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Review of recent literature concerning mixture toxicity of pesticides to aquatic organisms

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Abstract

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The simplest way to assess the effects of mixtures of pesticides is to add the effects of the individual substances to each other (concentration addition). In general, experiments show that substances do not enhance each other's action (no synergism). If there is still an enhanced effect, this will usually be small. Therefore, the concept of concentration addition is useful to estimate the adverse effects of mixtures of pesticides. This was concluded on the basis of a review of recent literature concerning the toxicity of mixtures of pesticides that was carried out by RIVM, together with the research institute Alterra. This inventory is an update from an analysis from 2000 and it confirms its conclusions. As a client, the ministry of VROM wanted to map out which new developments in the field of assessing the effects of pesticide mixtures are of importance.

Therefore, the study also describes the methodological improvements that can refine the risk assessment of mixtures. It is now possible to determine the effects of substances when they are used consecutively instead of simultaneously. This concept is relevant for pesticides because these substances are often used in succession. Further, the so-called species sensitivity distributions are now also applicable to mixtures of substances. These species sensitivity distributions describe the variation to which a group of different organisms is sensitive to the effects of substances. On the basis of these distributions, it is determined what concentrations are safe for the environment. For this method, however, a lot of data are required about the adverse effects of substances on organisms, which are usually not available.

So-called mesocosm studies, in which ecosystems are simulated in laboratories, show that synergetic effects are not to be expected if pesticides are used for the same biological groups, such as plants or insects. If several pesticides are applied for different biological groups, indirect effects that enhance each other are often noted, that is in the next level of the food web. When the practical application of pesticides for a particular crop is mimicked, the effects are mostly no larger than those of the most toxic substance. In that situation, enhanced effects are also not observed.

Key words:
Mixture toxicity, joint action, pesticides

Rapport in het kort

Overzicht van recente literatuur over mengseltoxiciteit van bestrijdingsmiddelen voor waterorganismen

De eenvoudigste manier om effecten van mengsels van bestrijdingsmiddelen te beoordelen is om de effecten van de individuele stoffen bij elkaar op te tellen (concentratieadditie). In het algemeen laten experimenten zien dat de stoffen elkaars werking niet versterken (geen synergisme). Als er toch sprake is van versterking, is dat effect doorgaans gering. Het concept concentratieadditie is daarom geschikt om de schadelijke effecten van mengsels van bestrijdingsmiddelen te schatten. Dit blijkt uit een overzicht van recente literatuur over de toxiciteit van mengsels van bestrijdingsmiddelen dat het RIVM met het kennisinstituut Alterra heeft gemaakt. De inventarisatie is een update van een analyse uit 2000 en bevestigt het beeld van toen. Het ministerie van VROM wilde als opdrachtgever in kaart brengen welke ontwikkelingen spelen op het gebied van het beoordelen van mengsels van bestrijdingsmiddelen.

De studie beschrijft daarom ook methodologische vernieuwingen die de risicoschatting van mengsels kunnen verfijnen. Zo is het mogelijk de effecten te bepalen van stoffen als ze achter elkaar worden gebruikt in plaats van tegelijkertijd. Dit concept is voor bestrijdingsmiddelen relevant aangezien deze middelen veelal achter elkaar worden gebruikt. Daarnaast zijn de zogeheten soortgevoeligheidsverdelingen nu ook geschikt gemaakt voor mengsels van stoffen. Deze verdelingen beschrijven de variatie waarin een groep van verschillende organismen gevoelig is voor effecten van stoffen. Op basis hiervan wordt bepaald welke concentraties veilig zijn voor het milieu. Voor deze methode zijn echter veel data nodig over de schadelijke effecten van stoffen op organismen, die in veel gevallen niet beschikbaar zijn.

Ook blijkt uit zogeheten mesocosmstudies, waarin ecosystemen in laboratoria worden nagebootst, dat er geen synergisme is te verwachten bij het gebruik van meerdere soorten bestrijdingsmiddelen voor dezelfde biologische groepen, zoals planten of insecten. Bij het gebruik van meerdere bestrijdingsmiddelen worden voor verschillende biologische groepen wel vaak indirecte effecten waargenomen, die elkaar versterken, namelijk in het volgende niveau van de voedselketen. Als het praktijkgebruik van bestrijdingsmiddelen voor een bepaald gewas in het veld wordt nagebootst, zijn de effecten meestal niet groter dan die van de meest giftige stof. Ook worden in die situatie geen versterkende effecten waargenomen.

Trefwoorden:
Mengsel toxiciteit, gezamenlijke werking, bestrijdingsmiddelen

Contents

Summary

In 2000, an important review of the scientific literature was made on the toxicity of mixtures of pesticides. In this report, an overview is given of the development of mixture toxicity of pesticides since then. Because of the large amount of studies considering mixture toxicity and pesticides, not all studies were evaluated. Special attention was paid to new developments in the field of mixture toxicity. Only the most recent of the remaining studies were considered, if no new concepts were introduced. In binary and multiple mixtures of pesticides, most often concentration addition was observed in the case of compounds with an equal mode of action or independent action (response addition) in the case of compounds with a different mode of action. In some cases, a response was noticed in between these two concepts. Synergistic or antagonistic effects were seldom observed. The combinations of an organophosphorus ester or a carbamate in combination with either another organophosphorus ester or a synthetic pyrethroid were the exceptional cases where synergism was sometimes observed. However, still the deviations from additivity were small. These observations confirmed earlier conclusions on mixture toxicity.

For similar reasons, an extensive report on mixture toxicity for the European Union recently proposed to use the concept of concentration addition in the risk assessment of mixtures. It was concluded that concentration addition was generally describing the data fairly well and that differences between concentration addition and independent action were generally small, especially in the case of multiple compounds. Moreover, the concept of concentration addition is generally the most cautious one. Further, by applying the toxic unit approach, concentration addition can make use of existing effect data such as NOECs, EC10s and EC50s. This viewpoint of using the concept of concentration addition is endorsed in the present study.

A few new developments in the field of assessing mixture toxicity were more thoroughly investigated. The first development is the modelling of toxicokinetics and toxicodynamics of a chemical in a species in order to predict the effects of multiple exposure to a single compound or mixtures of compounds in time. One of these models is called the Threshold Damage Model, which describes the cumulative (acute) toxicity of compounds that are not dosed simultaneously, but subsequently. This exposure regime is a common feature in agricultural practice. Because the parameters describing the toxicokinetics and toxicodynamics are generally not available, the use of the model will in first instance be limited to a few (sensitive) species and to certain specific sequences of pesticide applications for which these toxicokinetic and toxicodynamic parameters are determined. Other studies investigated the effect of hormesis for mixtures of substances in single species tests. It appeared that there was no effect of hormesis on the combined effect of compounds.

Another important development in the field of mixture toxicity is the application of species sensitivity distributions (SSDs). This method, which is often referred to as multi-substance potentially affected fraction (ms-PAF), calculates the percentile of species being affected by the exposure to multiple substances at the same time. The concepts of concentration addition and response addition can be applied to the potentially affected fraction in the SSD of the individual substances.

Finally, mixture toxicity in mesocosms was investigated, in which the direct effects and indirect interactions are studied of multiple species together. These studies showed no strong synergistic effects if compounds affecting the same biological groups were considered. The primary effects on individual biological groups as observed in mesocosms could usually be attributed to the most toxic compound. In these experiments, complex food web interactions can lead to secondary effects for biological groups that are not directly affected.

1 Introduction

1.1 Objective

The risk assessment of substances mostly focuses on the assessment of individual substances. As a consequence, the joint toxic action of mixtures of multiple compounds is often overseen, although recently much attention has been drawn to this subject. A situation where it is likely that more compounds may occur together or shortly after each other is the use for crop protection on the same or adjacent fields. In this report an overview is given of the recent literature on the mixture toxicity of pesticides.

The report is an update of the review that was carried out in 1999 by Deneer (2000). In this review, an assessment was made of the studies describing the mixture toxicity of mixtures containing pesticides. These data were solely based on laboratory single species studies. It was concluded that the concept of concentration addition was generally applicable to the mixtures studied. If deviations from concentration additivity occurred, the differences were generally small. If deviations were observed, this was usually for an organophosphorus ester or a carbamate in combination with either another organophosphorus ester or a synthetic pyrethroid. Still, the concept of mixture toxicity has been focused so far on simultaneous exposure to two or more compounds.

