Low-Level Exposure to Multiple Chemicals: Reason for Human Health Concerns?

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Background: A key question in the risk assessment of exposures to multiple chemicals is whether mixture effects may occur when chemicals are combined at low doses which individually do not induce observable effects. However, a systematic evaluation of experimental studies addressing this issue is missing.

Objectives: With this contribution, we wish to bridge this gap by providing a systematic assessment of published studies against well-defined quality criteria.

Results: On reviewing the low-dose mixture literature, we found good evidence demonstrating significant mixture effects with combinations of chemicals well below their individual no observable adverse effect levels (NOAELs), both with mixtures composed of similarly and dissimilarly acting agents.

Conclusions: The widely held view that mixtures of dissimilarly acting chemicals are "safe" at levels below NOAELs is not supported by empirical evidence. We show that this view is also based on the erroneous assumption that NOAELs can be equated with zero-effect levels. Thus, on the basis of published evidence, it is difficult to rule out the possibility of mixture effects from low-dose multiple exposures.

Key words: dose addition, independent action, low dose, mixture toxicity, multiple exposures, risk assessment. *Environ Health Perspect* 115(suppl 1):106–114 (2007). doi:10.1289/ehp.9358 available via *http://dx.doi.org/* [Online 8 June 2007]

With news of harmful chemicals in food and consumer goods appearing regularly in the media, the general public has come to realize that exposure is to multiple chemicals simultaneously, usually at low levels. Although awareness of the need to deal with combined exposures is growing among experts, judgments as to whether low-level exposures to multiple chemicals are a threat to human health present formidable challenges to risk assessment. Consequently, there is also a significant problem in communicating risk to the public.

It is no surprise that expert opinions about the topic are divided. In their recent *A European Environment and Health Strategy*, the European Commission has taken a cautious stance: "Even low level exposure over decades to a complex cocktail of pollutants in air, water, food, consumer products and buildings can have a significant effect on the health status of European citizens" [Commission of the European Communities (CEC) 2003]. An alternative view has been expressed by the European Crop Protection Association: "As a matter of fact, presently available data on exposure to mixtures of chemicals at doses well below the NOAELs [no observed adverse effect levels] of the individual constituents indicate that such exposure is of no health concern" (Carpy et al. 2000).

It has been argued that risks associated with low-level exposure to multiple chemicals cannot be assessed without considering the mode of action of the agents that make up the "cocktail of pollutants." According to this view, recently expressed by the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT 2002), a distinction should be made between similarly and dissimilarly acting agents. Similarly acting agents are assumed to show "dose additivity" over the entire dose range, including doses in the range of NOAELs. This suggests that combination effects are to be expected even at doses below NOAELs. In contrast, "adverse reactions" are assumed to be unlikely with mixtures of dissimilarly acting agents, when these are combined at doses below NOAELs. In view of the diversity of "real world" mixtures composed of numerous different chemicals with a multitude of different modes of action, it is suggestive to regard dissimilar action of mixture components as the default scenario. Consequently, so the presumption, mixtures pose no health concern, as long as the doses of each component stay below NOAELs (COT 2002; Feron et al. 1995).

This view is based on two premises: first, that NOAELs are a good approximation of "safe" doses of pollutants; and second, that the distinction between "similarly" and "dissimilarly" acting chemicals in a mixture is straightforward and of relevance to the risk assessment issue at hand. In this article, we review experimental studies that address the issue of mixture effects at low doses, for both similarly and dissimilarly acting chemicals. Because of the fundamental nature of the topic, we have not only reviewed studies with mammals, but have extended the scope to work with other organisms, such as fish, invertebrates, and microorganisms. This approach is justified because key toxicodynamic principles that govern the ways in which chemicals act in mixtures remain similar, regardless of organisms. On the other hand, care has to be exercised when making comparisons between *in vitro* and *in vivo* assays. To capture the effects of interacting pathways in mixture toxicology, the analysis of apical end points is often essential. Although many assays relevant to ecotoxicology easily lend themselves to such analyses, this is more complicated in mammalian toxicology, where emphasis is often on organ-specific toxicity.

Concepts and Terminology

Similar modes of action. It is well recognized that certain chemicals exert effects through similar modes of action. Examples include polychlorinated dioxins and furans (PCDD/Fs), to name but a few (van den Berg et al. 1998). Because these chemicals interact with well-defined molecular targets, it is thought that the same effect can be provoked by replacing one chemical with an equivalent dose of another. To deal with mixtures of such agents, the concept of dose addition (often also referred to as concentration addition) has been developed (Loewe and Muischnek 1926). Dose addition assumes that one chemical can be replaced totally or partly by an equal fraction of an equi-effective concentration of another, without changing the overall combined effect (see Table 1 for the mathematical formulation of this principle). If the assumption of dose addition holds true, these fractions of equi-effective concentrations, which are also called toxic units, sum up to a value of 1—therefore the name dose or concentration addition. A widely used application of dose addition is the "toxicity equivalence factor"

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(TEF) concept for the assessment of mixtures of PCDD/Fs (van den Berg et al. 1998). Under the additional assumption of parallel dose–response curves, doses of specific PCDD/F isomers are all expressed in terms of the dose of a reference chemical, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), needed to induce the same effect ("equivalent" or "equieffective" dose), and assessment of the resulting combined effect is obtained simply by adding up all equivalent TCDD doses.

The concept of dose addition implies that every toxicant in any concentration contributes, in proportion to its toxic unit, to the overall toxicity of a mixture. Whether the individual doses are also effective alone does not matter. Thus, combination effects should also result from toxicants at or below no observed effect levels (NOELs), provided sufficiently large numbers of components sum up to a suitably high total effect dose.

