single chemical HBGVs as a policy for protection against mixture risks?

As the only policy for delivering protection against mixture risks for human health, the continuous refinement of HBGVs for single substances has inherent limitations, to a point where this approach jeopardises essential protection goals.

First, the contention that the principles of independence of action apply by default to human exposure scenarios [1] is in contradiction to the available evidence. Accordingly, the claim that HBGVs for single chemicals alone can safeguard against mixture risks breaks down when independence of action does not apply.

Second, the research effort and data that have to go into the derivation of HBGV is enormous. Only for a small sub-set of chemicals, perhaps 2000−3,000, does sufficient knowledge exist to support the derivation of HBGVs. These numbers are dwarfed by the multitude of chemicals relevant for human exposures, estimated to be in the order of several tens of thousands. Most chemicals in current use are essentially untested.

Thus, the idea that HBGVs afford protection from mixture risks is only tenable scientifically when *all* of three conditions are fulfilled [1]: That all HBGV are zero-effect levels [2], that chemicals act together according to the principles of independent action, and [3] that the two conditions apply to all chemicals in a mixture, including untested chemicals. We have seen that these conditions are not met, and viewed from this perspective, continuous refinement of HBGVs as the sole safeguard against mixture risks, as advocated by the EU Scientific Committees and Herzler et al. [1,2] is a reckless policy doomed to failure.

Conclusions

Whether we accept DA as the default concept or prefer the (unrealistic) case of IA, the decisive factor that determines whether concerns about possible mixture risks are justified is the sheer number of chemicals that contribute to a common adverse outcome, their exposure levels and their potency. Fragmentary knowledge about the human exposome, and the fact that most chemicals are essentially untested, present formidable barriers to providing reassuring answers.

The inevitable conclusion is that the protection goals enshrined in HBGVs for single chemicals may not be realised when there is co-exposure to chemicals that produce the same effect, regardless of perceived MOA (see a recent mixture risk assessment study of male reproductive health [36]). HBGVs do not *per se* protect against mixture risks. While certain co-exposure scenarios may not present any risks, concerns about combined exposures cannot be ruled out without further investigation. Mixture risk assessments must therefore be at the heart of chemical risk assessment and be addressed as a matter of course, rather than be reserved for special cases, as argued by Herzler et al. [2]. Their four preconditions (common or interlinked MOA, hazards of high concern, exposures below regulatory limits for all single chemicals, and constancy of exposures during windows of vulnerability) for conducting mixture risk assessments are intended to define a very limited, arbitrarily circumcised chemical space for initiating mixture risk assessments. The inevitable result will be to leave the general population and wildlife largely unprotected against mixture risks.

As the political demand for considering mixture risks increases, regulatory authorities should emphatically embrace the challenge of dealing with mixture risks. First, more mixture risk assessments are needed, and this should be enshrined in all relevant chemical regulations, across regulatory domains [37]. Considerations

of MOAs should not be used to obfuscate and delay progress with mixture risk assessment. Second, additional risk management tools such as lowering of HBGVs by inclusion of so-called mixture assessment factors, or the introduction of group limit values, require urgent consideration.

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