This present review was initiated after the observation that many different pesticides are applied at the same time or one after the other on the same crops. It was noted that new scientific publications have become available in recent years that deal with sequential exposure of mixtures of pesticides. Ways to account for subsequent exposure to compounds have become available since the earlier-mentioned review from 1999. In this report, special attention will be paid to these new methodologies to account for mixture toxicity. Except for the new methodology to explain observed toxicity in single species, methods to describe mixture toxicity using species sensitivity distribution have been developed during recent years. Further, recent work on multiple substances in semi-field studies (mesocosms) is described as well.

2 Mixture toxicity in single species studies

2.1 Independent action, concentration addition, synergism and antagonism

There are two generally applied models to describe mixture toxicity: these are concentration addition (CA) and response addition or independent action (IA). In the first model, concentration ratios of the concentration divided by an effect concentration are summed. The underlying principle is that different compounds in a mixture have a similar mode of action and it is generally assumed that the compounds have the same target site in the organism. This description of mixture toxicity underlies the toxic unit approach for mixture toxicity to single species. The risk quotient in the risk assessment of multiple chemicals is also based on the same principle, by adding the risk characterisation ratios (ratio of predicted environmental concentration and predicted no-effect concentration). This type of additivity is sometimes denoted as Loewe additivity.

Sørensen et al. (2007) describe a method in which the deviation from concentration additivity can be tested. This is done by means of the following formula:

$$\left(\frac{d_1}{\delta_1}\right)^{\lambda} + \left(\frac{d_2}{\delta_2}\right)^{\lambda} = 1$$

In this equation d_1 and d_2 are the dose of the first and second compound, δ_1 and δ_2 are the effect dose (e.g., EC50) of the first and second compound, and λ is an interaction parameter, denoting the deviation from concentration addition. If $\lambda = 1$, the ratios of dose to effect dose are simply summed for the individual compounds, which refers to concentration addition. By means of a statistical F-test, it can be analysed if the parameter λ is significantly different from one.

When plotted as an isobologram for binary mixtures (i.e., a figure with the dose of one compound on the x-axis and the dose for another compound on the y-axis showing a line (isobole) that corresponds to a dose with a certain effect, e.g., EC50), this is a straight line for concentration addition. Visually, if there are deviations from concentration additivity, the resulting curve will not be a straight line (Figure 1).

Another advantage of the method of comparing toxicity is that it not only takes into account a single mixture ratio compared to the toxicity of the pure compounds, but also fits all data at the same time. This also means that the best fit not necessarily goes through the effect concentrations for the pure compounds but allows for some variability in the data.

Another possibility is that the compounds in the mixture have a completely dissimilar mode of action. This assumes that the target site of the individual compounds is not the same. The susceptibility of individuals to two compounds may then be fully positively or negatively correlated or in principle, anywhere in between. For more than two chemicals, a fully negatively correlation is impossible. The model that is most often used is that the response to two compounds is not correlated at all. This means that the fraction of organisms that responds to one compound is independent of the ratio that responds to other compounds. The unaffected fraction of the organisms exposed to a mixture of compounds can then be calculated as the product of the unaffected fractions of all compounds individually. This type of additivity is sometimes denoted as Bliss independence.

For compounds that have dose-response curves with log-logistic slope parameters around one, IA and CA predictions are similar. Concentration addition often leads to more conservative estimates than independent action, but this is not necessarily so. For example, in the activated sludge test, which can be considered as a bacterial community of multiple species, the independent action model yielded

consistently more conservative results but also described the data better than concentration addition (Cedergreen et al., 2008).

Antagonism and synergism are effects where the compounds in the mixture interact and the effects are respectively less or more than calculated from the models assuming no interaction. In principle, there are two models to account for no interaction, which are the simple similar action described by the concentration addition model and the independent action described by the response addition model. Anything in between thus is not necessarily the result of interaction between the compounds in the mixture. For this reason, combined effects less toxic than independent action will be considered as antagonism in this report (Bliss antagonism) and combined effects more toxic than concentration addition will be considered as synergism (Loewe synergism).
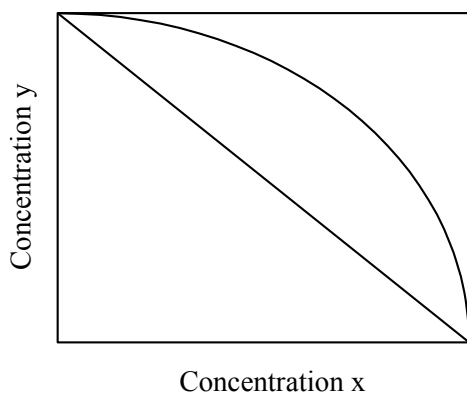

Figure 1: Example of an isobologram: Isoboles refer to a specific effect, e.g., 50% effect. A straight isobole is representative of concentration addition, a curved isobole refers to a situation with less effect at similar concentrations.

2.1.1 Range between concentration addition and independent action

Scheil et al. (2009) studied the combined effects of the organophosphate ester diazinon and the metabolite 3,4-dichloroaniline on the embryo and larvae of the zebrafish (*Danio rerio*). Although no detailed information is given and results can only be checked visually from presented figures, it is stated that concentration addition between the two compounds was observed for all endpoints (mortality, malformations, behaviour and stress protein).

Verro et al. (2009) indicate that for the application of independent action information on the dose response curve is necessary and that this information is usually lacking. Synergistic effects cannot be predicted until now and should be determined experimentally. Concentration addition can be performed with only the reported effect concentrations (e.g., EC50 data). Generally, this model predicts higher toxicity than the independent action model, but for complex mixtures these differences are within a factor of 10. Because concentration addition is generally in between independent action and synergism, it can be considered as a reasonable worst-case. Because of this fact and the practicality, concentration addition was used to model the combined effects of pesticides to algae, daphnids and fish in an Italian river basin (54 active ingredients applied over an area of 2817 ha). It was concluded that risk events (either due to run-off after rainfall or due to drift) caused toxic units for acute toxicity (mortality) to exceed 0.1 for algae and daphnids on a frequent basis and for daphnids the value of 1 is exceeded in July. The modelling shows that the toxicity is merely caused by only a few active ingredients, which renders concentration addition even more applicable. The modelling approach serves three goals: to estimate the total impact on the aquatic ecosystem of the mixtures of compounds, to identify the crops

that pose the largest risk for the aquatic ecosystem and to identify the most important compounds in this.

Sørensen et al. (2007) found that in a binary mixture of acifluorfen and diquat the growth rate of duckweed (*Lemna minor*) did not follow concentration addition. The mixture showed antagonism compared to concentration addition, with the parameter λ being 0.28. However, the deviation from this concentration additivity was only 65% for the equitoxic mixture, in other words, the ratio of the predicted EC50 and the observed EC50 was 61% (1/1.65). No comparison with independent action was presented.

In a second test with *Lemna minor* exposed to mixtures of mechlorprop and terbuthylazine, the obtained isobole was not symmetric, with the largest deviations from concentration addition up to a factor of two towards lower concentrations of terbuthylazine. The data showed antagonism compared to concentration addition. This deviation from this concentration additivity was for the equitoxic mixture 58%, in other words, the ratio of the predicted EC50 and the observed EC50 was 63%. No comparison with independent action was presented.

The third test was one with the algae *Pseudokirchneriella subcapitata* exposed to mixtures of glyphosate and metsulfuron-methyl. The data showed concentration addition. The equitoxic mixture was only 10% less toxic than concentration addition, which was not significant. In other words, the ratio of the predicted EC50 and the observed EC50 was 91%.

Bellas (2008) investigated the combined effects of zinc pyrithione, chlorothalonil and Sea-Nine 211 (active ingredient 4,5-dichloro-2-n-octyl-3-isothiazolinone) to embryos and larvae of the sea urchin *Paracentrotus lividus*. For the binary mixture of zinc pyrithione and chlorothalonil both concentration addition and independent action overestimated the toxic effect at the level of the EC50, and it was concluded that antagonism occurred. However, at lower concentrations at the NOEC/EC10 levels, these differences between predicted and observed concentrations were not present. Independent action predicted the observed toxicity of the binary mixture of zinc pyrithione and Sea-Nine 211 very well, especially for the larvae. For the embryonic development, concentration addition predicted the observed toxicity better at lower concentrations. The observed toxicity of the binary mixture of chlorothalonil and Sea-Nine 211, as well as the toxicity of the ternary mixture of the three compounds together, was in between concentration addition and independent action. On average, concentration addition underestimated the observed EC50s of the mixtures by 36% and independent action overestimated the EC50s by 30% (Table 1). Therefore, the use of concentration addition is considered as a reasonable worst-case.