Dissimilar modes of action. By activating differing effector chains, every component of a mixture of dissimilarly acting chemicals is thought to contribute to a common effect independent of all other agents in the mixture. By adopting the statistical concept of independent events for this situation, the resulting combined effect is assumed to be calculable from the effects caused by the individual mixture components, if present alone in the same concentration or dose as in the mixture (Bliss 1939) (for mathematics, see Table 1). This means that agents present at doses below effect thresholds (i.e., zero effect levels) will not contribute to the joint effect of the mixture, and if this condition is fulfilled for all components there will be no combination effect. This central tenet of the concept of independent action (often also called response addition) is commonly taken to mean that exposed subjects are protected from mixture effects as long as the doses of all agents in the combination do not exceed their NOAELs.

In practice, these two concepts are not always used in a mutually exclusive way, but rather according to circumstance and specific context. Especially when data for a mixture of interest are not available (which is often the case), certain default practices have to be used to bridge knowledge gaps, as is the case with the hazard index (based on dose addition), which is used as a screening tool to assess the potential for health hazards resulting from exposure to multiple chemicals in Superfund sites. For cancer risks, methods based on independent action are often employed. These and other details are described in key U.S. Environmental Protection Agency documents (U.S. EPA 1986, 1989, 2000).

Hypothesis Formulation and Experimental Design

We assess whether the above expectations are borne out by published experimental evidence. In view of received expert opinion, we put particular emphasis on examining the hypothesis that combinations of dissimilarly acting chemicals do not show mixture effects if the doses of all its components stay below their individual NOAELs. Before reviewing the relevant literature, however, it may be helpful to consider some requirements that must be met to ensure that experiments addressing the issue at hand are conclusive.

First, NOAELs for each mixture component should be estimated using the same assay system (and end point) chosen for the mixture study, under identical experimental conditions. Studies that fail to meet this requirement run the risk of administering mixture components at doses higher than NOAELs, in which case the experiment would miss the point entirely.

Alternatively, researchers may have to face the possibility that doses smaller than NOAELs were delivered. In this case, it is essential to consider the statistical power of the chosen experimental arrangement.

If dose addition applies, the expectation is that mixture effects may occur when the components are combined at doses equal to or below their NOAEL. To ensure that a low-dose mixture experiment is conclusive in this case, it becomes important to ascertain that an anticipated combination effect is sufficiently large to reach statistical significance, without violating the precondition that no single mixture component should exceed its NOAEL. The magnitude of the expected combination effect depends on factors such as number of mixture components, their concentration in the mixture, and the steepness of the dose–response curves of individual components (Drescher and Boedeker 1995). It would be trivial to attempt an experiment where, for example, two agents are combined at 1/100 of their individual NOAEL. The resulting mixture effect, although existing, would be too small to be detectable.

If independent action is valid, the situation is more problematic. In this case, the hypothesis is that combination effects should not occur if all mixture components are present at levels below their NOAEL. At the same time, the assumption is made that single doses below NOAEL do not induce effects. However, it is impossible to prove the latter proposition, because apparent absence of mixture effects always leaves the doubt that small, albeit statistically insignificant, effects may have been overlooked. Therefore, an experiment that conclusively proves this hypothesis is not easily designed, essentially because the hypothesis is formulated in the negative. As a matter of logic, negatives cannot be proven, and in this case, we must seek examples that falsify the hypothesis. A viable procedure in this case would be to first estimate NOAELs, and then to carry out regression analysis of the underlying dose–response function to obtain statistical estimates of effects associated with NOAELs. These can then be used to calculate an anticipated mixture effect under the independent action assumption. For the experiment to be conclusive, the expected mixture effect must reach statistical significance. Because of their greater statistical power, studies involving cell lines or microorganisms can be valuable tools to produce conclusive evidence.

Table 1. Mathematical formulation of concepts for predicting the toxicity of chemical mixtures.

	Dose addition (or concentration addition)	Independent action (also called response addition)
Binary mixtures	$\frac{c_1^*}{ECx_1}+\frac{c_2^*}{ECx_2}=1$	$E(c_{mix})=E(c_1)+E(c_2)-E(c_1)\bullet E(c_2)$
Multicomponent mixtures	$\sum_{i=1}^{n}\frac{c_i^*}{ECx_i}=1$	$E(c_{mix})=1-\prod_{i=1}^{n}\left[1-E(c_i)\right]$
Transformations for the prediction of effect concentrations (EC)[a]	$ECx_{mix}=\left(\sum_{i=1}^{n}\frac{p_i}{F_i^{-1}(x_i)}\right)^{-1}$	$X=1-\prod_{i=1}^{n}\left[1-F_i\left(p_i\bullet\left[ECx_{mix}\right]\right)\right]$

Notation: c_i = individual concentration of substance i in a mixture with n components (i = 1...n); c_i^* = individual concentration of substance i in a mixture eliciting the definite total effect X; c_{mix} = total concentration of substances 1...n in the mixture ($c_{mix} = c_1 + c_2 ... + c_n$); ECx_i = the concentration of substance i that causes the effect X if applied individually; ECx_{mix} = the total concentration of substances 1...n in a mixture that causes the total effect X and contains the mixture components in a given concentration ratio $p_1 : p_2 ... : p_n$; $E(c_i)$ = individual effect of substance i if present in the concentration c; $E(c_{mix})$ = total effect of the mixture with the total concentration c_{mix} if the mixture components are present in the concentration ratio $p_1 : p_2 ... : p_n$; X = definite value for the effect E; p_i = relative proportion of substance i expressed as a fraction of the total concentration of substances in the mixture ($p_i = c_i / c_{mix}$); F_i = concentration response function of substance i. Effects E denote the relative intensity or frequency of a response parameter (defined as fraction of a maximum possible value) and thus can only take values between 0% and 100%:$0 \le E \le 1$. If effects E are not considered as a function of concentrations c but of doses d, all formulas apply in an equivalent way (all c replaced by d).

[a]See Faust et al. (2003) for a full explanation of transformations.

