Joint toxic effects on *Caenorhabditis elegans*
on the analysis and interpretation of mixture toxicity data

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Voor mijn moeder en vader

Abstract

In polluted areas organisms are generally exposed to mixtures of toxic chemicals rather than a single toxicant only. Since the number of mixture toxicity studies with regard to soil systems is limited, the research in this thesis was focused on investigating ecotoxicological consequences of combined exposure for soil invertebrates. Two topics were studied: i) population-level consequences of chronic combined exposure of individual organisms, and ii) the influence of interactions between the toxic compounds in the soil matrix on their joint toxicity. The soil dwelling, free living nematode *Caenorhabditis elegans* was chosen as test species.

A quantitative description procedure was proposed for interpreting the joint toxicity of chemical mixtures, compared to the toxicity of the individual components. It enabled the identification of four biologically relevant deviation patterns from either the additive or the independent reference model, by means of likelihood analysis: i) no deviation, ii) synergism/antagonism, iii) toxicant ratio- and iiii) effect level dependent deviation. Using these models, sublethal effects of chronic stress of binary mixtures of copper and cadmium, and copper and carbendazim on life cycle events of the nematode *C. elegans* were studied. The cadmium-copper effect on reproduction was transient: it changed from synergistic, to a toxicant ratio dependent deviation from additivity. The effect of copper-carbendazim was synergistic at low effect levels and antagonistic at high effect levels. The juvenile period was a relatively non-sensitive parameter, whereas the length of the reproductive period was relatively more sensitive. In conclusion, it should be realised that mixture toxicity may be transient and that interactions may differ among life history traits.

It was discussed that the effect translation of combined effects to the population level depended on three factors: i) the sensitivity of each life history trait to each of the toxicants, ii) the combination effect of the toxicants on each life history trait, iii) the sensitivity of λ to changes in each life history trait. A detailed analysis of mixture effects on the life history of nematode *Caenorhabditis elegans* showed that synergistic effects on reproduction were transferred to the population level, despite the low sensitivity of λ to changes in this trait.

Subsequently single and combined toxicity of copper-zinc, copper-cadmium, cadmium-lead, copper-carbendazim and copper-carbendazim-iprodione to the nematode *Caenorhabditis elegans* were studied in LUFA 2.2 soil , and the one-week population increase was estimated as toxicity endpoint. Metals with the highest partition coefficient affected the sorption of metals with the lowest partition coefficient when both were combined. However, comparing soil sorption characteristics with joint toxicity patterns did not give general results. Nevertheless, it was discussed that for identifying fundamental principles of joint toxicity in ecotoxicology, and for developing predictive models, ecological “mechanisms” should be investigated.

CONTENTS

Chapter 1

General Introduction

Mixture Toxicity

In natural environments, organisms are frequently exposed to mixtures of pollutants (Strojan 1978) and it is relatively uncommon to find sites polluted with one toxicant only (Walker *et al.* 2001). Since nearly all regulatory toxicity testing is carried out using single compounds (Walker *et al.* 2001), it can be questioned whether ecological conclusions based on single toxicity studies suffice for protection against combined toxic effects that generally occur in contaminated sites.

This issue is relevant for ecotoxicology in general, but the number of mixture toxicity studies with regard to soil systems is limited. For instance, an extensive review on mixture toxicity research mentioned that only 4 out of 167 studies were concerned with soil invertebrates (Hensbergen and van Gestel 1995) indicating that current knowledge about combined effects in soil systems is scarce. However, combined exposure may alter in the ecophysiology of pollutants in invertebrates and influence their susceptibility to toxicants. Understanding these processes is required for successful protection of soil communities (Spurgeon *et al.* 1994), therefore in this thesis the research is focused on investigating ecotoxicological consequences of combined exposure for soil invertebrates.

For analysing the toxicological response to multiple chemical exposure, many different approaches have been developed over the last decades. Most confusingly, different terms are frequently used for the same principles, and different principles carry the same or similar terminology (Greco *et al.* 1995). Important for conceptualising mixture toxicity is the classification of combination mechanisms. These classifications have been based on biological, pharmacological, mathematical and graphical considerations, which leaded to confusing terminology. The topic has been discussed for many years, the earliest references date back to late 19th century. However, Loewe and Muischnek (1926) are often mentioned as the first that introduced a model for similar acting toxicants, and Bliss (1939) is considered as the first author who published a certain classification of combination mechanisms.

This introductory chapter starts with an overview of how mixture effects are taken into account in risk assessment. Then the analysis of the toxic effect of single chemicals will be discussed shortly, with some useful information to understand the development of mixture toxicity concepts. Different conceptual approaches and classifications will be discussed in the sections that follow. The overview is definitely not exhaustive, but the aim is to show that different approaches were based on different (overlapping) research goals, and that these goals should be kept in mind in model application. Therefore the most relevant representatives of these aims will be sorted and discussed. Special attention will be paid to the work of Hewlett and Plackett, who contributed significantly to the development of mixture

toxicity concepts with their publications between 1948 and 1979. Their classification is frequently found in mixture toxicity literature. The development of mixture toxicity concepts has always been food for discussion for theoretical biologists and statisticians, therefore some parts of the overview are somewhat technical. Yet, the standpoints are well understandable for the mathematically untrained reader, and probably the most important to read for taking note of the position chosen in the thesis. In the overview the analysis of a simple binary mixture is assumed for simplicity.

Risk Assessment and Joint Toxicity

To determine the maximally permissible concentration of a certain chemical in the environment, sensitivity data (i.e. L(E)C50, NEC or NOEC values) for different relevant taxonomic groups have to be compiled from literature. In deriving save concentrations it is assumed that the (log-transformed) sensitivity values of a set of species can be described by some distribution: the species sensitivity distribution (Posthuma *et al.* 2002b). The distribution enables the derivation of relevant quantities with regard to this group of organisms, like environmental quality criteria and potentially affected fractions. In general, the theoretical hazardous concentration for 5% of the species (HC5) is considered as the environmental quality criterion.

Mixture effects have been included in legislation by dividing environmental quality criteria by an arbitrarily chosen factor 100 (Hensbergen and van Gestel 1995). Alternatively, calculations of the species sensitivity distribution can be adapted for mixtures (Traas *et al.* 2002) by applying toxicological theories on joint effects of compounds (which are discussed below). A crucial step herein is the extrapolation from single compound effects to combined effects. The adequacy and robustness of current mixture models in predicting combined effects are still matter of debate.

Single Dose Response Relationships

The relationship between the dose of the chemical and response of the biological system (ranging from cellular systems to whole communities) is of particular interest in (eco)toxicology. This relationship is indicative for the effectiveness and thus the toxicity of the compound. It can be quantified by dividing a group of test subjects into subgroups, expose each subgroup to a different (and increasing) dose of the chemical of interest and measure the response of the test subjects. Here, the test subjects are assumed to be individual organisms, but the same concepts are generally applied. Drawing conclusions from dose response data requires inter- and extrapolations and therefore statistical operations. The statistical analysis depends on the characteristics of the response variable. Basically, one can distinguish quantal responses from graded, or continuous, responses.

Quantal responses

Quantal responses are binary: either a response or not. For instance, mortality data are quantal. It may be conceptualised by stating that if a certain internal concentration level is exceeded, i.e. the tolerance, the organism responds (by for instance dying). The individual test organisms may not have the same tolerance, and different tolerance values may be distributed in the test population. When the organisms are exposed to a certain concentration of a chemical, the probability of response can be expressed as:

$$p = \Pr\{\omega > \omega'\} \tag{1}$$

Here, p indicates the probability of response, ω indicates the concentration of the chemical at the internal site of action and ω' denotes the tolerance concentration. Pr indicates a probability function, to quantify the probability of response. If the relation between the exposure concentration and the internal concentration at the site of action is very simple, than equation 1 can be rewritten into:

$$p = \Pr\{c > c'\} \tag{2}$$

where c is the exposure concentration and c' is the tolerance concentration. The exact value of p depends on the assumed distribution of tolerance values in the population. While analysing the data, the probability distribution of the individual tolerances in the population are often empirically assumed to be log-normal, log-logistic, or log-weibull distributions. Thus, the distribution function quantifies the probability density of the log-transformed tolerance concentrations present in the test population (Christensen 1984). The dose response relationship represents the cumulative distribution of the log-transformed tolerance concentrations of the test organisms. The sigmoïdal form of these curves poses some problems when the parameters of the distribution have to be estimated. Yet, assuming a log-normal distribution, the data can be linearised by transforming the response into normal equivalent deviates (NEDs), which are units of the standard deviation of the standard normal distribution. However, traditionally Probits are more often used, which are NEDs + 5. After transformation the data can be analysed by weighted linear regression:

$$\text{Probit}(p) = \alpha + \beta \log c \tag{3}$$

here, Probit denotes the function to transform the observed effect probability into Probit values, c is the concentration of the chemical and α and β are parameters. An example is shown in figure 1. The mortality probabilities found in the experiment were transformed in Probit values and subjected to linear regression. The Probit value for $\log(c) = 1$ is approximately 6 (the secondary axis in the right hand denotes NED values). To calculate the

mortality probability for this concentration, one has to integrate the area under the normal curve until Probit = 6, as shown in the right hand site figure 1. For obtaining more biologically relevant information from the data it is often more useful to fit

$$\text{Probit}(p) = 5 + \frac{1}{\sigma}(\log c - \log EC50) \tag{4}$$

where σ is the standard deviation of normal distribution of the tolerances over the log-concentrations, and EC50 indicates 50% effect (in case of survival: LC50), which is a measure of toxicity. For other distributions other transformations are available (Christensen 1984).