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Table 1: Ratios of observed versus predicted toxicity for the embryonic development and larval growth of the sea urchin *Paracentrotus lividus*, expressed as the ratio EC50 predicted and EC50 observed

	Observed toxicity compared to CA		Observed toxicity compared to IA[1]	
Mixture	Embryonic development	Larval growth	Embryonic development	Larval growth
zinc pyrithione and chlorothalonil	60%	59%	70%	76%
zinc pyrithione and Sea-Nine 211	61%	54%	118%	98%
chlorothalonil and Sea-Nine 211	73%	75%	140%	136%
ternary mixture	63%	67%	144%	148%

[1]Lower predicted effect concentrations than observed effect concentrations might be attributable to antagonism.

Cedergreen et al. (2008) investigated the applicability of the concentration addition and independent action model to a large set of toxicity data for binary mixtures of two compounds, mostly pesticides, with dissimilar molecular target sites (dissimilar mode of action) at the level of the EC50. The set comprised 16, 9, 9, 21, 18, 15, and 10 different mixtures for the luminescent saltwater bacterium *Vibrio fischeri*, activated sludge bacteria, the waterflea *Daphnia magna*, the algal species *Pseudokirchneriella subcapitata*, the duckweed *Lemna minor* and the terrestrial plant species scentless mayweed (*Tripleurospermum inodorum*) and common chickweed (*Stellaria media*), respectively. It was concluded that in general, models accounting for synergy or antagonism (S/A) better predicted the data in about half of the considered mixtures. In general, the independent action model predicted the data somewhat better than concentration addition, except for the test with *Daphnia magna*, where concentration addition better described the data. The hypothesis that independent action would better describe the data was anticipated because this model describes the action of mixtures with a dissimilar mode of action, at least if binomially distributed endpoints (e.g., lethality) are considered instead of gradual endpoints (e.g., growth).

However, deviations of the concentration addition and independent action estimates from the observed toxicity were small and generally within a factor of two. Only in 6% of the cases the EC50 of the binary mixture was less than 50% of the values predicted by either concentration addition or independent action. Large synergistic effects are thus rather infrequent.

Munkegaard et al. (2008) tested the interaction between herbicides and organophosphorous insecticides with duckweed (*Lemna minor*) and the algae *Pseudokirchneriella subcapitata*. The tested herbicides were metsulfuron-methyl, terbuthylazine and bentazone; the insecticides were malathion, endosulfan and chlorpyrifos. The hypothesis was that the organophosphates could inhibit P450 in these species, which would lead to inhibition of metabolism of the herbicides and consequently, increased toxicity. Endosulfan is not an organophosphate. The insecticides were tested at half of the solubility and were not toxic to either duckweed or algae, except for malathion, which was toxic to algae. In none of the mixtures significant enhancement of toxicity was observed. However, the mixtures of bentazone with either of the pesticides showed antagonism, both in relation to concentration addition and independent action. The experiments with malathion and algae were performed in a mixture ratio study because malathion caused toxicity to algae. In the first test with metsulfuron-methyl, synergistic effects were observed. However, these effects could not be repeated in a second study, where the data could be described by either concentration addition or independent action. The studies with malathion and bentazone or terbuthylazine showed no deviation from concentration addition. However, the mixture of

bentazone and terbuthylazine deviated from independent action (more toxic), although given the structures and character of the compounds (herbicide and insecticide), independent action would be the most logical model.

De Zwart and Posthuma (2005) have evaluated the concepts of mixture toxicity for ecotoxicological endpoints. They cite a review by Warne, in which it is stated that 70% of the aquatic toxicity studies can be described by concentration addition and that 10–15% of the results showed more and 10–15% showed less toxicity than predicted by concentration addition. Only in 5% of the studies, the difference was larger than a factor of 2.5 and only in 1% the difference was more than a factor of 5. Although at the level of an organism, interactions are more complex than simple similar action and independent joint action, they conclude that the corresponding models concentration addition (CA) and response addition (independent action, IA) are still the most suitable models to predict ecotoxicity if the assumed mechanism of action is similar or dissimilar, respectively.

Syberg et al. (2008) studied the combined effects of three compounds in acute toxicity tests with *Daphnia magna*. This study was also included in the review by Cedergreen et al. (2008). The results show that based on isoboles, the two acetylcholine esterase inhibitors dimethoate and pirimicarb clearly follow concentration addition, both at the level of the EC50 and at the level of the EC10. For the combination of dimethoate with linear alkylbenzene sulfonate (LAS), no significant deviation from concentration addition was found at the level of both the EC50 and EC25. The mixture of pirimicarb and LAS was not statistically different from concentration addition at the level of the EC50, but the difference increased at the level of the EC10 to a significant difference and approximated independent action at this level.
The results are calculated based on the equation presented in the publication and these percentages are shown in Table 2. In the table it is clear that deviations from either model are at most a factor of two. It must be stressed that in such analysis the uncertainty in the dose response curve of the individual pure compounds plays a more important role than when isoboles are used, as is done in the publication.

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Table 2: Ratios of observed versus predicted toxicity for the immobilisation of water fleas (*Daphnia magna*), expressed as the ratio EC50 or EC10 predicted and EC50 or EC10 observed

Mixture (effect ratios)	Observed toxicity compared to CA [1]		Observed toxicity compared to IA [2]	
	EC50	EC10	EC50	EC10
75% dimethoate 25% pirimicarb	106%	107%	142%	143%
50% dimethoate 50% pirimicarb	76%	81%	140%	162%
25% dimethoate 75% pirimicarb	75%	69%	100%	122%
75% pirimicarb 25% LAS	103%	48%	134%	102%
50% pirimicarb 50% LAS	102%	58%	158%	113%
25% pirimicarb 75% LAS	100%	67%	133%	89%
75% dimethoate 25% LAS	114%	113%	148%	177%
50% dimethoate 50% LAS	108%	60%	171%	104%
25% dimethoate 75% LAS	162%	140%	216%	180%

[1] Higher predicted effect concentrations than observed effect concentrations might be attributable to synergism.
[2] Lower predicted effect concentrations than observed effect concentrations might be attributable to antagonism.

2.1.2 Synergism

Deneer (2000) already concluded that combinations of an organophosphorus ester or a carbamate with either another organophosphorus ester or a synthetic pyrethroid yielded the largest deviation from concentration addition. In a recent study on the acetylcholine-esterase (AChE), inhibition in the brain of Coho salmon with combinations of the organophosphates diazinon, malathion, chlorpyrifos or the carbamates carbaryl and carbofuran, severe cases of synergism were observed, especially between two organophosphate esters, but also between an organophosphate ester and a carbamate and to a lesser extent but still significant at EC50 levels for AChE reduction for two carbamates (Laetz et al., 2009). This study thus confirms the exception noted by Deneer (2000). However, the synergistic effects observed in this study were not small deviations from concentration addition. It was observed that at the highest exposure concentration, in which two chemicals were present at 0.5 times their EC50 each, statistically significant synergism was observed for all combinations of the five pesticides. The number of combinations resulting in statistically significant synergism increased with increasing concentrations. Synergism for phosphate ester and carbamates is explained by the fact that these compounds act on other biochemical targets. However, it is not stated if this could also be an explanation for the large synergistic effects of two phosphate esters or the smaller but still significant synergistic effects of two carbamates.

As an example, the combination of chlorpyrifos and malathion can be given. At nominal concentrations of chlorpyrifos and malathion that would be expected to result in 50% AChE inhibition if concentration

addition was applicable, 100% mortality was observed instead of 50% AChE inhibition. For the combination of diazinon and malathion, 100% mortality was already observed at concentrations that would be expected to result in 29% AChE inhibition if concentration addition was applicable. For comparison, the measured values for chlorpyrifos resulting in 100% mortality were slightly below 1.0 µg/L, while the quality standard set in the Water Framework Directive to protect the aquatic ecosystem against any adverse effects from short-term exposure is 0.1 µg/L (European Commission, 2005e).