Methods and Quality Criteria

Only papers published in international peer reviewed journals were considered for this review. Findings on joint effects of multicomponent mixtures at low doses or concentrations

of individual chemicals are listed in Tables 2–6 and discussed in the following sections. Many of these studies were designed for an assessment of observed joint effects in terms of agreement or disagreement with predictions based on the concept of DA (dose addition or concentration addition) or on the alternative concept of IA (independent action). Results of these comparisons are documented in Tables 2–5 as reported in the papers by using the following symbols: " = " indicates almost perfect agreement between observation and prediction; " ≈ " indicates that the observed joint effect differed slightly from the prediction, but under consideration of experimental errors this difference appeared to be insignificant; and " < " or " > " indicates that the observed joint effect was significantly smaller or greater than expected by the given predictive concept, respectively.

All studies were checked for compliance with a set of five different quality criteria for low-dose mixture studies, which can be derived from the preceding considerations on concepts and experimental design. Quality criteria fulfilled by the studies are indicated in Tables 2–6 by using the following letter codes: A: toxicity of individual mixture components was experimentally determined under identical conditions as the mixture [otherwise, estimates were derived from quantitative structure–activity relationship (QSAR) models or taken from the literature]; B: stability of test concentrations under test conditions was checked by analytical methods (does not apply to animal experiments with direct dosing); C: uncertainty of experimentally determined effects, effect concentrations, or effective doses was estimated by statistical methods; D: uncertainty of mixture toxicity predictions was estimated by statistical methods; and E: no observed effect concentrations (NOECs) or NOELs were determined for every individual substance, and individual concentrations or doses resulting in the given joint effect were demonstrated to be at or below these NOECs or NOELs, or insignificance of individual effects was demonstrated by other statistical approaches.

Codes given in parentheses indicate that the criterion was only partly fulfilled. Where quality criteria are not listed in the tables, the corresponding information was either not available from the original article cited or the criterion was not applicable to the specific type of study.

Results and Discussion

Early studies with unspecifically acting organic chemicals. In the 1980s, a series of studies of the effects of multicomponent mixtures of unspecifically acting organic chemicals on fish and other aquatic organisms was published (Table 2). Könemann (1980) combined 50 agents at concentrations of 2% of their median lethal concentration (LC_{50}) for fish and observed a joint mortality of 50%. Evaluating a broader range of end points, Hermens et al. (1984, 1985) and Broderius and Kahl (1985) were able to demonstrate strong mixture effects in experiments with 21–50 chemicals on daphnids, fish, and marine bacteria. In all these studies, a joint effect of 50% was observed when the mixture components were administered at concentrations equivalent to 2.4–9.6% of their individual median effective concentration (EC_{50}). In view of the evidence of the steepness of the concentration–response relationships of unspecifically acting organics in acute aquatic toxicity assays provided by Broderius and

Table 2. Significant joint effects of similarly acting toxicants at low concentrations: I. Evidence from early studies on the aquatic toxicity of mixtures of non-reactive organics with an unspecific "narcotic" mode of action.

Reference	Organism (species)	End point (exposure time)	No. of mixture components	Individual concentrations (% of EC_{50})	Joint effect (%)	Comparison with predictions[a]	Quality criteria fulfilled[a]
Könemann 1980	Fish (*Poecilia reticulata*)	Mortality (7 or 14 days)	50	2	50	= DA	A
Hermens et al. 1984	Waterfleas (*Daphnia magna*)	Immobilization (48 hr)	50	2.4[b]	50	≈ DA	(A),[c] B, (C, D)[d]
Hermens et al. 1984	Waterfleas (*Daphnia magna*)	Mortality and inhibition of reproduction (16 days)	25	6[b]	50	< DA	(A),[c] B, (C, D),[d] (E)[e]
Broderius and Kahl 1985	Fish (*Pimephales promelas*)	Acute mortality (96 hr)	21	5.9[b]	50	≈ DA	A, B, C
Hermens et al. 1985	Marine bacteria (*Vibrio fischeri*)[b]	Bioluminescence inhibition (15 min)	21	9.5[b]	50	< DA	A

[a]See explanation in "Methods and Quality Criteria." [b]Recalculated from the sum of toxic units reported in the article. [c]Individual EC_{50} values were determined experimentally for part of the components and estimated by a QSAR model for the remaining compounds. [d]Uncertainty in the comparison of observed and predicted mixture toxicity was assessed on the basis of a fixed estimate for the error in individual effect concentrations. [e]NOECs were determined for 5 out 25 mixture components; from the data reported in the paper it can be recalculated that in the case of these 5 substances 6% of the EC_{50} is always a concentration that is definitely lower than the corresponding NOEC. [f]Formerly *Photobacterium phosphoreum*.

Table 3. Significant joint effects of similarly acting toxicants at low concentrations: II. Evidence from studies on groups of substances with a common specific mechanism of action in mammalians or unicellular organisms.

Reference	Organism (species)	End point (exposure time/route)	Mixture components (mechanism of action)	Individual doses or concentrations	Joint effect	Comparison with predictions[a]	Quality criteria fulfilled[a]
Jonker et al. 1996	Rats (female Wistar rats)	Kidney toxicity examined by 40 different functional and morphological parameters (32 days/daily by oral gavage)	4 similarly acting nephrotoxicants (selective renal toxicity ascribed to a common bioactivation pathway following conjugation to glutathione)	Presumed NOEL (= 1/4 LOEL)	Increased kidney and liver weights; (other parameters did not show significant joint effects)	(= DA)[b]	A, C
Backhaus et al. 2000	Marine bacteria (*Vibrio fischeri*)	Bioluminescence inhibition (24 hr)	10 quinolone antibiotics (inhibition of bacterial DNA gyrase)	NOEC	99%	= DA, > IA	A, B, C, E
Faust et al. 2001	Algae (*Scenedesmus vacuolatus*)	Inhibition of reproduction (24 hr)	18 *s*-triazine herbicides (inhibition of photosynthetic electron transport)	4.7–60% of NOEC[c]	47%	≈ DA, > IA	A, B, C, E
Arrhenius et al. 2004	Natural marine microalgal communities (numerous species)	Photosynthesis inhibition (45 min)	12 phenylurea herbicides (inhibition of photosynthetic electron transport)	≤ NOEC[d]	28% and 37% (2 different communities)	≈ or < DA, > IA	A, B, C, E

LOEL, lowest observed effect level.
[a]See explanation in "Methods and Quality Criteria." [b]Qualitative assessment only referring to the fact that combined exposure to individual NOELs resulted in significant joint effects. In contrast to the other studies listed, experiments were not designed for a quantitative comparison between prediction and observation in terms of intensity or frequency of joint effects. [c]All mixture components were present at individual concentrations that were statistically estimated to exert mean individual effects of 1% only. These individual EC1 values were demonstrated to equal 4.7–60% of individual NOECs. [d]Mixture components were present at statistically estimated individual EC1 concentrations. These were demonstrated to be smaller or at most equal to individual NOECs.