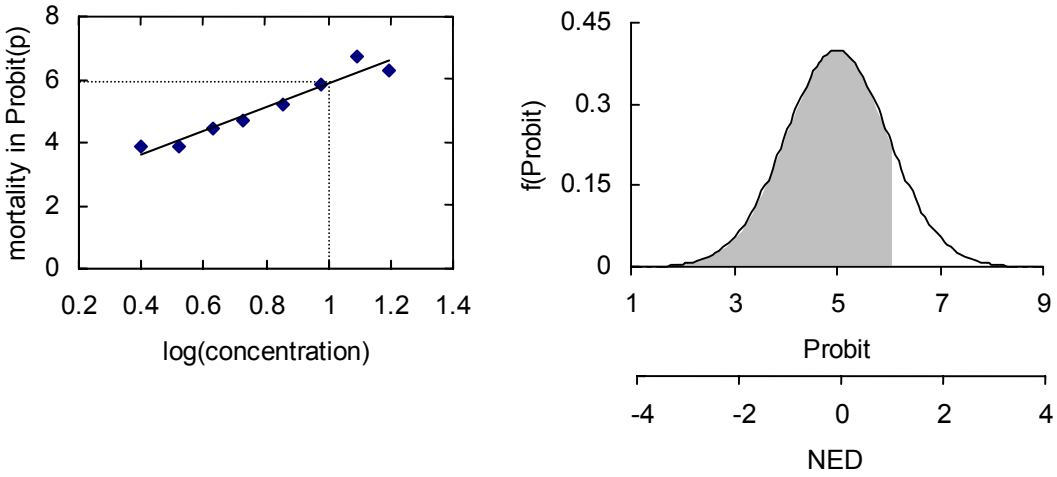

Figure 1. Example of Probit analysis. Left: an example a dose response analysis: the mortality of the beetle *Tribolium castaneum* after exposure to 1.2 % pyrethrins mg/10 sq.cm. (beetles were exposed on filter paper; data from Hewlett and Plackett (1950). The regression line is Probit(p) = 2.1 + 3.8log(c). Right: the area of integration (grey) to calculate the mortality probability for log(c) = 1. The secondary axis denotes the NED scale.

The concept of tolerance concentrations is not an absolute requirement for analysing dose responses, but should merely be considered as "a way of thinking". This concept is touched upon because is has been used in mixture toxicity concepts too. Alternatively, parameters of dose response curves (read: distribution functions) can be estimated by means of maximum likelihood methods (Kooijman 1981). Since the response variable is quantal, its distribution in every exposed subgroup is binomial:

$$f(p) = \binom{n}{k} p^k q^{n-k} \tag{5}$$

where p indicates the probability of responding, and q (= 1 - p) denotes the probability of not responding. The amount of exposed subjects in the subgroup is indicated by n, and the number of responding subjects is given by k. The values for p (and q) in every exposed

subgroup are defined by the dose response curve, and depend therefore on the model parameters. See Morgan (1992) for further details on maximum likelihood analysis of quantal data.

Continuous responses

Continuous responses are not binary, but can take (in principle) any value. The dose response relationship quantifies the effect of a chemical compound on some biological activity, like growth, number of offspring, or behaviour. For continuous responses the concept of a distribution of tolerances is not really applicable. However, the characteristics of continuous response variables usually correspond with the properties of a cumulative distribution function: they are sigmoïdal when related to log-concentrations, usually do not take negative values, and are frequently constrained between a maximum and a minimum value. Therefore cumulative distribution functions are often used to describe the relationship between the dose and a continuous response. Yet, probability based models based are constrained between 0 and 1, whereas continuous responses are not. The biological activity is usually high in the control group and decreasing due to the effect of the toxicant. Therefore, continuous responses are quantified by

$$Y = u_o f(c) \tag{6}$$

where u_o denotes the control response, and $f(c)$ is some algebraic form of a cumulative distribution function (Haanstra *et al.* 1985, Bruce and Versteeg 1992). The continuous response in a subgroup is usually assumed to be normally distributed. Assuming an equal variance in all m exposed subgroups reduces the negative log-likelihood function to

$$SS = \sum_{j=1}^{m} \left(Y_j - \hat{Y}\right)^2 \tag{7}$$

where $\hat{Y}$ indicates the modelled response in equation 6 and Y_j is the measured response. The minimum of equation 7, which is basically the calculation of the sum of squared residuals (SS), usually suffices as criterion for finding the best set of parameters for equation 6. Yet, to meet the assumption of equal variances among all subgroups various transformations of the response data can be considered.

Classification of combination mechanisms I
Primary goal: to identify the primary mode of joint action from dose response data

Combined effects
The first classification

The first generally accepted classification was developed by Bliss (1939). He recognised that the use of different definitions by different authors hampers a proper comparison of experimental results, and the derivation of general conclusions. Three types of joint action were distinguished:

- Independent joint action. The toxicants act independently and have different modes of toxic action. The tolerance to one component may or may not be correlated with the tolerance to the other. The toxicity of the mixture can be predicted from the dose response relationship for each constituent applied alone, and the correlation in tolerance to the two chemicals.
- Similar joint action. The toxicants produce similar but independent effects, so that one component can be substituted at a constant proportion for the other; variations in individual tolerance to the two components are completely correlated and parallel. The toxicity of the mixture is predictable directly from that of the constituents, if their relative proportions are known.
- Synergistic action. The effectiveness of the mixture cannot be assessed from that of the individual components, but depends upon knowledge of their combined toxicity when used in different proportions. One component synergises or antagonises the other.

The requirement for parallel dose response curves for similar joint action is often mentioned (Van der Geest *et al.* 2000). It can be justified from a pharmacological and a mathematical point of view. It will be discussed later in further detail. Bliss (1939) formulated similar joint action as

$$\text{Probit}(p)_{1,2} = \alpha + \beta \log(c_1 + kc_2) \tag{8}$$

where k indicates a ratio of toxicity and subscripts 1 and 2 indicate the two substances. Finney (1948) refined the theory of Bliss. Given two single dose response relationships:

$$\text{Probit}(p)_1 = \alpha_1 + \beta \log c_1 \qquad \text{Probit}(p)_2 = \alpha_2 + \beta \log c_2 \tag{9}$$

Finney stated that the following relationship holds, when the 50% effect level is considered (Probit$(p)_1$ = Probit$(p)_2$ = 5):

$$\log \psi = \log\left(\frac{LC50_1}{LC50_2}\right) = \frac{\alpha_2 - \alpha_1}{\beta} \tag{10}$$

where ψ denotes the relative toxicity of one substance compared with the other. Thus, the concentration of one component can be formulated in terms of an equivalent dose of the other:

$$\text{Probit}(p)_2 = \alpha_1 + \beta \log(\Psi c_2) \tag{11}$$

In addition, the concentration of a mixture of two chemicals in the relative amounts of f_1 and f_2 can be written in terms of an equivalent dose of one of the substances:

$$c_1 = (f_1 + \Psi f_2) c_{1,2} \tag{12}$$

Consequently, the effect of the mixture can be calculated from

$$\text{Probit}(p)_{1,2} = \alpha_1 + \beta \log((f_1 + \Psi f_2) c_{1,2}) \tag{13}$$

and the LC50 of the mixture can be deduced from:

$$\log((f_1 + \Psi f_2) LC50_{1,2}) = \frac{5 - \alpha_1}{\beta} \tag{14}$$

This way of quantifying mixture effects requires parallel dose response curves of both mixture constituents individually, and their joint effect.

A unified theory for non-interactive action

Hewlett and Plackett extended the conceptual framework introduced by Bliss (1939). Their most important contributions were published in Plackett and Hewlett (1948), Plackett and Hewlett (1952), and Hewlett and Plackett (1959). These theories still serve as the basis of today's thinking about mixture toxicity, although the technical execution of the models has rarely been performed. Yet, the work on non-interactive action has successfully been generalised and applied by Christensen and Chen (1985) and Chen and Chiou (1995).

According to Hewlett and Plackett the toxic effect of a mixture can be characterised by four types of joint action, which should be considered as extreme classifications on a continuous biological scale (table 1).

Table 1. The four possible combination mechanisms for the joint action of toxicants, as defined by Hewlett and Plackett (1959).

	Similar	Dissimilar
Non-interactive	Simple similar	Independent
Interactive	Complex similar	Dependent

Thus, the actual combination mechanism can be partially similar or partially interactive. Hewlett and Plackett defined the four extremes very strictly in order to enable the development of biologically based models. The action of a single drug is defined as follows: a part reaches the site of action, this part produces the biochemical and physiological changes that, if large enough, lead to the response. The remainder drains of to "sites of loss", which includes storage in tissues, metabolising by the organism and excretion. Two toxicants have a similar joint action when they elicit a certain quantal response by causing the same physiological system to react or fail, whether administered separately or jointly. Two toxicants have a dissimilar joint action when they elicit a certain quantal response by causing respectively different and distinct physiological systems to react or fail, whether administered separately or jointly. Interaction between two toxicants, A and B, occurs when the presence of A influences the amount of B reaching B's site of action; and/or the reversal. The extent of correlation of tolerances is explained from possible differences in sites of loss. So, two chemicals can have the same site of action, but they can differ in the way that they are metabolised to an inactive substance. Negative correlation might occur if the same enzyme system promotes one chemical to a more active compound, and another to an inactive one. The theoretical framework will be explained by first considering independent action, and then by simple similar action.