Synergistic action

The influence of the herbicide atrazine on the toxicity of organophosphate insecticides to the amphipod *Hyalella azteca* was examined (Anderson and Lydy, 2002). Atrazine is in itself essentially not toxic to *Hyalella*. However, when dosed together in combination with the insecticides chlorpyrifos, methyl parathion and diazinon, which are all acetylcholine-esterase inhibitors, the toxicity of these insecticides, expressed as their LC50 values, increased. Without atrazine the LC50 for chlorpyrifos was 0.0427 µg/L, the LC50 for methyl parathion was 2.1 µg/L, and the LC50 for diazinon was 4.3 µg/L. For all three insecticides, the combined presence of 10 µg/L had no influence on the toxicity, i.e., the synergistic ratio was 1.0. Increasing concentrations of atrazine increased the toxicity of the three organophosphate insecticides up to a factor of 3 at 200 µg/L (Table 3).

The mechanism of synergistic action is probably caused by the fact that atrazine is inducing P450 isoenzymes and, as a consequence of the enhanced metabolism, increases the conversion of the organophosphates in the more potent *o*-analog metabolites. The inhibition of acetylcholine-esterase was increased in the presence of atrazine.

It was also tested whether the consecutive exposure to atrazine and the organophosphates showed the same effects. This was indeed observed, however, only when the exposure to atrazine had lasted for 144 h. It is suggested that the exposure to atrazine needs to be long enough to induce cytochrome P450 induction. However, in the simultaneous exposure the exposure period was only 96 h, which would then not be enough to induce cytochrome P450. Given the reported kinetics of atrazine in *Hyalella*, it might also be that the remaining atrazine in *Hyalella* is below the critical level if the organisms were exposed for less than 144 h.

Table 3: Overview of synergistic ratios for the toxicity (LC50) of organophosphate substances to the amphipod *Hyalella azteca* in the presence of a range of atrazine concentrations

	Atrazine concentration			
	10 µg/L	40 µg/L	80 µg/L	200 µg/L
Chlorpyrifos	1.0	1.6	2.0	2.8
Methyl parathion	1.0	1.0	1.7	2.9
Diazinon	1.0	1.0	2.0	3.0

This is a clear example of synergistic action. The concentrations of atrazine to cause this synergistic effect are 40 to 80 µg/L. Although these concentrations are well above the maximum acceptable quality standard of 2.0 µg/L and the annual average quality standard of 0.6 µg/L derived for atrazine as a priority substance in the Water Framework Directive, the onset of effects is at a concentration only two times higher than the lowest EC50 for atrazine of 20.5 µg/L (European Commission, 2005d).

The majority of the synergistic effects observed in the review by Cedergreen et al. (2008) were tests with *Vibrio fischeri* or *Daphnia magna*, where either prochloraz or the organophosphate insecticide chlorfenvinphos was used in the binary mixtures. Both substances can potentially interact with the P450 system, which may explain the observed toxicity.

2.1.3 Antagonism

Belz et al. (2008) studied the effect of the combination of pesticides on duckweed (*Lemna minor*). Two combinations were tested: acifluorfen in combination with mesotrione and acifluorfen in combination with terbuthylazine. It was stated that the combination of acifluorfen with mesotrione showed large antagonistic effects. However, antagonism was in this case defined as a deviation from concentration addition. By analysing the response curves, it appears that indeed for this mixture effect concentrations are higher than expected, both on the basis of concentration addition and on the basis of independent action. For the mixture of acifluorfen and terbuthylazine, such an effect is only observed at relatively high concentrations of acifluorfen (>50%) at the level of the EC50. For the rest of the combinations, no antagonistic effects were observed and toxicity was close to independent action or even in between concentration addition and independent action (see Table 4).

The concentration at which 50% effect occurred, at the concentration where hormetic effects vanished (LDS) and at the concentration with maximum hormetic effect (see 2.2), all showed the same isoboles for joint action of mixtures at different concentration ratios. A similar observation can be made from Table 4, where no large differences between the analyses of the EC50 and EC10 are observed.

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Table 4: Ratios of observed versus predicted toxicity for the growth of duckweed (*Lemna minor*), expressed as the ratio EC50 or EC10 predicted and EC50 or EC10 observed

	Observed toxicity compared to CA		Observed toxicity compared to IA [1]	
Mixture	EC50	EC10	EC50	EC10
83% acifluorfen 17% mesotrione	71%	67%	82%	78%
67% acifluorfen 33% mesotrione	62%	59%	79%	80%
50% acifluorfen 50% mesotrione	45%	38%	61%	61%
33% acifluorfen 67% mesotrione	60%	45%	83%	83%
17% acifluorfen 83% mesotrione	80%	68%	116%	134%
83% acifluorfen 17% terbuthylazin	80%	90%	87%	102%
67% acifluorfen 33% terbuthylazin	74%	65%	88%	85%
50% acifluorfen 50% terbuthylazin	94%	83%	120%	129%
33% acifluorfen 67% terbuthylazin	88%	81%	121%	158%
17% acifluorfen 83% terbuthylazin	65%	52%	96%	125%

[1] Lower predicted effect concentrations than observed effect concentrations might be attributable to antagonism.

As mentioned above, Munkegaard et al. (2008) tested the interaction between herbicides and organophosphorous insecticides to duckweed (*Lemna minor*) and the algae *Pseudokirchneriella subcapitata*. The mixtures of bentazone with either malathion, endosulfan or chlorpyrifos, which were not toxic by themselves, showed antagonism both in relation to concentration addition and independent action.

2.2 Hormesis

The herbicide acifluorfen studied by Belz et al. (2008) caused hormesis at low concentrations. At these low concentrations the herbicide had a positive effect on the growth of duckweed compared to the control instead of a negative effect. All binary mixtures of acifluorfen with mesotrione or terbuthylazine also showed significant hormesis, with on average 41% (±31%), ranging from 6 to 128%. The concentration at which hormesis disappeared (designated as LDS, i.e., the concentration at which the response curve falls below the control value) was 46±7% of the EC50. Although it is mentioned that the slope of the dose response curve was less steep than natural phytotoxins tested with lettuce, the range between no-effect and 50% effect is thus very small (a factor of two).
The study was set up to investigate whether the mixture effects of binary mixtures led to a linear interpolation of the hormesis effects caused by the individual compounds. Although this was observed for the hormetic effects of phytotoxins to lettuce, this was not observed for the mixtures of acifluorfen

with mesotrione or terbuthylazine, where the hormetic effect was larger in the mixtures with 50% acifluorfen than with the pure compound itself, although the other compounds mesotrione or terbuthylazine did not induce significant hormetic effects. The relevance of this finding is however not clear and it might be that this is attributable to a relatively large susceptibility to the experimental variance in determining the maximum hormetic effect.

Although hormetic effects of the mixture occurred, the use of a monotonic decrease model or a model accounting for hormetic effects only led to marginal differences in assessing the toxic effects of the mixture. Further, it should be noted that that the combined dose of the compounds is a measure of the total effect and that the hormetic effect of one chemical when applied alone does not alleviate the toxic effect of another compound.

2.3 Modelling of effects and subsequent exposure to multiple chemicals

Methodologies to model the effects of sequential and pulsed exposure of single substances and mixtures are reviewed by Ashauer et al. (2006a). The reviewed and proposed models are two-step models. The first part is toxicokinetic modelling. All models discussed apply first-order kinetics for uptake and the process is thus defined by an uptake rate constant and elimination rate constant, to estimate the internal concentration of a pesticide. The second part is the toxicodynamic part. Once the substance has been taken up, the toxicodynamics describe the processes injury and recovery.

Two general models are proposed. These are the modified damage assessment model (DAM) and the threshold hazard model (THM). In short, the modified DAM models damage accrual (e.g., mortality) and recovery/repair by means of two first-order rate constants. The critical body residue (CBR) model and the critical target occupation (CTO) or critical area under the curve (CAUC) models are the extremes of this modified DAM, with instantaneous and no recovery, respectively. The THM defines a threshold below which no toxicity is supposed to occur. Above the threshold the hazard increases linearly with the internal concentration. The recovery is modelled by the toxicokinetics only. If the internal concentration drops below the threshold, the hazard reduces to zero.

At present, the models are suitable for describing observed toxicity data but not yet for modelling the toxicity of substances and especially not of mixtures of substances, because the parameters of the model are not known beforehand. However, once a set of toxicity data applied with pulsed exposure with different (standard) organisms is performed and evaluated with this kind of model, the models can be applied quantitatively in risk assessment. When such a data set is available for a vast set of chemicals, the parameters could be estimated for different types of chemicals (e.g., with different modes of toxic action). However, it is obvious that these data are not yet available.