Kahl (1985), it seems reasonable to assume that these concentrations were below the NOEC of each chemical. However, the validity of this assumption was confirmed by actual determinations of NOEC values in just one of these studies and for only five of the mixture components (Hermens et al. 1984). For all the other substances and studies this ultimate proof is missing. It is therefore necessary to consider mixture studies where NOEL/NOEC estimates for every mixture component were provided explicitly.

Mixtures composed of agents with similar specific modes of action. Table 3 shows a compilation of low-dose mixture experiments involving agents with a common specific mode of action. Jonker et al. (1996) tested the dose additivity assumption with a mixture of four nephrotoxicants—tetrachloroethylene, trichloroethylene, hexachloro-1,3-butadiene, and 1,1,2-trichloro-3,3,3-trifluoropropene—administered to female rats. All four chemicals produce kidney toxicity through a pathway involving conjugation to glutathione. Increased kidney and liver weights were observed in rats that received the agents at 25% of their individual lowest observed nephrotoxic effect level, which the authors presumed to be equivalent to NOELs. This study is suggestive of combination effects at doses around NOELs, but it suffers from a lack of proof that the chosen doses were indeed NOELs.

Backhaus et al. (2000), Faust et al. (2001), and Arrhenius et al. (2004) have presented mixture studies on marine bacteria, algae, and algal communities where combinations of chemicals were selected according to very strict similarity criteria. The mixtures included 10 quinolone antibiotics (inhibitors of bacterial DNA gyrase), 18 *s*-triazines, and 12 phenylurea herbicides (inhibitors of photosynthetic electron transport). NOECs were estimated by using Dunnett's test, and all agents were administered at concentrations equal to or below their individual NOECs. In all cases, significant mixture effects ranging from 28 to 99% of a maximally possible effect were observed, and these effects could be predicted quite accurately by application of the dose addition concept.

Table 4 lists studies with different groups of endocrine-active chemicals that show evidence for joint effects in the low-dose range. Silva et al. (2002) have assessed the effects of eight xenoestrogens in a yeast reporter gene assay based on estrogen receptor alpha. All chosen chemicals were able to bind to and activate the estrogen receptor alpha. NOECs were estimated by using Dunnett's test (Rajapakse et al. 2002), and joint effects of up to 40% of a maximal estrogenic effect were seen at concentrations around or below NOECs. Again, the observed combined effects agreed well with the additivity expectation of dose addition. Tinwell and Ashby (2004) analyzed mixtures of eight estrogenic chemicals in the rat uterotrophic assay. Combinations of all agents at doses that gave no significant responses when tested individually produced quite strong uterotrophic effects. Very recent mixture experiments with five estrogenic chemicals in fathead minnows (*Pimephales promelas*) presented by Brian et al. (2005) also demonstrated combination effects at concentrations that individually did not induce a significant response. The induction of the egg yolk protein vitellogenin, an estrogen receptor-mediated response, matched the dose addition expectation. Crofton et al. (2005) conducted an in-depth study of a mixture of 18 polyhalogenated hydrocarbons [2 PCDDs, 4 PCDFs, and 12 coplanar and noncoplanar polychlorinated biphenyls (PCBs)] where

Table 4. Significant joint effects of similarly acting toxicants at low concentrations: III. Evidence from studies with different groups of endocrine-active chemicals.

Reference	Organism and/or assay (species)	End point (exposure time/route)	Mixture components	Individual doses or concentrations	Joint effect	Comparison with Predictions[a]	Quality criteria fulfilled[a]
Silva et al. 2002	YES: recombinant yeast estrogen screen (*Saccharomyces cerevisiae* genetically modified to express the human estrogen receptor)	Estrogen receptor activation (72 hr)	8 xenoestrogens	43–100% of NOEC[b]	Significant estrogenic activity	= DA	A, C, E
Tinwell and Ashby 2004	Rats, uterotrophic assay (immature female AP rats)	Uterine weight increase (3 days/daily by subcutaneous injection)	8 estrogens and xenoestrogens	≤ NOEL[c]	Significant uterotrophic activity		A, C, E
Brian et al. 2005	Fish (male *Pimephales promelas*)	Vitellogenin induction (14 days)	5 estrogens and xenoestrogens	≤ NOEC[c]	Significant vitellogenin induction (~ 50% of maximum possible effect)	≈ DA	A, B, C, D, E
Crofton et al. 2005	Rats (young female Long Evans rats)	Decrease of serum total T_4 concentrations (4 days/daily by oral gavage)	18 thyroid-disrupting chemicals	≤ NOEL	Significant T_4 decrease	DA[d]	A, C, D, E

T_4, thyroxine.
[a]See explanation in "Methods and Quality Criteria." [b]Individual concentrations equalled 50% of statistically estimated individual EC1 values. These concentrations were demonstrated to equal 43–100% of individual NOECs. [c]Tests were not designed for conventional NOEL or NOEC determinations. However, individual doses or concentrations in the mixture were demonstrated to provoke no effects significantly different from untreated controls (i.e., they must have been ≤ NOEL or NOEC). [d]Dose-dependent additivity and synergism.

Table 5. Significant joint effects of dissimilarly acting toxicants at or below individual NOECs.