Independent joint action

In dissimilar action the actions of the individual chemicals might in fact be separate in time or (as well as) separate biochemically. Bliss (1939) stated that the correlation of the tolerances should be taken into account. Consider two toxicants A and B, to which a population of organisms is exposed. Their proportional effect (kill) are indicated by respectively p_1 and p_2, and their joint proportional effect by p. Hence, the proportions of surviving are $q_1 = 1 - p_1$, $q_2 = 1 - p_2$ and $q = 1 - p$. Suppose that $p_1 \geq p_2$. If the tolerance for A and the tolerance for B are completely and positively correlated, then the proportion that would have been killed by B are already killed by toxicant A. The opposite is true if $p_2 \geq p_1$. Thus,

$$p = p_1 \quad (p_1 > p_2) \qquad\qquad p = p_2 \quad (p_2 > p_1) \tag{15}$$

Suppose that the tolerance for A and the tolerance for B are uncorrelated. Then a proportion p_1 of the population will receive a lethal dose of A and will not survive. Since the

tolerances are uncorrelated, q_1 is a random sample with respect to its tolerance for B. Hence, a proportion of $p_2q_1 = p_2 - p_1p_2$ will receive a lethal dose of B. The total proportional response is therefore

$$p = p_1 + p_2 - p_1p_2 \qquad \leftrightarrow \qquad q = q_1q_2 \tag{16}$$

Plackett and Hewlett (1948) also allow for a negative correlation between the tolerance for A and the tolerance for B. Then, the proportion that survives toxicant A are those organisms that are most susceptible for toxicant B. As a consequence:

$$p = p_1 + p_2 \quad (p_1 + p_2 \le 1) \qquad\qquad p = 1 \quad (p_1 + p_2 \ge 1) \tag{17}$$

In conclusion, the proportional effect of the mixture can be considered as a function of the toxicity of the individual components in the mixture, combined with the degree of correlation of the tolerances present in the population. Therefore, a general expression should reduce to equation 15, 16 and 17 if the correlation of the tolerance for A and the tolerance for B are 1, 0 and –1 respectively. This can be accomplished by assuming that the joint distribution of log-tolerances in the population is bivariate normal distributed, which is basically extension of the concepts regarding single toxicant probit curves. In addition, in case of more than two toxic chemicals it can be assumed that the tolerance concentrations follow a multivariate normal distribution (Christensen and Chen 1985). To enable calculations, the probability of response can be expressed in normal equivalent deviates (NED), which are units of the standard deviation of the standard normal distribution. Let y'_1, y'_2 and y' be the NEDs corresponding to p_1, p_2 and p. For the response to compound A one can calculate:

$$p_1 = F(y'_1) = \int_{-\infty}^{y'_1} f(u)du \quad \text{, where } f(u) = \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{1}{2}u^2\right) \tag{18}$$

and y'_1 can be related to the concentration of the toxicant by (weighted) linear regression, as indicated in figure1. Thus, F denotes the inverse NED function and u the NED scale. Figure 1 also shows that the following relations hold: $p_1 = F(y'_1)$, $q_1 = F(-y'_1)$, $p_2 = F(y'_2)$, $q_2 = F(-y'_2)$, which do not hold for Probit values. The formula quantifying the non-responding fraction is simpler than the responding fraction. Assuming a bivariate normal distribution of tolerances, the proportion of organisms not responding can be calculated from:

$$q = \int_{-\infty}^{-y'_1} \int_{-\infty}^{-y'_2} \frac{1}{2\pi\sqrt{(1-\rho^2)}} \exp\left(-\frac{u_1^2 - 2\rho u_1 u_2 + u_2^2}{2(1-\rho^2)}\right) du_1 du_2 \tag{19}$$

The correlation of tolerances is indicated by ρ. The equation determines the area of integration of the bivariate distribution of log-transformed tolerance concentrations, to

calculate the proportions of organisms that did not respond to the toxicants. This is illustrated in figure 2. Figure 2A and 2B show two versions of the bivariate normal distribution as a function of their normal equivalent deviates. The NED scale is indicated u_1 and u_2. Figure 2A shows the bivariate normal distribution without correlation, and figure 2B shows the bivariate normal distribution with correlation ρ = 0.9. Figure 2C shows the u_1-u_2 plain, thus figure 2A or 2B from the top. If a certain concentration of toxicant A yields NED = 2 and toxicant B yields NED = 1 when the proportions of survivors are calculated, then the grey area represents the area of integration for calculating the survival in the mixture. From equation 19 it can be seen that if ρ = 0, figure 2A, the equation becomes the product of two single integrals, so that $q = F(-y'_1)F(-y'_1) = q_1q_2$ (equation 16).

Figure 2A

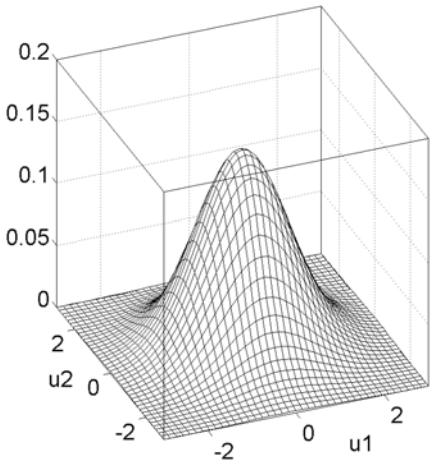

Figure 2B

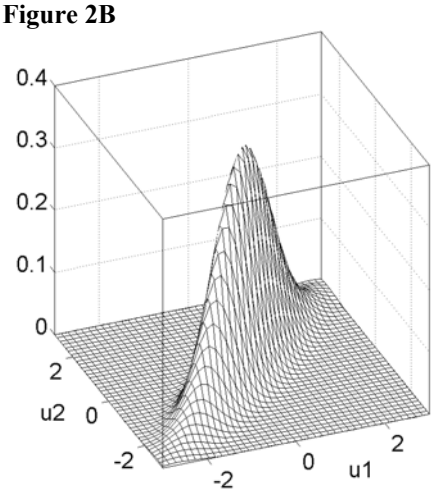

Figure 2C

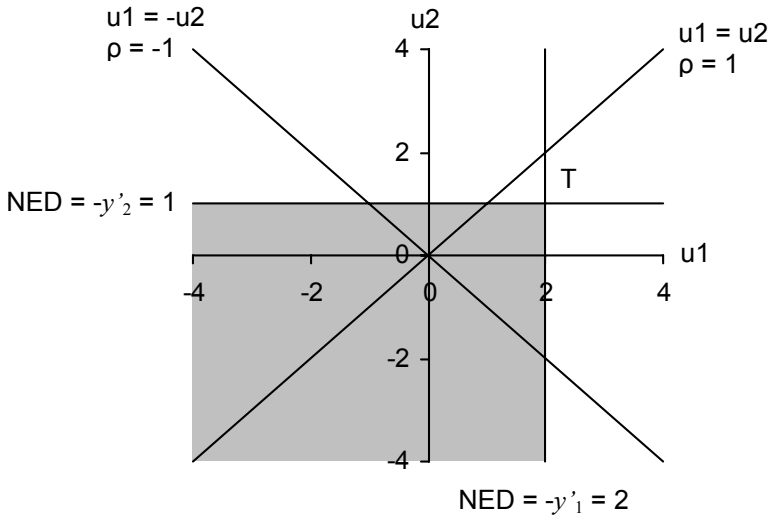

Figure 2. Two versions of the bivariate normal distribution as a function of its normal equivalent deviates (NEDs), and an integration region in the NED plain. The NED scale is indicated u1 and u2. Figure 2A shows the bivariate normal distribution without correlation, and figure 2B shows the bivariate normal distribution with correlation ρ = 0.9. Figure 2C shows the u1-u2 plain, which should be projected on the horizontal plain in figure 2A and figure 2B. See text for further explanations.

If $\rho \rightarrow 1$, the bivariate distribution shrinks to an univariate distribution above the line $u_1 = u_2$, as is illustrated in figure 2B. Considering figure 2C, the integration of the area above the line $u_1 = u_2$ is determined by the piece if this line that is located in the grey integration area. It can be seen that the length of this piece of line only depends on the smallest NED, thus the smallest q_i and therefore the largest p_i. Therefore, $q = F(-y'_1)$ if $p_1 \geq p_2$ and , $q = F(-y'_2)$ if $p_1 \leq p_2$, as described in equation 15. If $\rho \rightarrow -1$, the bivariate distribution shrinks to an univariate distribution above the line $u_1 = -u_2$. In figure 2C it can be seen that the area above the line $u_1 = -u_2$ can only be integrated if point T is located on the right site of this line, otherwise the line is not crossing the grey integration area at all. As long as T is located at the right site of the line $u_1 = -u_2$ the sum of the p_i's is smaller than 1 (equation 17). The proportion of survivors is represented by the piece of this line that is located in the grey integration area. The two pieces outside this area represent the proportional mortality. Summing the two proportions (after integration of the area above the lines) to calculate total mortality is equivalent to $p = F(y'_1) + F(y'_2)$, as in equation 17.