In a following paper (Ashauer et al., 2007b), the DAM and THM were combined into one model, with a killing and recovery rate as well as a threshold for toxicity. The recovery rate is for recovery or repair at the receptor level, while the threshold is explained as a value for translating effects on the receptor level to effects on whole organisms. The model is referred to as the threshold damage model (TDM). In this paper, the model was applied to a series of (pulsed) exposure regimes of chlorpyrifos, a substance that inhibits the enzyme acetylcholine esterase and pentachlorophenol, which uncouples the oxidative phosphorylation process. The model has five parameters. For the toxicokinetics these are the uptake rate and the elimination rate. These were fitted earlier in a separate experiment (Ashauer et al., 2006b). The three remaining parameters describe the toxicodynamics and are the killing rate, the recovery rate and the threshold. These three parameters were fitted to the data from the pulsed exposure regimes.

In the paper, the data are also fitted to the survival probability deduced from time-weighted concentrations in the pulsed exposure regimes, as well as the concentration in a 48-h LC50 experiment. The time-weighted average model based on the 48-h LC50 yielded poor results and is not useful. Contrary to that, the time-weighted average model based on pulsed exposure was able to be

extrapolated to other experiments rather well. The fits were comparable to the TDM. However, the model remains empirical and is expected to have less predictive power than the TDM, if other exposure regimes are to be assessed (Ashauer et al., 2007b).

In another paper, the same experiment was performed with a third chemical. The chemical used was the carbamate carbaryl, which also inhibits acetylcholine esterase. Again, both the TDM and the TWA model based on pulsed exposure gave rather good predictions of the toxicity in different exposure profiles. However, the TDM performed better that the TWA model with pulsed exposure (Ashauer et al., 2007c). It appeared that carbaryl had a higher toxic potency, expressed in the killing rate than chlorpyrifos and pentachlorophenol, but the threshold was also higher than that of chlorpyrifos. Further, the recovery rate constant of carbaryl was higher than that of chlorpyrifos but substantially lower than that of chlorophenol, for which effects disappear once the chemical is eliminated from the organism.

In a last publication in this series, the results on chlorpyrifos and carbaryl from the former publications were used to estimate the effects of sequential pulses of these two chemicals. The model is able to distinguish the differences in toxicity between two exposure scenarios, which only differ in the order in which the two substances are applied. Because the rate constant for damage repair or recovery is longer for chlorpyrifos than for carbaryl, a 1-d pulse of chlorpyrifos 15 days prior to the pulse of carbaryl is of influence on the toxicity of carbaryl, while a 1-d pulse of carbaryl applied 15 days before the pulse of chlorpyrifos has no effect on the effects of chlorpyrifos. It is also shown that this is not a toxicokinetic effect because after 14 days of clean water, the internal concentrations of both substances have already declined to insignificant levels. The observed effects should be attributed to cumulative damage and the difference in the rate of repair or recovery from this damage between the two substances (Ashauer et al., 2007a). It should be noted that the cumulative damage is modelled for two substances that although different in chemical structure, act on the same receptor (acetylcholine-esterase). It remains unknown how the model performs with substances that act by different modes of toxic action.

Short description of the threshold damage model (TDM):

Toxicokinetics:

$$\frac{dC_{\text{int}}(t)}{dt} = k_{\text{in}} \times C(t) - k_{\text{out}} \times C_{\text{int}}(t)$$

where C_{int} is the internal concentration [amount $\times$ mass^{-1}], C the concentration in the water [amount $\times$ volume^{-1}] and k_{in} and k_{out} the uptake rate constant [volume $\times$ mass^{-1} $\times$ time^{-1}] and the elimination rate constant [time^{-1}], respectively.

Toxicodynamics:

$$\frac{dD(t)}{dt} = k_{\text{k}} \times C_{\text{int}}(t) - k_{\text{r}} \times D(t)$$

where k_{k} is a killing rate constant [mass$\times$amount$^{-1}\times$time^{-1}], k_{r} is the rate constant for damage recovery or repair [time^{-1}] and $D(t)$ is damage [-]

$$\frac{dH(t)}{dt} = \max[D(t) \text{ - threshold},0]$$

where $H(t)$ cumulative hazard, threshold is a dimensionless threshold parameter [-]. The differential of $H(t)$ is the hazard rate, which is the probability of the organisms dying at a given time.

$$S(t) = e^{-H(t)} \times S_{\text{background}}(t)$$

where $S(t)$ is the survival probability [-] (probability of an organism surviving until time t) and $S_{\text{background}}(t)$ is the survival probability resulting purely from the background (or control) mortality [-]

multiple substances:

Single substances are denoted by the superscript i

$$\frac{dC_{\text{int,i}}(t)}{dt} = k_{\text{in,i}} \times C_{\text{i}}(t) - k_{\text{out,i}} \times C_{\text{int,i}}(t)$$

$$\frac{dD_{\text{i}}(t)}{dt} = k_{\text{k,i}} \times C_{\text{int,i}}(t) - k_{\text{r,i}} \times D_{\text{i}}(t)$$

$$D_{\text{mix}}(t) = \sum_i D_{\text{i}}(t)$$

$$\text{threshold}_{\text{mix}}(t) = \sum_i \left(\text{threshold}_{\text{i}} \times \frac{D_{\text{i}}(t)}{D_{\text{mix}}(t)} \right)$$

$$\frac{dH(t)}{dt} = \max[D_{\text{mix}}(t) \text{ - threshold}_{\text{mix}}(t),0]$$

$$S(t) = e^{-H(t)} \times S_{\text{background}}(t)$$

3   Mixture toxicity in species sensitivity distributions

To assess the mixture toxicity of substances that have a similar mode of action, De Zwart and Posthuma (2005) propose as a criterion that the shape of the dose-response curves (slopes) for different compounds must be equal. The combined effect level can then be estimated from the sum of the toxic units (based on EC50s) and the uniform slope of the dose response curves (i.e., the dose response curve is described by the EC50, which is different for each compound and a common slope of the curve, which is equal for substances with the same mode of action). This is based on toxicity tests with single species and single compounds. In principle, they state that this could be extrapolated to the species assemblage in the environment as well. This means that for compounds with the same mode of toxic action, the species sensitivity distributions have the same slope. De Zwart & Posthuma (2005) propose a maximum deviation of ±10% in the slope β of the log-logistic distribution as acceptable.

The proposed method (De Zwart and Posthuma, 2005; Traas et al., 2002) then looks at the combinations of groups of similar acting compounds, for which the SSD curves are assumed to be parallel to each other. The fraction affected for a group of compounds can be calculated based on the hazard units of the individual effect data for mixtures (Traas et al., 2002):

$$PAF_{TMoA} = \frac{1}{1 + e^{-\log(\sum HU_{TMoA})/\beta_{TMoA}}}$$

The hazard unit can be seen as the measure of concentration relative to toxicity.

For purely narcotic compounds, for which the mode of toxic action is the same for all species, this approach can be followed by taking into account the species sensitivity distribution over all species. In the case of mixtures of compounds that have a specific mode of action for a certain taxonomic group (i.e., the mode of action within one taxonomic group remains the same), the species sensitivity distribution might be better restricted to all species from one taxonomic group, for which the mode of action remains the same and the potentially affected fraction calculated per taxonomic group (Posthuma et al., 2002). The reasons for doing so are multiple. First, the SSD does not take into account that there are several groups in the ecosystem, of which some are susceptible to the stress of the pollutants present and others not. This means that although the affected fraction of total species in the ecosystem can be small, the affected fraction of a susceptible fraction may be quite substantial. This can lead to chain reactions in the ecosystem that are not predicted by the species sensitivity distribution based on single species laboratory studies. Although this argument is very valid, it is not restricted to the SSD of multiple substances but applies to SSDs for single compounds as well. The next argument is that splitting the data into taxonomic groups for which the group of compounds has a unique mode of action will more likely result in unimodal instead of polymodal distribution. As the PAF for one group of substances is described by the equation above, it is necessary that the data fit well to a log-logistic distribution.