Reference	Organism or cell type (species)	End point (exposure time)	Mixture components	Individual concentrations	Joint effect	Comparison with predictions[a]	Quality criteria fulfilled[a]
Hermens et al. 1985	Fish (*Poecilia reticulata*)	Mortality (14 days)	33 aquatic pollutants from 3 groups with probably different modes of action	4% of EC_{50} (assumed to be below NOEC)	50%	≈ DA or < DA[b]	A
Payne et al. 2001	MCF-7 human breast cancer cells	Stimulation of cell proliferation (7 days)	4 persistent organochlorine pesticides exerting effects on cell proliferation in different ways	25–100% of NOEC[c]	Significant proliferative effect	= DA, = IA[d]	A, C, E
Walter et al. 2002	Algae (*Scenedesmus vacuolatus*)	Inhibition of reproduction (24 hr)	11 aquatic priority pollutants selected for structural diversity by chemometric analysis	NOEC	64%	< DA, ≈ IA	A, B, C, E
Faust et al. 2003	Algae (*Scenedesmus vacuolatus*)	Inhibition of reproduction (24 hr)	16 toxicants known to interact with completely different molecular target sites in algae	6.6–66% of NOEC[e]	18%	< DA, ≈ IA	A, B, C, D, E

[a]See explanation in "Methods and Quality Criteria." [b]Observed mixture toxicity was slightly lower than predicted by DA, but significance of the difference was not assessed by statistical means. [c]Recalculated from individual concentrations and NOECs reported in the study. [d]Both predictive concepts, DA and IA, gave nearly identical and accurate predictions. [e]Mixture components were present at statistically estimated individual EC1 concentrations. These were demonstrated to equal 6.6–66% of individual NOECs.

young female rats were treated for 4 days. Altered serum total thyroxine levels were recorded, and the mixture ratio was chosen to be proportional to the levels of the chemicals reported in breast milk, fish, and other human food sources. There was no deviation from dose additivity at the lowest tested doses of the mixture, but at higher test doses the additivity model underpredicted the empirical effects by a factor of 2–3. Significant joint effects were observed at doses of the individual mixture components equivalent to their individual NOELs, or even below.

All these studies were well designed to address the issue of combination effects at low doses. Taken together, there is very good empirical support for the notion that chemicals with a similar mode of action may produce combination effects at doses below NOEL/NOEC. Is the same true for mixtures composed of chemicals with dissimilar modes of action?

Experimental studies providing evidence for mixture effects of dissimilarly acting chemicals at low doses. There is evidence that dissimilarly acting agents, when combined at doses below their NOAELs, may also produce significant mixture effects (Table 5).

In an early study Hermens et al. (1985) combined 33 chemicals that can be grouped into three classes with presumably differing modes of action. The mixture produced 50% mortality in fish when all components were present at 4% of their individual EC_{50}. It was assumed that these concentrations were below NOECs, although NOECs were not estimated in this study. It is therefore conceivable that some chemicals may have been present at levels above their NOECs, and this point may be particularly relevant with compounds that exhibit shallow dose–response curves. These weaknesses have been overcome in later studies of mixture toxicity from multicomponent mixtures of dissimilarly acting chemicals.

In a study using a cell-proliferation assay with human breast cancer MCF-7 cells, Payne et al. (2001) tested a mixture of two estrogen receptor agonists [*o,p′*-dichlorodiphenyltrichloroethane (DDT), *p,p′*-DDT], one anti-androgenic agent (*p,p′*-dichlorodiphenyldichloroethene) and a chemical that induces cell division by as yet poorly defined mechanisms (β-hexachlorocyclohexane). A significant proliferative effect was observed when these chemicals were present at concentrations equivalent to 25–100% of their individual NOECs. Independent action and dose addition predicted the observed effect equally well.

Walter et al. (2002) assessed the effect of a mixture of 11 aquatic priority pollutants on algal reproduction. The chemicals were selected for structural diversity by using chemometric methods, and their NOECs estimated by hypothesis testing methods. In this study, statistical estimates of effect concentrations lower than the corresponding NOECs were derived by regression analysis of concentration response data, down to effect levels of 1%. Based on these estimates of low effects, independent action yielded quite accurate predictions of mixture toxicity. Combined at their NOECs, the pollutants produced a joint effect of 64%.

All these studies used groups of similarly acting chemicals, where each group had a different presumed mode of action. Often, dissimilarity was inferred on the basis of diverse chemical structures, but proof of dissimilar action could not be provided because the actual mechanisms involved were unclear. There is the possibility that many of these experiments in fact used chemicals that at least partly acted in similar ways. Thus, there is a

Table 6. Rat studies providing no strong evidence for significant joint effects of dissimilarly acting toxicants at or below individual NOELs.

Reference	No. and type of rats	End point (exposure time/route)	Mixture components	Individual doses	Joint effects	Authors' conclusions	Quality criteria fulfilled[a]
Jonker et al. 1990	10 male and 10 female Wistar rats per dose group	Hematology, clinical chemistry, urinalysis, and pathology examined by 76 parameters (4 weeks/via diet)	8 diverse chemicals, arbitrarily chosen	1/10 NOAEL 1/3 NOAEL NOAEL	No clearly treatment-related effects No clearly treatment-related effects Slight increase in relative kidney weights and decrease of hemoglobin in males; swollen or dark livers in 3/10 males; no other clearly treatment-related effects	"Some, but no convincing evidence for an increased risk from exposure to a combination of chemicals when each chemical is administered at its own individual NOAEL"	A, C, E
Jonker et al. 1993	10 male and 10 female Wistar rats per dose group	Hematology, clinical chemistry, urinalysis, and pathology examined by 45 parameters (4 weeks/via diet)	4 kidney toxicants damaging epithelial cells of the proximal tubules by different mechanisms	1/4 NNEL NNEL	No clearly treatment-related effects Slight growth retardation in males; findings on increased relative kidney weights and epithelial cells in urine in males were inconclusive	"Simultaneous administration of the four nephrotoxins at their NNEL produced only weak indications of increased toxicity"	A, C, E
Ito et al. 1995	19 or 18 male F344 rats per dose group	Enhancement of liver preneoplastic lesion development initiated by DEN (6 weeks, via diet)	20 pesticides not classified as carcinogens and permitted for use in Japan	ADI[b] 100 x ADI[b]	No effect Enhanced development of preneoplastic lesions	"The present safety factor approach is appropriate for the risk evaluation of environmental chemicals"	C
Groten et al. 1997	8 male Wistar rats per dose group	Hematology, clinical chemistry, biochemistry, and pathology examined by 47 parameters (4 weeks/inhalatory and via diet)	9 chemicals with diverse MoA, relevant to the general human population in terms of use pattern and exposure	1/3 NOAEL NOAEL	Increase in relative kidney weights Hyperplasia and metaplasia of nasal epithelium, hepatocellular hypertrophy, decreased plasma triglyceride concentrations, altered ALP enzyme activities, increased relative kidney weights	"Simultaneous exposure to the nine chemicals does not constitute an evidently increased hazard …, provided the exposure level of each chemical in the mixture is at most similar to or lower than its own NOAEL"	A, B, C, E
Wade et al. 2002	10 sexually mature male Sprague-Dawley rats (9 controls)	General physiology, liver, reproductive organs and immune system examined by 54 parameters (70 days/by gavage daily)	18 contaminants of human reproductive tissues with diverse MoA	TCDD ≤ NOAEL,[c] other 17 toxicants at MRL, RfD, TDI, or PTDI levels	No adverse effects	"MRLs, TDIs, or RfD … provide adequate protection for adult male animals, for those systems examined"	C, (E)