Simple similar joint action

For quantifying simple similar action, it can be assumed that at the common site of action the first toxicant is κ times as active as the second is. Thus, ω_1 of the first chemical (A) will have the same effect as $\kappa\omega_1$ of the second (B). Consequently, ω_1 of the first chemical together with ω_2 of the second can be expected to have a physiological effect equal to $(\kappa\omega_1 + \omega_2)$ of the second. For simple similar action one can therefore state:

$$p = \Pr\{\kappa\omega_1 + \omega_2 > \omega'_2\} \tag{20}$$

Now, κ is organism specific (because the tolerances are organism specific) and can be interpreted as follows. If an individual is twice as tolerant for B than for A, then A is twice as toxic. Consequently, ω_1 of chemical A will have the same effect as $2\omega_1$ of chemical B. The tolerance concentration is thus a measure of toxicant activity at the site of action, and κ can be defined as $\kappa = \omega'_2/\omega'_1$. Substituting this in equation 20 and dividing by ω'_2 yields

$$p = \Pr\left\{\frac{\omega_1}{\omega'_1} + \frac{\omega_2}{\omega'_2} > 1\right\} \tag{21}$$

and assuming a simple relationship between the concentration at the site of action ω and the exposure concentration c:

$$p = \Pr\left\{\frac{c_1}{c'_1} + \frac{c_2}{c'_2} > 1\right\} \tag{22}$$

Hewlett and Plackett (1959) assume some functional relationship between ω and c, which is not relevant for explaining the non-interactive mixture concepts. It is therefore omitted here.

Similar to individual dose response curves, and the model for independent joint action, the exact value of p depends on the assumed (joint) distribution of the tolerance for A and the tolerance for B. The calculation of p requires the quantification of the region of this distribution that has to be integrated, similar to the independent joint action model. Again, the formula quantifying the non-responding fraction is simpler than the responding fraction. Therefore the integration region defined by

$$q = \Pr\left\{\frac{c_1}{c'_1} + \frac{c_2}{c'_2} \le 1\right\} \tag{23}$$

will be evaluated. In the same way as in case of independent joint action, a bivariate log-normal distribution of tolerances can be assumed, and the effect of the individual toxicants can be quantified by transforming the proportional effects to NEDs. For toxicant i one can fit the following dose response curve:

$$y'_i = \alpha_i + \beta_i \log c_i \tag{24}$$

Similarly, the proportion of surviving organisms represents those individuals where the exposure concentration did not exceed the tolerance concentration, thus the proportional survival is functionally related to c'. Therefore, also the tolerance concentrations can be expressed on the NED scale, indicated by u (as in equation 18):

$$-u_i = \alpha_i + \beta_i \log c'_i \tag{25}$$

These functions can be substituted in equation 23 in order to calculate the proportional survival (q). Therefore the inverse relationship between effect and dose has to be calculated. Combining equation 24 and equation 25 yields:

$$\frac{c_i}{c'_i} = 10^{\frac{(y'_i + u_i)}{\beta_i}} \tag{26}$$

Assuming that the joint distribution of log tolerance concentrations in the population is bivariate normal, one can calculate the proportion of survivors from

$$q = \iint_R \frac{1}{2\pi\sqrt{\left(1-\rho^2\right)}} \exp\left(-\frac{u_1^2 - 2\rho u_1 u_2 + u_2^2}{2\left(1-\rho^2\right)}\right) du_1 du_2 \tag{27}$$

where the region of integration (R) is defined by:

$$10^{\frac{(y'_1+u_1)}{\beta_1}}+10^{\frac{(y'_2+u_2)}{\beta_2}}\leq 1 \tag{28}$$

What does this expression actually mean? Recall figure 2C, showing that for the independent model the calculation of the unaffected fraction proceeds by integrating volume above the region that is defined by $-y'_1$ and $-y'_2$ in the (u_1, u_2) plane. These were both calculated from the individual dose response curves. For the similar action model, one has to calculate the line in the (u_1, u_2) plane that is defined by equation 28. Figure 3A shows an example similar as figure 2C, it shows the integration region defined by equation 28 when y'_1 = -2, y'_2 = -1, β_1 = 4.5, β_2 = 8. Hence, calculating the proportion that survived the mixture exposure requires integration of the volume of the bivariate normal distribution above the grey region, which is the region defined by equation 28. This is generally smaller than the region the one defined by independent action. By introducing an extra parameter in equation 28 the region of integration defined by the similar action model can approach the region defined by the independent action model. The generalisation can be performed as follows (Hewlett and Plackett 1959):

$$10^{\frac{(y'_1+u_1)}{\lambda\beta_1}}+10^{\frac{(y'_2+u_2)}{\lambda\beta_2}}\leq 1 \tag{29}$$

Figure 3B shows that if $\lambda \rightarrow 0$, the region of integration defined by the similar action model approaches the region of integration defined by the independent model. Therefore, λ can be considered as a similarity parameter. If the correlation between the tolerances is complete and positive (ρ = 1), then the bivariate distribution shrinks to an univariate distribution above the line $u_1 = u_2$. If the NED of the mixture that corresponds with the mixture effect (p) is denoted by y', then (if λ = 1 and ρ = 1) one can calculate the mixture effect from

$$10^{\frac{(y'_1-y')}{\beta_1}}+10^{\frac{(y'_2-y')}{\beta_2}}=1 \tag{30}$$

In conclusion, the model for a binary mixture effect unifying the simple similar action model and the independent model is

$$q=\Pr\left\{\left(\frac{c_1}{c'_1}\right)^{1/\lambda}+\left(\frac{c_2}{c'_2}\right)^{1/\lambda}\leq 1\right\} \tag{31}$$

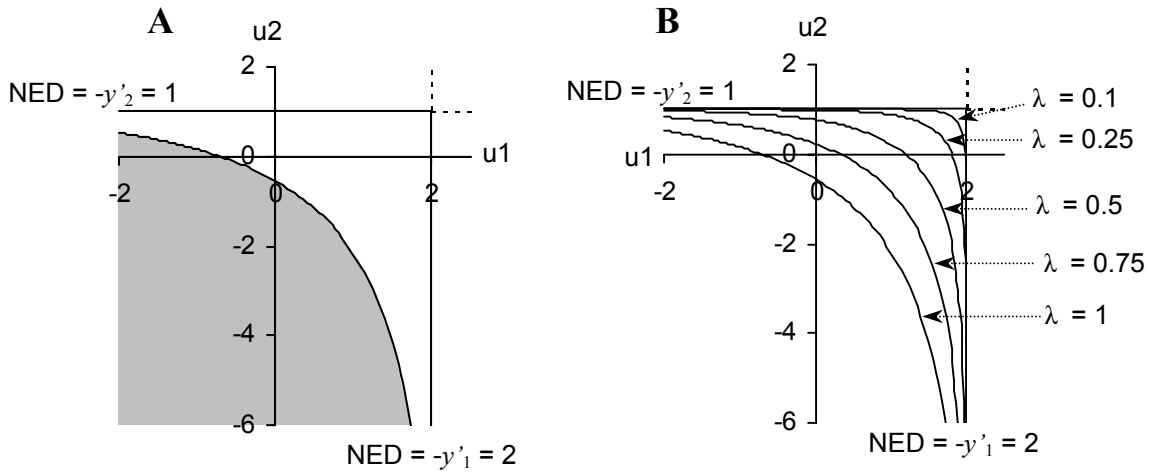

Figure 3. The integration region in the NED plain of the simple similar action model compared with the independent model. In figure 3A the integration region defined by simple similar action equation 28 is indicated by the grey colour. Figure 3B shows the effect of the similarity parameter (equation 29). If $\lambda \rightarrow 0$, the region of integration defined by the similar action model approaches the region of integration defined by the independent model.

Equation 31 is very general. It sets no restrictions on the distribution functions, (dis)similarity of action, correlations between the tolerances to the individual compounds and, most importantly, the slopes of the individual dose response relationships. Drescher and Boedeker (1995) used this expression to explore the quantitative relationship between the independent action and the simple similar action model.

Plackett and Hewlett discuss interaction intensively (Plackett and Hewlett 1952), but they never succeeded in formulating a satisfying biologically based expression for interactive chemicals.

Non-quantal mixture responses

The classification of Hewlett and Plackett was criticised by Ashford and Smith (1965). Subsequently, Ashford and Cobby (1974) and Ashford (1981) presented an alternative classification. They stated that the models available in literature involve the implicit or explicit assumption of a monotonic dose response relationship with regard to the individual constituents in the mixture. Yet, experimental data indicate that dose response relations are not always monotonic, such as in case of hormesis (Van Ewijk and Hoekstra 1993). Ashford (1981) proposed a framework of models accompanied by a classification of combination mechanisms, based on physiological/pharmacological considerations. The classification agrees partly with the work of Hewlett and Plackett. However, the models are explicitly developed for non-quantal, i.e. continuous, responses, and based on binding kinetics and the law of mass action. Within this modelling concept a biological system (for instance an organism) is divided into physiological subsystems (like nerve system, endocrine system,

cardio-vascular system) and active sites (enzyme systems), that influence the subsystem. The resulting classification is shown in table 2.

Table 2. The six possible combination mechanisms for the joint action of toxicants, as defined by Ashford (1981).

	None	Some	All
Common sites of action (similarity)	Dissimilar (noninteractive)	Partially similar	Similar
Common subsystems (dependence)	Independent (noninteractive)	Partially dependent	Fully dependent

For dissimilar action, the components of the mixture have no common sites of action. For partially similar joint action, the mixture components share some common sites. For similar joint action, each component affects each site. Independent joint action applies when the components affect no subsystem in common, whereas all other combination mechanisms should be considered as examples of dependent joint action.