Per taxonomic group, the potentially affected fractions due to multiple groups of compounds with different modes of action can then be summed in a way that is comparable to response addition, to construct the overall potentially affected fraction for multi substances (ms-PAF) (Traas et al., 2002):

$$PAF_{RA} = 1 - \prod_i \left(1 - PAF_i\right)$$

Chèvre et al. (2006) developed an approach to use species sensitivity distribution in the assessment of mixtures of similar compounds. In brief, species sensitivity distributions were first constructed using the acute toxicity data of the compounds considered, assuming a common slope of the SSD for all substances. The relative potency compared to the most data rich substance was then determined on the

basis of the lower confidence interval (5%) of the hazardous concentration to 5% of the species (HC5). The SSD for the most data rich substance was then constructed with a slope that was assumed to be equal to that for the acute toxicity data. The HC5-5 was determined from this SSD curve and the HC5-5 for the rest of the compounds was derived by applying the relative potency factors to this HC5, assuming that the relative potencies for the SSD based on NOECs and the SSD based on EC50s are equal.

This concept was tested with a series triazine (atrazine, terbuthylazine, simazine, cyanazine, metribuzine, and terbuthryn) and phenylurea (linuron, chlortoluron, metoxuron, diuron and isoproturon) herbicides. These substances are believed to have similar mode of action and in the risk assessment of the mixture of these substances, the risk quotients (e.g., measured concentration divided by the lower limit of the HC5) can be summed up to obtain the overall risk. The hypothesis of a common slope of the species sensitivity distribution was rejected for four of the eleven compounds, including the reference compound atrazine, for which the most extensive data set was available. The other substances were metribuzine, linuron, and isproturon. However, the species sensitivity distribution for the chronic data for atrazine showed a slope that was equal to the common slope for the acute toxicity data.

The authors propose the HC5-5 derived in this way as the water quality criterion for these substances and claim that because of the consistent way in which they are derived, this approach is better than the derivation of water quality criteria for single substances, as is usually done. However, in the derivation of single substance quality criteria, the data search is generally much more exhaustive and data are far better evaluated than those used in the study. Therefore, some of the HC5-5 values derived are compared with the quality standards that have been proposed or set under the Water Framework Directive as national specific, river basin specific or priority substances. The HC5-5 for atrazine was 1.8 µg/L. The value for atrazine as a priority substance under the Water Framework Directive is 0.6 µg/L (European Commission, 2005d), which differs only by a factor of three. For simazine, the same assessment can be made. The HC5-5 for simazine was 2.8 µg/L. The value for simazine as a priority substance under the Water Framework Directive is 1 µg/L (European Commission, 2005c), which differs only by a factor of three too. For the phenylurea herbicides diuron and isoproturon, this assessment can also be made. The HC5-5 for diuron was 0.1 µg/L. The value for diuron as a priority substance under the Water Framework Directive is 0.2 µg/L (European Commission, 2005a), which differs only by a factor of two. The HC5-5 for isoproturon was 0.3 µg/L. The value for diuron as a priority substance under the Water Framework Directive is 0.3 µg/L as well (European Commission, 2005b). The proposed value for linuron derived in the UK of 0.5 µg/L (Crane et al., 2007) is less than a factor of two higher than the HC5-5 of 0.3 µg/L. It must be concluded that the method yields values that are comparable with the values derived in international frameworks, for which a robust literature search has been performed.

In a case study, the implications of the combined effect for the total risk of these herbicides was tested. In general, the single compounds do not lead to risk quotients exceeding one, however, the combined effect of all herbicides lead to a regular exceeding of the risk quotient one. This stresses the importance of taking mixture toxicity into account. For the analysis, the risk quotients of the individual compounds were summed. Alternatively, independent action was used to assess the total risk resulting from the herbicides. Normally, these methods are applied to the response in single species studies, here it is extended to the potentially affected fraction of species in the species sensitivity distribution. It appears that the difference between concentration addition and independent action is only a factor of 1.3.

This concept was also applied to a mixture of six organophosphates (chlorpyrifos, diazinon, dichlorvos, dimethoate, parathion-ethyl, parathion-methyl) and another mixture with three beta blockers (Chèvre et al., 2008). Four of the six insecticides had a slope that was significantly different from the uniform slope. Only for chlorpyrifos and diazinon the hypothesis that the slope of the species sensitivity distribution for acute data was not different from the common slope for the six insecticides was not

rejected. For the chronic SSD, the hypothesis that the slope was not different from the common slope for the acute toxicity was not rejected for only two chemicals, which were diazinon and parathion-ethyl. For dichlorvos, there were not enough data to make this comparison. Even for the most data-rich compound chlorpyrifos, which is considered as the reference compound for the other substances, the slope of the SSD for the chronic toxicity data appeared to have a slope that was significantly different from the comment slope of the SSDs for acute toxicity.

The results are nevertheless encouraging: the values for the 5th percentile of the HC5 are HC5-95% 0.0008 µg/L for chlorpyrifos, 0.0027 µg/L for diazinon, 0.0009 µg/L for dichlorvos, 0.026 µg/L for dimethoate, 0.0016 µg/L for parathion-ethyl, and 0.0031 µg/L for parathion-methyl. These limits are mostly within a factor of 10 from environmental quality standards that have been proposed or set under the Water Framework Directive as national specific, river basin specific or priority substances. These values are assumed to be based on a more thorough and exhaustive assessment of the available data. The proposed value for diazinon derived in the UK of 0.01 µg/L is a factor of four higher than the HC5-5 (Lepper et al., 2007). Dichlorvos has been considered for the substances relevant for the river Rhine. The value of 0.0006 µg/L (ICPR, 2007) is very similar to the HC5-5 of 0.0009 µg/L. For dimethoate a proposal was made by the UK (Johnson et al., 2007) and by the Netherlands (Moermond et al., 2008). The value from the UK was 0.48 µg/L, the Dutch value was 0.07 µg/L. The HC5-5 was thus in between these values.

The value for chlorpyrifos, which is priority substance as set by the WFD, is however 0.03 µg/L (European Commission, 2005e), which is a factor of 40 higher than the HC5-5. This value, set under the WFD, seems rather high in comparison with the other data, especially because chlorpyrifos was the reference substance and thus the substance with the most data available in the publication by Chèvre et al. (2008).

4 Mixture toxicity in semi-field studies

This section summarises the results of experiments performed with microcosm and mesocosm (cosm) experiments in which mixtures of pesticides were evaluated. We only included studies that addressed community responses, not included are studies dealing with the fate of mixtures (e.g., Bouldin et al., 2005; Lytle and Lytle, 2002), single species studies performed in cosms with amphibians (e.g., Boone and James, 2003), microbes (e.g., Downing et al., 2004), and macrophytes (e.g., Hanson et al., 2002), and not field studies (e.g., Berenzen et al., 2005; Liess et al., 2005).

Table 5 summarises all studies included in this review and classifies them into two groups, one group containing experiments in which pesticides were applied together as mixtures (mixture approach) and one containing experiments in which the crop approach was used and pesticides are applied over time, mimicking exposure by spray drift from a typical pesticide application programme for a particular crop. The 'mixture approach' group is further subdivided into studies dealing with mixtures of only herbicides or insecticides and mixtures of herbicides and insecticides together.

We retrieved four cosm studies from the literature evaluating the effects of herbicide mixtures (Carder and Hoagland, 1998; Hartgers et al., 1998; Knauert, 2008; Knauert et al., 2008; Relyea, 2009).

Carder et al. (1998) assessed the combined effects of three environmentally realistic levels of alachlor (ranging from 0.7 to 93.5 µg/L) and atrazine (ranging from 2.9 to 155.4 µg/L) on indigenous epipelagic algae in recirculating laboratory streams over a four-week period. The effects of atrazine and alachlor together on algal community biovolume appeared to be additive rather than synergistic, most likely a result of the difference in modes of action of the two herbicides.

Hartgers et al. (1998) exposed indoor microcosms to chronic levels of a mixture of three herbicides (atrazine, diuron, and metolachlor in equitoxic ratios up to the EC50s) for 28 days to evaluate the safety factors as used in the first tier of the registration process in Europe (European Union, 1997). The plankton communities in this aquatic ecosystem were, except for one phytoplankton taxon, sufficiently protected against the mixture of herbicides by the safety factors, in this case assessed against a safety factor of 10 on the lowest NOEC.

The mixture toxicity of three photosystem II inhibitors toward photosynthesis as well as the structure of primary producers was studied in outdoor mesocosms (Knauert, 2008; Knauert et al., 2008). This experiment confirmed the applicability of the concept of concentration addition for three PSII inhibitors when considering their effects on a natural algal community (Knauert et al., 2008) and on the macrophyte *Myriophyllum spicatum* (Knauert, 2008) under environmental conditions. The substances atrazine, isoproturon and diuron were individually applied at their HC30 from short-term laboratory EC50s and as a mixture of 1/3 of their HC30. For the algal communities, the photosynthetic inhibition was 45.6%, 35.6%, 47.7% for atrazine, isoproturon, and diuron, respectively and 48.6% for the equitoxic mixture. For *Myriophyllum spicatum* no significant differences were observed either.