Abbreviations: ALP, alkaline phosphatase; ALAT, alanine aminotransferase; DEN, diethylnitrosamine; MoA, mode(s) of action; MRL, maximum residue level estimated by ATSDR (Agency for Toxic Substances and Disease Registry of the U.S. Department of Health and Human Services); NNEL, no nephrotoxic effect level; PTDI, provisional tolerable daily intake established by Health Canada (Wade et al. 2002); RfD, reference dose established by U.S. EPA (Wade et al. 2002); TDI, tolerable daily intake established under the Canadian Environmental Protection Act (Wade et al. 2002).

[a]See explanation in "Methods and Quality Criteria." [b]ADIs are based on NOAELs for noncarcinogenic effects, provided by the Japanese Ministry of Health and Welfare (Ito et al. 1995) or taken from a FAO/WHO report (Ito et al. 1995). [c]NOAEL not determined in the study, but taken from the literature.

need to consider studies that have employed very strict criteria for dissimilar action.

A diverse mixture of 16 chemicals, all known to specifically interact with different target sites in algae, was assessed for inhibition of reproduction in algae by Faust et al. (2003). When these chemicals were combined at concentrations equivalent to 6.6–66% of their NOECs, a combined effect of 18% was observed. Similar to the approach taken by Walter et al. (2002), estimates of low effects, down to 1%, were produced by regression analysis of concentration–response data of individual chemicals. These estimates were used to calculate mixture effect predictions according to independent action. This yielded fairly accurate predictions of the observed combination effects, although dose addition fell well short of observations. Similar results were obtained with a mixture of specifically dissimilarly acting chemicals in bacterial systems (Grimme et al. 1998).

In demonstrating that dissimilarly acting chemicals too have the propensity to produce significant mixture effects when combined at levels below NOECs, these studies contradict received expert opinion and falsify the hypothesis we set out to examine. However, before we continue, let us review the papers often quoted (COT 2002) in support of the notion that mixtures of dissimilarly acting chemicals are safe at doses below NOAELs. The relevant studies are listed in Table 6.

Weak or lacking evidence of low-dose combination effects with dissimilarly acting agents? The first of these studies was published by Jonker et al. (1990), who prepared mixtures of eight arbitrarily chosen chemicals which they fed to rats. Each chemical affected a different target organ, by differing modes of action. In one mixture, the agents were combined at doses equivalent to their NOAEL, and two further mixtures representing 1/3 and 1/10 NOAEL were investigated. Rats exposed to the NOAEL mixture for 4 weeks showed darkened livers, decreased hemoglobin levels, and increased kidney weights. The experiment with the 1/3 NOAEL mixture yielded increased kidney weights, which the authors interpreted as "chance finding." No effects became apparent with the 1/10 NOAEL mixture. Although the authors concluded that there was "some, but no convincing evidence for an increased risk from exposure to a combination of chemicals when each chemical is administered at its own individual NOAEL," it is debatable whether the NOAEL and 1/3 NOAEL mixtures were entirely devoid of effects. In fairness to the authors, however, it is important to point out that the chosen end points are quite difficult to quantify.

Jonker et al. (1993) also examined a mixture of toxicants that act by differing mechanisms but affect the same target organ. This mixture included four different kidney toxicants. The chemicals were combined at doses presumed to be NOAELs on the basis of range finding tests, and at 1/4 of NOAELs. Rats exposed to the NOAEL combination experienced slight growth retardations, increased relative kidney weights and elevated numbers of epithelial cells in their urine. However, rats given one of the individual chemicals at doses equal to the presumed NOAEL showed similar effects. Thus, at least one dose higher than its actual NOAEL was used in the mixture experiment. The combination of 1/4 of NOAEL did not provoke significant observable effects.

Ito et al. (1995) explored the effects of 19 organophosphates and one organochlorine on the formation of preneoplastic lesions in the livers of rats pre-treated with the liver carcinogen diethylnitrosamine (DEN). The 20 chemicals were combined at doses equivalent to their acceptable daily intakes (ADI) and to 100 times their ADI. There were increased preneoplastic lesions with the 100-times-ADI mixture, but the ADI mixture did not induce observable effects. None of the selected chemicals were tested individually, and the doses in this study were based on ADI values proposed by the Japanese government, reflecting a diversity of end points. Thus, it is impossible to assess how close the doses of the chemicals in the two mixtures were to their NOAELs for preneoplastic lesions. It cannot be ruled out that the individual doses in the ADI mixture were far below their NOAELs; therefore, even in combination, significant effects might not be expectable. On the other hand, it is likely that some of the chemicals in the 100-times-ADI mixture exceeded their individual NOAELs (in relation to preneoplastic lesions). This might explain why effects were seen with this mixture.