Median effect models

Chou and Talalay approached combined toxicity from a pharmacological point of view, and published many articles on the subject between 1977 and 1996. They based their modelling exercise on enzyme kinetics, where equations were based on inhibitions of Michaelis-Menten and higher order kinetic systems (Chou and Talalay 1977, 1981). Their classification was based on a distinction between mutually exclusive reversible inhibitors (in our context: toxic chemicals) on enzyme systems and mutually nonexclusive reversible inhibitors. Their formulae on mutually nonexclusive reversible inhibitors were found to be incorrect for mathematical reasons by Greco *et al.* (1995), and are therefore omitted here. However, The formula for two mutually exclusive reversible inhibitors was (Chou and Talalay 1983):

$$\left[\frac{(f_a)_{1,2}}{(f_u)_{1,2}}\right]^{\frac{1}{m}} = \left[\frac{(f_a)_1}{(f_u)_1}\right]^{\frac{1}{m}} + \left[\frac{(f_a)_2}{(f_u)_2}\right]^{\frac{1}{m}} = \frac{(D)_1}{(D_m)_1} + \frac{(D)_2}{(D_m)_2} \tag{32}$$

where $(f_a)_1$, $(f_a)_1$, $(f_a)_{1,2}$ denote the affected fraction by respectively drug (or chemical) 1, 2 and their combination, f_u denotes the unaffected fraction, m the slope of the dose response curves, D the dose of a drug in the mixture and D_m the individual dose required for the medium (50%) effect. In this expression it can be seen that for quantifying the dose response of the mixture, the dose response curves of the individual compounds and the mixture need to be parallel, just as in the work of Finney (1948) (equation 14). Therefore, Chou and Talalay stated that similar

slopes indicated mutually exclusive inhibitors and that a dissimilar slope of the mixture indicated mutually nonexclusive inhibitors. However, Chou and Talalay rewrote the median effect model to the more general expression (Chou and Talalay 1984):

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \tag{33}$$

where D_x denotes the dose required for x% effect and CI is the combination index, where $CI <$ 1 indicates synergism, $CI = 1$ indicates summation and $CI > 1$ indicates antagonism. Greco *et al.* (1995) show that if this expression is not constraint by a slope of the mixture dose response curve, parallel dose response curves of the individual mixture components is not required for solving the equation. This is equivalent to the procedure outlined in chapter 2, and will therefore be discussed there. Unsatisfactorily, no robust criteria for identifying primary modes of joint action were formulated.

What does non-interaction mean?

There are many publications on defining interaction by attempting to identify the best model for quantifying pharmacological non-interaction (Hertzberg and MacDonell 2002). Terms like response multiplication, response addition, non-addition, and different interpretations of interaction (Calabrese 1995) have lead to confusion in this field (Hensbergen and van Gestel 1995). See Greco *et al.* (1995) for an extensive review on the subject and a list of publications where different terminologic frameworks are proposed. A list of terms as defined by the US EPA (2000) is presented in appendix I, to show the currently used jargon. In order to attempt to unify various approaches in one simple framework a classification was proposed at the Fifth International Conference on the Combined Effects of Environmental Factors in Saaresilkä, Finland, September 1992 (Greco *et al.* 1992). The classification, based on two toxic chemicals is shown in table 3.

Table 3. The eleven possible combination mechanisms for the joint action of toxicants, as published by Greco *et al.* (1992).

	2 chemicals active individually	2 chemicals active individually	1 chemicals active individually	no chemicals active individually
Effect greater than predicted	Loewe synergism	Bliss synergism	Synergism	coalism
Effect equal than predicted	Loewe additivity	Bliss independence	inertism	inertism
Effect less than predicted	Loewe antagonism	Bliss antagonism	antagonism	

The names Loewe and Bliss refer to the authors who were considered as the first who introduced the non-interaction models equation 33 (Loewe and Muischnek 1926) and equation 16 (Bliss 1939). Various authors have proposed these models as suitable references for quantifying pharmacological non-interaction, but there is no agreement on which one is best (Greco *et al.* 1995). The classification in table 3 is based on physiological considerations: the primary aim was to make statements on joint modes of action by identifying non-interaction. It may therefore be considered as an intermediate classification between the identification of the primary modes of joint action above and characterising dose response data below. The term interaction as used in this thesis will be defined in chapter 2.

Classification of combination mechanisms II
Primary goal: Characterising dose response data

Most of the modelling work discussed above was highly theoretical and rather technical. The low accessibility for experimental toxicologists and biologists hampered general use. As a result other researchers brought up ideas to handle combined toxicity data, more or less independently from the theoretical biologists.

Classification based on Isoboles

Mixture toxicity data can be depicted in an isobologram. In an isobologram for binary combinations, iso-effect lines are drawn to connect the points that yield the same response level. For a mixture of *n* substances it results in an iso-effect hyperplane in an *n*-dimensional space, where each axis represents concentrations of the individual compounds in the mixture. However, isoboles for mixtures of two substances are easy to depict, and therefore frequently used for the classification of mixture effects. Figure 4A shows the isoboles when chemical A is toxic and chemical B is not (Hewlett 1969). The horizontal line represents the effect of substance A if B does not influence the toxicity of A. If the presence of B enhances the toxicity of A, then the iso-effect line is represented by line 2, which is called synergistic. If the toxicity of A is diminished by the presence of B, the effect is represented by line 3, which is classified as antagonistic. If both A and B are toxic, then the isoboles can be represented by figure 4B (Sprague 1970). When a straight line connects both effect levels, the mixture effect is classified as additive. Line 2 shows increased toxicity, hence synergism. Line 3 is sometimes classified as partial additivity. Line 4 shows the isobole if chemical A is toxic and is not influenced by chemical B, and the reverse. Isoboles outside this square can be classified as antagonistic (line 5).

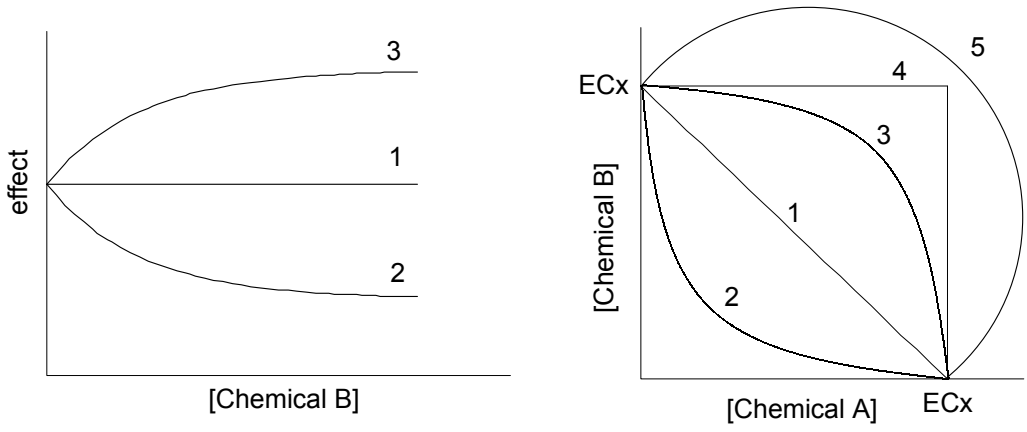

Figure 4. Isobolic representation of mixture effects. Figure 4A shows the effect of chemical B on chemical A: 1: no effect, 2: synergism, 3: antagonism. Figure 4B shows the isoboles when both chemical A and chemical B are toxic: 1: additive, 2: synergism, 3: partial additivity, 4: one chemical is non-toxic, without influencing the other, 5: antagonism.

The Toxic Unit Concept

In mixture toxicity research several quantification procedures have been developed which can be grouped by the term toxic-unit concept (Sprague 1970). The toxic unit is defined as the actual exposure concentration divided by its effect concentration, which is calculated from the individual dose response relationship. It scales all toxicants in the mixture relative to their toxicity. Since the *EC50* is the easiest effect concentration to calculate, it is the most frequently used quantity for scaling. The toxic units are added and the outcome is called toxic strength (*M*). Additive action is defined by a toxic strength of 1. For *n* chemicals the toxic unit model can be written as:

$$\sum_{i=1}^{n} TU_i = M \quad \text{, where} \quad TU_i = \frac{c_i}{EC50_i} \tag{34}$$

Thus, the *EC50* for calculating toxic units can also be *LC50*, or any other concentration defining a certain effect level, such as *EC10*, *EC20*, *EC25* etc. Therefore, *EC50* in equation 34 can be replaced by *ECx*, where *x* denotes the effect level of interest. Since additive action is defined by **Σ**TU = 1, equation 34 is equivalent to equation 30 and equation 33, thus simple similar action with ρ = 1, and the model for mutually exclusive reversible inhibitors.