Relyea (2009) evaluates the effects of a cocktail of five herbicides (glyphosate, atrazine, acetochlor, metolachlor, and 2,4-D), as well as their individual effects to aquatic outdoor mesocosms to investigate how mixtures of pesticides at low concentrations (2–16 ppb) affect plankton and amphibian communities. The individual herbicides showed occasional impacts on individual plankton taxa but there was no clear indication of any indirect effects from the addition of the herbicides, except from acetochlor. The addition of the herbicide mixture caused a reduction in phytoplankton that was also observed in the acetochlor treatment (Table 5). In this study, the impact of the herbicide mixtures could largely be predicted from the impacts of the individual herbicides.

Five different studies evaluated the mixture toxicity of insecticides (Cuppen et al., 2002; George et al., 2003; Relyea, 2009; Sibley et al., 2000; Van Den Brink et al., 2002a).

Cuppen et al. (2002) and Van den Brink et al. (2002a) studied the effects of chronic exposure to different concentrations of equitoxic mixtures of the insecticides chlorpyrifos and lindane on

invertebrate and algae communities in indoor plankton microcosms. The observed effects could be explained from the individual toxicity of the insecticides to the invertebrates and did not indicate synergistic effects. They also concluded that the safety factors set by the Uniform Principles for individual compounds, in this case 0.01 times the EC50, also ensure protection against chronic exposure to a mixture of insecticides at community level, though not always at species level. However, the reported LOECs for the invertebrate community are 0.044 to 0.067 for chlorpyrifos, with lindane concentrations varying from 1.84 to 2.12 µg/L. The LOECs for chlorpyrifos are of the same order of magnitude as the derived AA-EQS (European Commission, 2005e), set for chlorpyrifos as a priority substance under the Water Frame Directive.

George et al. (2003) conducted two experiments in outdoor microcosms, one evaluating the effects of insecticides with a similar mode of action on zooplankton and one using insecticides with a dissimilar mode of action. The binary organophosphorous mixtures of chlorpyrifos and diazinon were equitoxic and conformed to the concentration addition model. The observed response of zooplankton exposed to the mixture of chemicals with different modes of action, consisting of chlorpyrifos, endosulfan and trifluralin, was a result of the susceptibility of individual taxa to the dominating pesticide in each mixture.

Besides herbicides, Relyea (2009) also evaluated the effects of a cocktail of five insecticides (malathion, carbaryl, chlorpyrifos, diazinon and endosulfan), as well as their individual effects on plankton and amphibian communities in aquatic outdoor mesocosms. For amphibians, there was an apparent direct toxic effect of 6.4 µg/L endosulfan that caused 84% mortality in leopard frogs and an indirect effect induced by diazinon (2.1 µg/L), which caused 24% mortality in leopard frogs. The mix of insecticides eliminated 99% of leopard frogs, while the gray tree frogs were not affected and grew nearly twice as large due to reduced competition. In all cases, the mix of insecticides impacted the zooplankton to a degree that was nearly identical to the individual effects of one or more of the individual insecticides. The conceptual models of concentration addition and independent action were not investigated. However, no strong contraindication results from the presented data.

The study (14 days) by Sibley et al. (2000) used a regression design to assess direct and indirect population-level responses of zooplankton and phytoplankton to a binary mixture of diazinon and chlorpyrifos at nominal concentrations of 0.44–44.0 µg/L (chlorpyrifos varying from 0.01 to 1.14 µg/L and diazinon from 0.43 to 43.2 µg/L). It concluded that no synergetic direct effects were observed and highlighted the complexity that often accompanies ecological interpretation of ecosystem-level impacts.

Effects of herbicide/insecticide mixtures were also evaluated in five studies (Fairchild et al., 1994; Grünwald, 2003; Hoagland et al., 1993; Relyea, 2009; Van Den Brink et al., 2009).

Fairchild et al. (1994) investigated the effects of a herbicide and insecticide mixture in mesocosms. The application of atrazine (50 µg/L) had a strong effect on the composition of the macrophyte community but had no clear effect on the macrophyte biomass. Therefore, the hypothesised ecological synergisms did not occur because of the rapid aqueous dissipation rate of the pyrethroid insecticide esfenvalerate, which was dosed twice in concentrations up to 1.71 µg/L with an interval of six weeks. The functional redundancy of the macrophyte community thus had no influence on the dissipation and the bioavailability of the insecticide.

Grunwald (2003) conducted separate mesocosm experiments with isoproturon and alpha-cypermethrin and their mixture (initial concentration of isoproturon up to 316 µg/L and initial concentrations of cypermethrin up to 1.9 µg/L). He found that taxa sensitive to either active ingredient showed no difference in their reaction to the mixture while the response of moderately susceptible taxa was altered via food web interactions. The level of indirect effects was also higher in the study, which evaluated the mixture compared to the studies with the individual compounds.

The individual and combined effects of atrazine and bifenthrin (up to a few µg/L each) on lake communities were investigated by Hoagland et al. (1993). The observed interaction effects indicated

that when either compound was introduced at ecologically realistic levels, its effects were masked if the other toxicant was present at high concentrations, and that the two pesticides did not act synergistically. Relyea (2009) also studied the combined effects of the five herbicides and five insecticides together on plankton and amphibian communities in aquatic outdoor mesocosms (see above for the effects of the mixtures of the five herbicides and five insecticides separately). The effects of the mixture on the amphibians, phytoplankton and zooplankton could largely be explained from the effects of the individual pesticides and large synergetic interactions were absent.

Van den Brink et al. (2009) studied the effects of chronic application of a mixture of the herbicide atrazine (up to 260 μg/L) and the insecticide lindane (up to 150 μg/L) in indoor freshwater plankton-dominated microcosms. For lindane, predictable effects based on literature values were reported while atrazine produced fewer effects than expected, probably due to decreased grazer stress on the algae as a result of the lindane application. Macroinvertebrates were the most sensitive group with a NOEC for community effects at 0.01 toxic units. Thus, the safety factors set by the EU for individual compounds were also found to ensure protection at community level in this mixture toxicity study, though not always at the population level. Both substances are also priority substances under the Water Framework Directive. The concentration of atrazine in the 0.01 TU treatment is comparable to the annual average quality standard under the WFD (European Commission, 2005d). However, the concentration of lindane at the NOEC in the mesocosm was a factor of 15 higher than the AA-EQS under the WFD (European Commission, 2005f), which aims to protect species at the population level as well.

Arts et al. (2006) treated mesocosms with a range of pesticides to simulate various spray drift rates resulting from a typical potatoes crop protection programme. A total of 15 treatments were made in the sequence typical of the spray calendar for potatoes with applications of prosulfocarb, metribuzin (both herbicides), lambda-cyhalothrin (insecticide), chlorothalonil and fluazinam (both fungicides). Multi and repeated stress played a small role within the applied pesticide package because of rapid dissipation of most substances and the absence of many simultaneous applications. At 5% spray drift of the recommended dose, long-term effects on zooplankton and macroinvertebrates were observed, some of which did not fully recover by the end of the present study. At 1% spray drift of the recommended dose, only slight transient effects were observed. This suggests that in this case risk assessments based on the individual compounds with an assumed spray drift of 1% would have been sufficiently protective for their uses in a crop protection programme.

These conclusions are largely supported by Van Wijngaarden et al. (2004), who evaluated the effects of a realistic pesticide application scenario in tulip cultivation in indoor microcosms and Wendt-Rasch et al. (2004), who evaluated a pesticide application scenario on outdoor microcosms differing in trophic status, although in both studies small effects were observed at the community level at 0.5% of the recommended dose. The pesticides used in these studies were the fungicide fluazinam, the insecticide lambda-cyhalothrin and the herbicides asulam and metamitron. In the study by Wendt-Rasch et al. (2004), lambda-cyhalothrin was applied in a dose that was ten times higher (i.e., 5% instead of 0.5% and so on). The observed effect on the species composition of periphytic algae, following the application of the 0.5% dose, could therefore be an indirect effect of the higher dose of lambda-cyhalthrin 5%. In the study by Van Wijngaarden et al. (2004), the dissipation of the substances from the water phase was fast, except for asulam. At 0.5% of the recommended doses short-term effects occurred at the community level on macro-invertebrates, mainly due to the presence of lambda-cyhalothrin. Pronounced effects were observed at the 2% and 5% of the recommended doses.