Groten et al. (1997) selected nine chemicals with differing target organ toxicity and modes of action and exposed rats to two combinations. A mixture composed of doses equivalent to the NOAELs of each chemical produced increased relative kidney weights, hepatocellular hypertrophy, and hyperplasia of nasal epithelial cells. Administered at 1/3 of their NOAELs, the nine chemicals induced increased relative kidney weights. This study would suggest that there were effects in the low dose range. The authors' conclusion that "simultaneous exposure to the nine chemicals does not constitute an evidently increased hazard …, provided the exposure level of each chemical in the mixture is at most similar to or lower than its own NOAEL" may have to be tempered in the light of a discussion about the toxicological relevance of the observed effects.

The effects on rats of mixtures of 18 organochlorine pesticides and environmental contaminants, including TCDD, were analyzed by Wade et al. (2002). The animals were exposed for 70 days to a combination of all agents at their respective maximum residue level (MRL) or ADI level. This ADI mixture failed to produce observable effects. However, this experiment is difficult to interpret because none of the chemicals were tested individually and information about their NOAELs in relation to the end points examined is missing. Given that only 10 animals per group were used, it is likely that the study was of relatively low statistical power. A combination equivalent to doses 10 times higher than those in the ADI mixture was also examined and decreases in epididymus weights were observed. However, TCDD alone, at the dose present in the mixture, produced the same effect. This indicates that the observed effects were attributable solely to TCDD, and that the contribution of all other chemicals to the overall joint effect was negligible.

Although some of the studies in Table 6 provide evidence for combination effects (Groten et al. 1997; Jonker et al. 1990), the apparent absence of effects in the remaining papers can be explained in terms of insufficient statistical power or flawed selections of dose levels. The question arises as to whether the outcome of these studies is in conflict with the experimental work demonstrating clear mixture effects with dissimilarly acting chemicals below NOAEL or NOEL (Table 5). To address this point we have to turn to theory.

Theory expectations. The formulas in Table 1 express that under the assumption of independent action a mixture effect will not occur if all components fail to induce an effect. Especially with mixtures composed of a very large number of components, this proposition forces clear distinctions between zero effects and small, albeit statistically insignificant effects:

Consider the hypothetical case of 100 chemicals, all present at zero effect levels. With the formula in Table 1, the expected mixture effect is

$$E_1 = 1 - [(1 - 0)^{100}], \qquad [1]$$

which indeed resolves to zero. If, however, these 100 chemicals all produce an effect of 1%, we obtain

$$E_2 = 1 - [(1 - 0.01)^{100}], \qquad [2]$$

which equals 0.63, or 63% of a maximally inducible effect. Should each of the 100 agents produce an effect of only 0.1%, the expected combined response will be 9.5%.

Therefore, to assess whether fairly large combination effects can be expected from multicomponent mixtures, even if all components are only present at low doses, very small

effects of the individual components need to be detected. However, with most *in vivo* bioassays it is very difficult to demonstrate reliably an effect of 1%, let alone effects < 1%. In environmental toxicology, usually effects of between 10 and 30% cannot be distinguished with certainty from control responses (Moore and Caux 1997; U.S. EPA 1991), and a reanalysis of developmental toxicity bioassays yielded statistical detection limits equivalent to effects of about 5–20% on average (Allen et al. 1994).

In view of these difficulties, it has long been acknowledged that no effect levels (NELs) (in the strict sense of zero effect levels) cannot be determined empirically. As a way out of this dilemma, NOELs and later NOAELs were introduced with the intention to provide an approximation of zero effect levels (Zbinden 1979). Since then, NOAELs have become the linchpin of statutory chemicals risk assessment in the European Union. How reliable are NOAELs as approximations of zero effect levels?

NOAELs are not zero effect levels. The studies listed in Table 5 demonstrate the induction of significant mixture effects with dissimilarly acting chemicals combined at levels below their NOEL. Because theory predicts that combination effects should not occur when all mixture components are present at zero effect levels, the doses used in these studies must have exceeded zero effect levels. If this is correct, it follows that even fractions of NOELs and NOAELs are not zero effect levels. Alternatively, the chemicals in these mixtures, although presumed to be dissimilarly acting, were in fact acting similarly, and the reason why combination effects were seen lies in the well-established ability of similarly acting agents to produce mixture effects below dose thresholds. Although this latter possibility cannot entirely be ruled out as an explanation for the outcome of the first two studies in Table 5 (Hermens et al. 1985; Payne et al. 2001), its relevance appears to be negligible in the case of Walter et al. (2002) and Faust et al. (2003), who have employed the best available dissimilarity criteria during the selection of mixture components.

Nevertheless, it remains that NOAELs are not to be equated with true NELs. Although familiar to statisticians (Chapman et al. 1996; Moore and Caux 1997), this notion may be confusing to others. It should be borne in mind, however, that NOAELs are derived by hypothesis-testing procedures. These examine whether the null hypothesis "controls and treated groups do not differ" can be rejected. NOAELs are the highest tested dose that did not produce a statistically significant effect. Only when the responses in the treated group exceed a certain limit (defined by significance criteria) can the hypothesis be rejected, and consequently, the tested dose is deemed larger than NOAEL. However, this cannot be taken to mean that NOAELs are devoid of effects. At and below NOAELs, effects may either be truly absent or remain undetected, due to lack of statistical power. Therefore, rather than being a genuine reflection of zero effects, NOAELs ("one of the most misunderstood notions in ecotoxicology"; Moore and Caux 1997) define a gray area, where it is impossible to distinguish whether effects are present or not. This realization has led to harsh criticism by the European Commission (EC 1996) and has motivated the search for alternatives to NOAELs—for example, the benchmark approach (Crump 1984).

Thus, the view that mixtures of dissimilarly acting chemicals are "safe" at doses below NOAEL does not only lack empirical support, it is also based on the erroneous assumption that NOAELs are indeed zero effect levels. How should risk assessment and regulation take account of this insight?