Various methods have been developed to evaluate whether *M* deviates significantly from 1 (e.g. Van Wijk *et al.* 1994, Ince *et al.* 1999). In addition various mixture toxicity indices have been developed (MTI) based in the toxic-unit concept (De March 1987). For instance,

Könemann (1981) developed an index where the similar action model and the independent action model (ρ = 1) were taken as reference.

$$\mathrm{MTI} = \frac{\log M_0 - \log M}{\log M_0} \quad \text{, where} \quad M_0 = \frac{M}{TU_{\max}} \tag{35}$$

TU_{max} denotes the largest TU in the mixture. In case of independent action (ρ = 1) the MTI returns 0, which is called "no addition" by Könemann (1981). In case of similar action (concentration addition), the MTI is 1. In fact, Könemann (1981) based a mixture toxicity classification on this system:

Antagonism:	$M > M_0$	$TU_{max} > 1$
No addition:	$M = M_0$	$TU_{max} = 1$
Partial addition:	$M_0 > M > 1$	$TU_{max} < 1$
Concentration addition:	$M = 1$	$TU_{max} < 1$
Supra addition:	$M < 1$	$TU_{max} < 1$

And comparing the MTI with this classification yields the following scale:

antagonism	no addition	partial addition	concentration addition	supra addition
	↓		↓	
	0		1	→ MTI

Multiple linear regression

Alternatively, toxic mixtures have frequently been investigated by means of a factorial experimental design in combination with multiple regression (Narotsky *et al.* 1995, Nesnow *et al.* 1995, Groten *et al.* 1996). The advantage of this approach is that the experimental design and data analyses are statistically well developed (Neter *et al.* 1996). Deviations from the model can be quantified and tested for significance by including interaction parameters in the model. It can be shown that the model for multiple regression, without interaction terms, and the similar action model with ρ = 1 are equivalent (Gennings 1995). Consider the equation for multiple regression:

$$g(y) = \beta_0 + \sum_{i=1}^{n} \beta_i c_i \tag{36}$$

where $g(y)$ is a link function, y is the modelled response, and β_0 and β_i are parameters. The concentration of one toxicant resulting in a specified effect, y^*, can be calculated from

$$ECx_i = \frac{g(y^*) - \beta_0}{\beta_i} \tag{37}$$

where ECx_i denotes the concentration of toxicant i that results in effect y^*. Equivalently, equation 36 can be rewritten into

$$1 = \sum_{i=1}^{n} \frac{c_i}{(g(y^*) - \beta_0)/\beta_i} \tag{38}$$

Substituting equation 37 in equation 38 yields the similar action model with $\rho = 1$, the toxic unit concept, and the model for mutually exclusive reversible inhibitors. Note that the model allows for different slopes of the individual dose response relationships, indicated by $ß_i$.

Analysing mixture toxicity data with multiple regression is statistically robust, but the approach also raises questions. If the response variable is not quantal, it is difficult to decide which link function should be used. If the experiment is conducted at low effect levels, it can be assumed that this part of the dose response curve is linear, but at such low toxicity levels slopes might be flat and interactions hard to detect. In addition, the biological interpretation of higher order interaction terms can be difficult. It can however be an efficient design to detect deviations from additivity (Gennings 1995).

Classification of combination mechanisms III
Primary goal: Predicting mixture effects

An ultimate goal of mixture toxicity research is to predict combined effects when the effect of the individual compounds is known. However modelling tools are limited and approaches and classifications are mainly based on the modelling work presented above. At the moment a lot of work has been performed to determine empirically which model, the independent or the additive model, predicts combined toxicity data best. In general, the additive model seems to yield the best predictions. However, it was shown that the independent model predicted the effects of independent acting chemicals substantially better than the additive model (Backhaus *et al.* 2000). Therefore environmental researchers started to develop a "mixed modelling approach" for effect prediction (Posthuma *et al.* 2002a). The basic idea is to use the additive prediction if compounds are expected to act similar and the independent prediction if compounds are expected to act dissimilar (Junghans *et al.* 2002). It may be a useful approach for toxicants with a very specific mode of action, like organic pesticides. Yet, the interactions that may lead to increased or decreased toxicity are ignored.

Another method to quantify a prediction of a combined effect is to calculate toxic equivalency factors (TEF) of each compound in a mixture (Hensbergen and van Gestel 1995, Neumann 1996). It should actually be considered as a tool for risk assessment, rather than a biological model, and it is assumed to be best suitable for chemical congeners. It has for instance been worked out in detail for PCB's (Van Zorge *et al.* 1989). The TEF of chemical *i* in a mixture is defined as follows:

$$\mathrm{TEF}_i = \frac{\text{toxicity of the most toxic compound}}{\text{toxicity of compound } i} \tag{39}$$

where the toxicity can be quantified by NOECs, LOECs, L(E)C50s, etc. Thus, all chemicals are scaled relative to the most toxic compound in the mixture. The toxicity of the mixture can be quantified by the total toxic equivalency (TEQ):

$$\mathrm{TEQ} = \sum_i^n c_i * \mathrm{TEF}_i \tag{40}$$

where c_i denotes the concentration of chemical *i* in the mixture. It quantifies the toxicity of the mixture as a concentration of the most toxic one. It is basically an additive model where interactions are ignored.

Standpoints With Regard to Mixture Concepts

- *Different models?*

Various authors have proposed the same (or similar) ideas for mixture toxicity analysis. The toxic unit model was, in various forms, attributed to Loewe and Muischnek (1926), Hewlett and Plackett (1959), Sprague (1970), Chou and Talalay (1981), and Berenbaum (1985). It is equivalent to multiple regression, and graphically represented by the straight-line isoboles connecting the iso-effect levels in graph 4B (Appendix II). Since many authors applied it in proposals for mixture toxicity frameworks it carries many names like the additive model, the toxic unit model, the (simple) similar action model, concentration addition and model for mutually exclusive inhibitors. Plackett and Hewlett (1952) showed that the additive model and the independent model can be unified. It can therefore be concluded that, the expressions found in literature are basically not so different. The variability is based on how the models are used, rather than which model is used.

- *Use of the models*

When applying mixture models, the research aim should be clearly defined. It is frequently stated that the additive model can only be used appropriately in case of similar acting chemicals, and the independent model in case of dissimilar acting chemicals. This statement is

only true when the aim of the modelling exercise is to predict combined effects, because then the implicit model assumptions have to be satisfied. For characterising dose response data without a priori knowledge about modes of joint action, any model can be used irrespective of its mechanistic underpinning. Note however that different comparisons have different descriptive meanings because of the different mathematical structure of the expressions. The additive model compares relative toxicities, whereas the independent model compares probabilities of response.

Identifying the primary mode of joint action from dose response data is not possible. The problem is that different combination mechanisms can yield the same response patterns. Thus, a certain response pattern observed in the data cannot exclusively be assigned to a certain combination mechanism. The consequences of this phenomenon for data analysis are discussed in chapter 2.

- *Dose response curves*

It is also frequently stated that the individual dose response curves need to be parallel for applying the additive model (Hensbergen and van Gestel 1995, Van der Geest *et al.* 2000). However, the algebraic formulations used by Hewlett and Plackett (1959), Greco *et al.* (1995), Haas *et al.* (1996), and in chapter 2 show that it is not required mathematically, if the additive model is not constraint by a specific slope for the mixture effect. Yet, pharmacologically it can be argued that if two substances act on the same receptors and if they are stored, excreted and/or metabolised in the same way, this similarity should be expressed in parallel dose response curves. But what does the slope of the dose response curve actually mean? For the Weibull function, the slope was identified as the average number of toxicant molecules per receptor (Christensen and Chen 1985). Similarly, for the log-logistic model the slope was found to represent the number of molecules of inhibitor, interacting with one molecule of an enzyme species (Chou and Talalay 1981). Thus, if two molecules interact with the **same** receptor instead of only one, the slope is twice as flat. More generally, if twice as much molecules of substance A than substance B act on the same receptors and if twice as much is stored, excreted and/or metabolised in the same way, then the dose response curve for substance B can be expected to be twice as steep as the dose response curve for A. Therefore, parallel individual dose response curves is **no** requirement for similar modes of joint action, and hence not indicative.

Aim of the Study

The mixture toxicity concepts are based on toxicological considerations. Yet, the research interest as introduced in the first paragraph is ecotoxicological, thus ecological aspects should be incorporated. Soil invertebrates may be exposed to chemical mixtures throughout their whole life, and various life cycle events may be altered. The response of an organism to a chemical mixture may dependent on the response parameter analysed. For instance, an organism might experience a synergistic effect on body size and an antagonistic effect on

reproduction. What is the relevance of such an observation? Yet, it can be hypothesised that ecological consequences of combined effects on individuals depend on the translation to higher levels of biological organisation, such as populations or communities. From that perspective it is required to understand if and how mixture effects on individuals are translated to the level above.

Furthermore for soil systems it was recognised that combined effects encompass several levels of interaction (Calamari and Alabaster 1980). First, the combined effect is subject to chemical interactions in the soil matrix, influencing sorption and hence bioavailability. Second, physiological interactions during the uptake processes by the organism can affect the joint effect of the chemicals. Third, interactions at the intoxication processes, at the receptors and target sites can occur, which affect toxicodynamics and therefore the combined effect. Insight in the relative importance of these interaction levels is of great value (Posthuma *et al.* 1997, Van Gestel and Hensbergen 1997, Jason and Lanno 2000).

The aim of the research described in this thesis was to include these ecological aspects in mixture toxicity analysis, in order to improve the understanding of the ecological consequences of combined exposure. The soil dwelling, bacterivorous nematode *Caenorhabditis elegans* was chosen as test organism. It is frequently used in ecotoxicity testing and may be considered to represent the metazoa which are important for decomposition and nutrient cycling in soil systems (Freeman *et al.* 1999).

In order to enable the analysis of complex biological interactions, it was decided to limit the research to relatively simple (mainly binary) mixtures. Six model test chemicals were chosen: copper, zinc, cadmium, lead, carbendazim and iprodione. Copper is an essential metal, because of its specific incorporation into a large number of enzymatic and structural proteins. It has the ability to function as an electron transfer intermediate, therefore it is important for oxidation/reduction enzyme activities. On the other hand, copper intoxication can lead to direct protein damage, structural impairment of essential metal binding sites (Alt *et al.* 1990), and to the production of oxyradicals, hence cellular injury (Goldstein and Czapski 1986). Zinc is also an essential metal, it is important in membrane stability, in over 300 enzymes, and in the metabolism of proteins and nucleic acids (Simon-Hettich 2001). Mechanistic studies on zinc toxicity often yield contradictory results (Simon-Hettich 2001), but cytotoxic and teratogenetic effects have been reported (Klaassen *et al.* 1986). Cadmium is a non-essential metal, it replaces zinc in various proteins (Vallee and Ulmer 1972) and causes cellular lesions such as disrupted cytosomes and shortened microvilli (Popham and Webster 1979). Lead is also a non-essential metal. From both *in vivo* and *in vitro* studies, lead exposure has been reported to have effects on virtually all neurotransmitter systems (US EPA 1986). The nervous system is therefore an important target site (Klaassen *et al.* 1986). Carbendazim (MBC; methyl-2-benzimidazole carbamate) is a systemic fungicide. Benzimidazoles interfere with DNA synthesis and inhibit of cellular development (Tomlin 1997). Iprodione belongs to the group of dicarboximides and is also a systemic fungicide. It inhibits germination of spores and growth of fungal mycelium (Tomlin 1997). The biochemical mode of action is not known. At high dosage, uncoupling of mitochondrial electron transport has been recorded, but this is not thought to be the primary mode of action.