In short, most studies confirm that when pesticides affect the same biological groups, synergetic effects are not to be expected. When mixtures of pesticides that affect different biological endpoints (e.g., insecticides and herbicides) are evaluated, often increased indirect effects are noted due to food web interactions. When pesticides are applied over time, mimicking exposure by spray drift from a typical

pesticide application programme for a particular crop, the effects are often governed by the most toxic one and synergetic effects were not observed.

The PERPEST model predicts the effects of a given concentration of a pesticide based on the outcome of already performed experiments using experimental ecosystems (Van den Brink et al., 2006; Van Den Brink et al., 2002b). Currently, there are three versions of the PERPEST model, version 1 is the original version that was released in 2003 and described in Van den Brink et al. (2006; 2002b) and Van Nes and Van den Brink (2003). Version 2 was released in 2007 and has an extended data base and can also evaluate the effects of insecticide, herbicide and fungicide mixtures, separately. For this version, no updated manual is available but it has been thoroughly tested. Version 3 was released in 2009 and can predict the effects based on more complex exposure endpoints than the peak alone. It can predict the effects of single and multiple applications on the basis of the highest peak concentration and 7-day and 21-day Time Weighted Average concentrations. In 2010, the model will be elaborated so that it can also deal with mixtures of insecticides, herbicides and fungicides, after which it will be thoroughly tested. In 2010, an updated manual will also be available. All versions of the PERPEST model are available via www.perpest.wur.nl.

Table 5: Overview of microcosm and mesocosm experiments performed with pesticide mixtures. Results are discussed in the text

Reference	Systems	Pesticides	Endpoints	Duration (w)
Mixture approach				
Herbicides				
(Carder and Hoagland, 1998)	Indoor streams	Alachlor Atrazine	Algae	4
(Hartgers et al., 1998)	Indoor microcosms	Atrazine Diuron Metolachlor	Community metabolism Algae Macroinvertebrates Zooplankton	8
(Knauert, 2008; Knauert et al., 2008)	Mesocosms	Atrazine Isoproturon Diuron	Community metabolism Algae and macrophytes	25
(Relyea, 2009)	Mesocosms	Acetochlor Metolachlor Glyphosate, 2,4-D Atrazine	Community metabolism Algae Zooplankton Amphibians	5
Insecticides				
(Cuppen et al., 2002; Van Den Brink et al., 2002a)	Indoor microcosms	Lindane Chlorpyrifos	Community metabolism Algae Macroinvertebrates Zooplankton	14
(George et al., 2003)	Outdoor microcosms	Chlorpyrifos Diazinon	Zooplankton	1-4
(George et al., 2003)	Outdoor microcosms	Chlorpyrifos Endosulfan Trifluralin	Zooplankton	1-4

(Relyea, 2009)	Mesocosms	Carbaryl Malathion Chlorpyrifos Diazinon Endosulfan	Community metabolism Algae Zooplankton Amphibians	5
(Sibley et al., 2000)	Outdoor microcosms	Diazinon Chlorpyrifos	Algae Zooplankton	2
Herbicide/Insecticide				
(Fairchild et al., 1994)	Mesocosms	Atrazine Esfenvalerate	Community metabolism Algae Zooplankton Fish	20
(Grünwald, 2003)	Outdoor enclosures	Alpha-cypermethrin Isoproturon	Community metabolism Algae and macrophytes Zooplankton	15
(Hoagland et al., 1993)	Mesocosms	Atrazine Bifenthrin	Community metabolism Algae Zooplankton Fish	3
(Relyea, 2009)	Mesocosms	5 Herbicides 5 Insecticides (see above)	Community metabolism Algae Zooplankton Amphibians	5
(Van Den Brink et al., 2009)	Indoor microcosms	Atrazine Lindane	Community metabolism Algae Macroinvertebrates Zooplankton	16
Crop approach				
(Arts et al., 2006)	Experimental ditches	Prosulfocarb Metribuzin Lambda-cyhalothrin Chlorothalonil Fluazinam	Community metabolism Algae and macrophytes Macroinvertebrates Zooplankton	30
(Van Wijngaarden et al., 2004)	Indoor microcosms	Lambda-cyhalothrin Fluazinam Asulam Metramitron	Community metabolism Algae and macrophytes Macroinvertebrates Zooplankton	15
(Wendt-Rasch et al., 2004)	Outdoor microcosms	Lambda-cyhalothrin Fluazinam Asulam Metramitron	Community metabolism Algae and macrophytes	10

5 Conclusions and recommendations

Many studies have been performed on the mixture toxicity of pesticides during the last decade. In laboratory studies with simultaneous exposure to binary mixtures, synergism was seldom observed. In almost all cases the results could be explained by response addition or concentration addition or were somewhere in between. Deneer (2000) concluded in his review that combinations of an organophosphorus ester or a carbamate with either another organophosphorus ester or a synthetic pyrethroid yielded the largest deviation from concentrations addition. Again, the most obvious deviations from concentration addition were found for these combinations.

In a recent review for the European Commission, the use of the concentration addition model was proposed as the concept of mixture toxicity that is most convenient to be incorporated in policy (Kortenkamp et al., 2009). The reasons for this are twofold from a policy point of view. First, the model concept is generally more cautious than the concept of response addition. Nevertheless, the differences are usually small and hence, the outcome will not be overly conservative. Because synergism is only seldom observed, the risk of being insufficiently protective is low as well. Therefore, it can be concluded that concentration addition usually gives reasonable estimates of the joint toxicity of multiple compounds, especially if the number of compounds increases (i.e., not binary or ternary mixtures). A second reason for the use of concentration addition is that the model concept can make use of existing data such as NOEC, EC10, or EC50s by applying the toxic unit approach. This is different from the response addition concept, which needs full dose-response relationships for all species and all compounds to be assessed. Usually, this information is not available, which makes the response addition model less suitable for the risk assessment of mixtures. Concentration addition is therefore proposed to be implemented in policy frameworks.

New developments in the field of mixture toxicity include the assessment of the role of hormesis in mixture toxicity, the modelling of sequential exposure instead of simultaneous exposure, the application of species sensitivity distribution to mixtures of compounds and the effect of mixtures in mesocosms. The effect of hormesis (stimulation instead of inhibition at low concentrations) appeared to have no effect on mixture toxicity. It was concluded that dose-response curves of individual compounds were similar to those of mixtures of compounds with similar modes of action.

The modelling of the effects of sequential exposure to different compounds is a promising new development. It clearly shows that even when effects due to previous exposure are absent, this previous exposure can have effects on a subsequent exposure with the same or another substance. The model that is used for this purpose (Threshold Damage Model), however, needs not only dose-response functions, but also the time-dependent toxicity in the form of toxicokinetic and toxicodynamic parameters. Because these input parameters are not readily available but need to be generated, this modelling application will in first instance be applicable to a specific application of a sequence of pesticides, for which the target organisms have been investigated and the model input has been generated.

The application of species sensitivity distribution to mixtures of compounds requires that for every compound a species sensitivity distribution is available. If no full distribution is available, the slope of the SSD can be derived from a structurally related compound, for example. The application of the SSD method to mixtures is promising but the realism of the outcome is strongly dependent on the quality of the underlying SSDs for the single compounds. The SSD method only takes into account simultaneous exposure. The method is capable of dealing with concentration addition for groups of similar compounds and response addition for groups of compounds with different modes of toxic action at the same time. In this approach, the concepts of concentration addition and response addition are transferred to the level of species sensitivity of the SSD, instead of the response of individuals in a dose response relationship.

In mesocosm studies performed with mixtures of compounds, increased indirect effects are often observed due to food web interactions. These effects are rather complex and concepts such as response addition and concentration addition are difficult to assess. However, synergistic effects were not observed in the mesocosm studies with compounds that affect the same biological groups. This was the case in most laboratory studies as well.

To study the possibilities for implementation of these concepts for mixture toxicity in the risk assessment of pesticides, another RIVM project is ongoing. In this framework, the actual use of pesticides on different crops over time is examined, which gives better insight into the combination of pesticides and the sequence they are applied in. This will enable a better assessment of the combined effects of common practice in pesticide use.

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