Joint effects from low-dose combinations. Both under the assumptions of dose addition and independent action combination effects may result from chemicals that each produce very small effects, if they are present in large numbers. In current regulatory practice NOAELs are combined with so-called safety factors, to derive ADIs. The safety factors are intended to deal with statistical uncertainties in the estimation of NOAELs, species–species extrapolations, interindividual variations, and sometimes even extrapolations from acute to chronic effects. In human risk assessment, safety factors ranging from 10 to 1,000 are commonly used. The claim is that the ADIs derived for single chemicals signify exposure levels that can be tolerated for a lifetime without harmful effects [World Health Organization (WHO) 1978]. The question is whether this claim is viable when exposure is to large numbers of chemicals, all at levels around their individual ADIs. On the basis of the available evidence, it is hard to generally rule out the possibility of combination effects, quite independent of whether exposure is to similarly or dissimilarly acting agents.

However, the fact that joint effects cannot generally be ruled out gives little indication about the likelihood with which they might occur. To come closer to an answer, more information about at least one key aspect is required:

Improved knowledge about the relevant exposure scenarios, in terms of the nature of active chemicals, and their number, is essential. It is widely acknowledged that this information is at best fragmentary for most human exposure scenarios, but indications are that it may involve several hundred or more chemicals. To fill this knowledge gap is of utmost importance. Lack of information about relevant exposure scenarios represents perhaps the most serious obstacle to making progress with mixtures risk assessment both in human and ecological toxicology. Exposure assessment strategies that adopt a more holistic approach, instead of focusing on individual chemicals, are needed to overcome this situation. However, it is likely that incomplete knowledge about this aspect will remain an obstacle for the foreseeable future. How should chemicals regulation deal with multiple exposures in the face of these uncertainties?

Perspectives for chemicals regulation and dealing with uncertainty. It seems to us that a change in the paradigms that govern human (and ecologic) risk assessment is required. First, risk assessment should recognize the limitations of the current chemical-by-chemical approach and should embrace fully the reality of mixture effects. There are encouraging signs of moves in this direction. In a recent opinion paper, the European Scientific Committee on Toxicology, Ecotoxicology and the Environment (SCTEE 2004) pointed out that "for compounds with identical mode of action, such as oestrogenic hormones and xenoestrogens … the performance of individual risk assessments is problematic. …The effects may be additive, especially since these chemicals co-occur in the aquatic environment."

Second, it is necessary to abandon an obsession with synergisms that is still fairly widespread when it comes to justify the need for mixture studies. The overemphasis on synergisms in recent years has diverted attention away from the realization that additivity matters too, especially when considering multiple exposures in the low dose range. So far, evidence of synergism with multicomponent mixtures (in the sense of effects larger than anticipated with dose addition or independent action) awaits publication.

Third, attempts should be made to reach a consensus about a default approach for dealing with mixtures in human risk assessment. Such default approaches are often adopted out of necessity—for example, to bridge data gaps about mixtures of relevance or to deal with extrapolation issues such as from high to low dose or between species (U.S. EPA 1986, 1989, 2000). Various proposals exist for deciding on the basis of presumed mechanisms which of the two concepts, dose addition or independent action, should be used to assess mixtures [Agency for Toxic Substances and Disease Registry (ATSDR) 2002; Groten et al. 2001; Mileson et al. 1998]. For example, the U.S. EPA (2002) has proposed a single approach for the regulation of pesticides that share a common mode of action. The COT (2002) has suggested to adopt independent action as the default approach, and to use dose addition only in specific cases. Such dichotomous approaches are problematic, for

several reasons: First, unambiguous criteria for what should constitute "similar" or "dissimilar" action do not exist and are currently difficult to define. Sometimes, the induction of the same phenomenological effect is deemed sufficient for similar action. At the other extreme of the spectrum of opinions, an identical toxic mechanism, involving the same toxic intermediate is required to fulfill the similarity assumption. A middle position is occupied by the view that interactions with the same site or tissue should qualify for similarity. Second, in most cases, the precise mechanisms of action are unknown. Exceptions are very few groups of chemicals, perhaps including some organophosphorus and carbamate pesticides and PCDD/Fs. Thus, it is the rule rather than the exception that agreement about similarity or dissimilarity of action cannot be reached. This situation is likely to remain unchanged in the foreseeable future. Third, knowledge about mechanisms changes and expectations about presumed modes of action does not necessarily match biological observation. For example, we have recently found that the effects of mixtures of anticancer drugs with different sites of actions were described better by dose addition and not independent action, as originally expected (Phul P, Kortenkamp A, unpublished data). Thus, serious doubts exist to what degree knowledge about specific molecular mechanisms can be used constructively in mixtures risk assessment.

Therefore, lack of knowledge about the mode of action of mixture components should not block choices between the two concepts for risk assessment purposes. Instead, in the absence of information, precaution should be the overriding concern. Thus, which concept yields the more conservative mixture effect prediction?

In the ecotoxicologic arena, systematic comparative studies of the mixture effect predictions produced by dose addition and independent action have shown that dose addition yielded the more conservative predictions, but that overall the quantitative differences between both concepts were relatively small (Backhaus et al. 2000; Faust et al. 2003). Here, the case can be made for using dose addition as the default approach for mixture assessments. This would avoid lengthy and largely fruitless discussions about establishing modes of action. Such a modus operandi would have two advantages: First, the data requirements for proper use of dose addition are less stringent than those for independent action. Although the former works well on the basis of effect doses, the use of independent action usually requires knowledge of entire dose–response curves, particularly in the low effect range. Second, prospective mixture effect assessments should be compliant with the precautionary principle. This favors the concept that typically yields the more conservative predictions, such as dose addition.

Although the case for dose addition is validated in ecotoxicology, the situation is not so clear-cut in human toxicology. Here, the relevant information is largely missing and research efforts are currently directed into conducting studies to fill these gaps. In the interim, human risk assessment could work on the basis of the rebuttable hypothesis that dose addition is applicable, but should rapidly modify this practice as soon as evidence to the contrary becomes available.

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