The chapters that follow will consider the effects of combined exposure in settings of increased biological complexity. Chapter 2 should be considered as a continuation of the given overview of the mixture toxicity concepts. It discusses the application of the mixture models in bioassay studies and the interpretation of the results, after considering the standpoints. In chapter 3 combined effects at the individual level are studied and in chapter 4 the translation of mixture effects from the individual to the population level is discussed. In chapter 5 mixture effects on population performance of *C. elegans* in soil are analysed. Chapter 6 summarises the obtained results, and discusses some implications for risk assessment and possibilities for future research.

Acknowledgements
The literature on mixture toxicity concepts is incredibly confusing. My writing of this chapter has significantly benefited from literature studies provided by Kees van Gestel and Bill Greco.

Appendix I

Mixture toxicity terminology as defined by the US EPA (2000).

Additivity
When the "effect" of of the combination is estimated by the sum of the exposure levels or the effects of the individual chemicals. The terms "effect" and "sum" must be explicitly defined. Effect may refer to the measured response or the incidence of adversely affected animals. The sum may be a weighted sum (see "dose addition") or conditional sum (see "response addition").

Antagonism
When the effect of the combination is less than that suggested by the component toxic effects. Antagonism must be defined in the context of the definition of "no interaction", which is usually dose or response addition.

Chemical antagonism
When a reaction between the chemicals has occurred and a new chemical is formed. The toxic effect produced is less than that suggested by the component toxic effects.

Chemical synergism
When a reaction between the chemicals has occurred and a different chemical is formed. The toxic effect produced is greater than that suggested by the component toxic effects, and may be different from effects produced by either chemical by itself.

Complex interaction
When three or more compounds combined produce an interaction that cannot be assessed according to the other interaction definitions.

Dose additivity
When each chemical behaves as a concentration or dilution of every other chemical in the mixture. The response of the combination is the response expected from the equivalent dose of an index chemical. The equivalent dose is the sum of component doses scaled by their toxic potency relative to the index chemical.

Index chemical
The chemical selected as the basis for standardisation of toxicity of components in a mixture. The index chemical must have a clearly defined dose-response relationship.

Inhibition
When one substance does not have a toxic effect on a certain organ system, but when added to a toxic chemical, it makes the latter less toxic.

Masking
When the compounds produce opposite or functionally competing effects at the same site or sites, so that the effects produced by the combination are less than suggested by the component toxic effects.

No apparent influence
When one substance does not have a toxic effect on a certain organ or system, and when added to a toxic chemical, it has no influence, positive or negative, on the toxicity of the latter chemical.

No observed interaction
When neither compound by itself produces an effect, and no effect is seen when they are administered together.

Potentiation
When one substance does not have a toxic effect on a certain organ or system, but when added to a toxic chemical, it makes the latter more toxic.

Response additivity
When the toxic response (rate, incidence, risk or probability of effects) from the combination is equal to the conditional sum of component responses as defined by the formula for the sum of independent event probabilities. For two chemical mixtures the body's response to the first chemical is the same whether or not the second chemical is present.

Synergism
When the effect of the combination is greater than that suggested by the component toxic effects. Synergism must be defined in the context of the definition of "no interaction", which is usually dose or response addition.

Unable to assess
Effect cannot be placed in one of the above classifications. Common reasons include lack of proper control groups, lack of statistical significance, and poor, inconsistent, or inconclusive data.

Chemical mixture
Any set of multiple chemical substances that may or may not be identifiable, regardless of their sources, that may jointly contribute to toxicity in the target population. May also be referred to as a "whole mixture" or as the "mixture of concern".

Components
Single chemicals that make up a chemical mixture that may be further classified as systemic toxicants, carcinogens, or both.

Complex mixture
A mixture containing so many components that any estimation of its toxicity based on its components' toxicities contains too much uncertainty and error to be useful. The chemical composition may vary over time or with different conditions under which the mixture is produced. Complex mixture components may be generated simultaneously as by-products from a single source or process, intentionally produced as a commercial product, or may coexist because of disposal practices. Risk assessments of complex mixtures are preferably based on toxicity and exposure data on the complete mixture. Gasoline is an example.

Similar components
Single chemicals that cause the same biologic activity or are expected to cause a type of biologic activity based on chemical structure. Evidence of similarity may include similarly shaped dose-response curves, or parallel log dose-probit curves for quantal data on the number of animals (people) responding, and the same mechanism of action or toxic endpoint.

Similar mixtures
Mixtures that are slightly different, but are expected to have comparable characteristics for fate, transport, physiologic processes and toxicity. These mixtures may have the same components but in slightly different proportions, or have most components in nearly the same proportions with only a few different (more or fewer) components. Similar mixtures cause the same biologic activity or are expected to cause the same type of biologic activity due to chemical composition. Similar mixtures act by the same mechanism of action or affect the same toxic endpoint. Diesel exhausts from different engines are an example.

Chemical classes
Groups of components that are similar in chemical structure and biologic activity, and that frequently occur together in environmental samples, usually because they are generated by the same commercial process. The composition of these mixtures is often well controlled, so that the mixture can be treated as a single chemical. Dibenzo-dioxins are an example.

Appendix II

Because equation 34 can be generalised to all effect levels, the binary toxic unit model can be written as:

$$1 = \frac{c_1}{ECx_1} + \frac{c_2}{ECx_2}$$

where x denotes a certain effect level. We may write c_2 as a function of c_1:

$$\frac{c_2}{ECx_2} = 1 - \frac{c_1}{ECx_1}$$

$$c_2 = ECx_2 - \frac{ECx_2}{ECx_1} c_1$$

In this way, the formula takes the form y = a + bx, thus the relation between c_1 and c_2 is a linear one, where the intercept is defined by ECx_2, and the slope by the ratio of ECx_2 to ECx_1. This is mathematical expression for the straight-line isobole (line 1) in figure 4B. The reader is invited to check the formula if either c_1 or c_2 is 0.

Chapter 2

Mixture toxicity of environmental contaminants: revisiting strategies for data analysis and interpretation

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Abstract

The environmental effect of pollution is caused by the simultaneous exposure to different toxic compounds. For studying interactions between toxic chemicals a biologically meaningful quantitative description of mixture toxicity data is essential. Therefore a quantitative description procedure is proposed for interpreting the joint toxicity of chemical mixtures, compared to the toxicity of the individual components. Mixture toxicity classification concepts are based on two non-interaction models: additive joint action and independent joint action. Since combination mechanisms cannot be identified from dose response data a rigid separation between these models is justified. The independent model is a statement about relationships between probabilities of response, and the additive model is a statement about the relative toxicities. They do not constitute real alternatives and can therefore both be used as a reference from which mixture toxicity data can deviate, thus enabling the characterisation of the actual effect. Four biologically relevant deviation patterns can be identified by means of likelihood analysis: no deviation, absolute deviation (synergism/antagonism), toxicant ratio dependent deviation, and effect level dependent deviation. Using data sets from cell lines, invertebrates and fish, we demonstrate the versatility of the likelihood-testing framework for data quantification and interpretation of interactive joint action.

Introduction

Since environmental pollution is caused by the presence of various contaminants simultaneously, ecotoxicologists have increasingly focused on the development of a firm scientific framework to address joint toxic effects. The analysis of the adverse effects of chemical mixtures comprises two main principles: the principle of additive (or similar) action (Loewe and Muischnek 1926) and the principle of independent action (Bliss 1939). Both principles assume non-interactive joint action and are embedded in a mechanistic context that enables the prediction of a combined effect, based on the effect of the individual chemicals (Hewlett and Plackett 1959). The additive action principle applies to compounds having similar modes of action *i.e.* they act as if they share the same pathway to the site of action, affecting the same physiological system. This is supported by a profound theoretical study based on enzyme kinetics (Chou and Talalay 1977, 1981). The independent action principle relates to independent modes of action of the chemicals. The individual compounds do not interfere with each other during exposure, uptake and toxic action. Theoretical support is based on statistical reasoning (Plackett and Hewlett 1948). Indeed, experiments have shown that both model concepts predict the combined toxic effect quite well (Altenburger *et al.* 2000, Backhaus *et al.* 2000). However, if mechanistic knowledge is absent and the joint effect has to be judged from the dose response data, the application of the models is by no means straightforward. Here, we propose a robust framework for the analysis of mixture toxicity data to enable a detailed quantitative description of how joint effects deviate from a non-interaction model of interest. The practical application and the biological interpretation are emphasised.

Modelling approaches

In order to select which quantitative approach is most suitable for analysing mixture toxicity data the aim of the analysis should be clearly defined. This may seem obvious, but the strong link between aim and modelling approach is not always thoroughly considered when quantitative procedures in mixture toxicity are discussed. It is therefore worthwhile to examine a few (of course partly overlapping) aims found in literature briefly, before presenting our perspectives.

The classification of combination mechanisms presented by Bliss (1939) was an important stimulus for quantitative mixture toxicity research. This classification was adopted and worked out in detail by Plackett and Hewlett (1948, 1952) and Hewlett and Plackett (1959). They compiled a set of mathematical formulae within a framework of four clearly defined combination mechanisms, unifying independent and similar joint action. Their aim, and therefore the initial aim of the classification, was to identify the primary mode of joint action from dose response data by means of biologically based modelling.

The pitfalls of this approach are still not always fully recognised. There are no satisfying criteria to identify a certain combination mechanism in the data. Although it is possible to model an expected effect supposing some biological assumption (*e.g.* similar joint action), it cannot be claimed that this assumption actually applies if the expected effect has been found. On the one hand there is no reason to reject the assumption, but on the other hand the underlying cause might be completely different (*i.e.* mechanistic interaction). Thus, if similar action explains the data well, then there is no indication that the mode of joint action was actually similar, which limits the mechanistic interpretation and extrapolation of the results.

Another aim of mixture toxicity analyses was to identify a scientifically sound reference model, again focusing on the independent action and additive joint action models. In essence, two research questions needed to be addressed. First, the qualification of interaction by identifying which model represents the non-interactive response best (Berenbaum 1985, Greco *et al.* 1995). Second, the determination of the most suitable model for predicting combined effects empirically, without knowledge about interactions between the individual compounds (Payne *et al.* 2000). These analyses require a sound comparison between the independent action model, the similar action model and the data (Drescher and Boedeker 1995). Particularly the second research question is primarily relevant for modelling exercises in risk assessment.

Another type of toxic mixture research is to study the interactive effects experimentally. Two approaches are frequently found in literature: 1] factorial design with multiple regression or 2] application of mixture toxicity indices, like the Toxic Strength. The aim of these studies and mixture toxicity analysis in general is understanding the biological and chemical processes that drive joint toxic effects (Feron and Groten 2002, Gennings *et al.* 2002). For this type of research a biologically meaningful and robust quantitative description of mixture toxicity data is essential.

It is exactly at this point where we would like to make our contribution. We have derived a set of equations, aiming at a relatively easy interpretation of the joint toxicity of chemical mixtures, compared to the toxicity of the individual components. Therefore we have applied a rigid separation between the additive joint action model and independent joint action model. Although mathematically related, combining them has no practical advantages. If mechanistic knowledge is poor it is impossible to decide whether an observed deviation from a non-interaction model is due to interaction or due to (partial) similarity. A more pragmatic approach is to judge the non-interaction models on their mathematical properties, without a mechanistic interpretation. In their mathematical definitions they do not constitute real alternatives (De March 1987b). The independent model is a statement about relationships between probabilities of response, whereas the additive model is a statement about the relative toxicities. From that perspective they are just two different methods to compare the actual toxicity of the mixture with a reference, and they are mutually exclusive.

Comparing dose response data with a reference enables the determination whether the reference model can describe the actual effect adequately. More importantly, it enables the characterisation of the actual mixture effect by identifying and quantifying of the pattern of deviation from the reference, subject to the specific combinations of the individual

components in the mixture. This deviation pattern is thus equivalent to statistical interaction. For quantitative analyses, the most relevant deviation patterns to identify in the data have to be defined a priori. The following deviation patterns may be considered as biologically most important (figure. 1):

- No deviation. The actual effect of the mixture is well described by one of the chosen reference situations (fig. 1A).
- Absolute synergism or antagonism. When all combinations of a mixture are either more toxic or less toxic than the reference situation, synergism or antagonism is observed (fig. 1B).
- Toxicant ratio dependent deviation. When the deviation pattern is subject to the relative amount of (one of) the toxicants in the mixture, the effect is toxicant ratio dependent. Considering two substances, synergism can be observed if toxicant A is relatively dominant in the mixture whereas antagonism can be observed if toxicant B is relatively dominant in the mixture (fig. 1C).
- Effect level dependent deviation. When, for example, antagonism is observed at low effect levels and synergism at high effect levels, an effect level dependent deviation from the reference is identified (fig. 1D).

A quantification procedure requires mathematical formulae and a robust method of statistical testing. Haas and Stirling (1994) and Haas *et al.* (1996) have introduced a nested modelling approach, which is adopted and expanded in this study. Two data sets serve as example for the technical execution of the analysis. Yet, a characterised effect should be considered as the outcome from a "black box". To study interaction effects in detail, researchers are increasingly interested in how a given mixture influences different response variables (*e.g.* Khalil *et al.* 1996, Forget *et al.* 1999, Fernandez and Beiras 2001), and how combined effects depend on (bio)chemical factors. Previous studies have for instance focused on the influence of bioavailability and uptake characteristics of metals on their joint toxic effects (Posthuma *et al.* 1997, Van Gestel and Hensbergen 1997, Jason and Lanno 2000), and on the role of abiotic (stress) factors, as low oxygen, temperature, pH, or salinity (*e.g.* Roberts *et al.* 1990). Within this modelling context this means comparing characterised effects, which is illustrated by a reanalysis of a joint toxicity time series experiment (Chang *et al.* 1985) and the quantification of mixture effects at the DNA, RNA and protein level (Ryan *et al.* 1992).

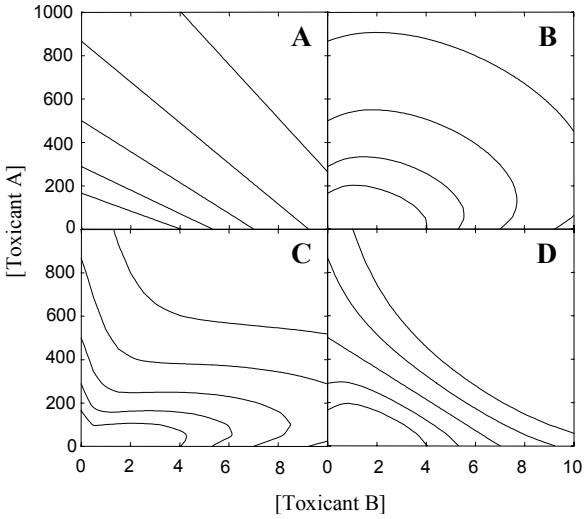

Figure 1. Isobolic representation of the toxic effect of a binary mixture, the additive model is chosen as reference. In each graph the lines indicate (from left to right) 10%, 25%, 50%, 75% and 90% effect. Figure 1A depicts no deviation from additivity, 1B depicts an antagonistic deviation from additivity, 1C depicts a toxicant ratio dependent deviation from additivity and 1D depicts an effect level dependent deviation from additivity.

Analysis Procedure

Reference models. In this derivation a usual (non-quantal) dose response relationship is assumed functionally related the toxicant concentration ($f(c)$), *i.e.* a certain biological response is measured, which is high in the control group, and non-linearly decreasing down to zero due to an increased concentration of the toxicant. Thus, the biological response is defined as the opposite of the toxicological effect. In terms of the correlation of tolerances for the individual compounds (which is actually more relevant for quantal response data), we just assume full correlation for the additive model, and no correlation for the independent model, as is frequently (implicitly) done in literature.

The additive joint action model is defined as $\Sigma_i^n TUx_i = 1$, where TUx_i is the dimensionless toxic unit that quantifies the relative toxicity of the individual component i in the mixture of n chemicals, from $TUx_i = c_i(ECx_i)^{-1}$ (Sprague 1970, Berenbaum 1985). Here, c_i denotes the concentration of each chemical in the mixture, and ECx_i is the effect concentration resulting in the same effect as the mixture, where x indicates the effect level of interest. The toxic units should add up to one to describe straight-line isoboles. The additive model can be generalised and rewritten as

$$\sum_{i=1}^{n} \frac{c_i}{f_i^{-1}(Y)} = \exp\left(G\right) \qquad (1)$$

to describe deviations from additivity for a mixture of n compounds (Haas and Stirling 1994, Haas *et al.* 1996). Here, the effect concentration resulting in biological response Y, is calculated from the inverse function of the dose response relationship (f_i^{-1}). The inverse dose response relationship represents ECx_i, and quantifies the concentration of the individual compound that results in the same effect as the mixture if it would have been administered alone. Deviations from additivity are quantified by the extent function (G). Note that if $G = 0$, the right hand sight becomes 1, *i.e.* the additive function. Equation 1 gives an implicit relationship between the concentrations of the individual compounds and the combined biological response, and should be solved by iteration.

The dose response relationship for independent joint action can be calculated by multiplying the complements of the proportional toxic effects of the individual compounds in the mixture ($h_i(c_i)$). Yet, the control response (u_0) should be estimated as well. Consequently, the independent model may be defined as:

$$Y = u_0 \prod_{i=1}^{n} h_i(c_i) \tag{2}$$

Quantifying deviation patterns from the independent model requires the incorporation of an extent function in equation 2. However, since the biological response has been assumed to be restricted between a control response value and 0, extending equation 2 easily results in extrapolation problems. Therefore a transformation step has to be applied. Thus, the generalised independent model may be written as:

$$Y = u_0 \Phi\left(\Phi^{-1}\left(\prod_{i=1}^{n} h_i(c_i) \right) + G \right) \tag{3}$$

where the deviation function is added to the unaffected fraction of the mixture, transformed by the inverse of the standard cumulative normal distribution function (Φ^{-1}). After transforming back (by applying the standard cumulative normal distribution function (Φ)), the divergence of the mixture effect from the independent reference can be quantified. The standard cumulative normal distribution function is an arbitrarily chosen transformation function; any other cumulative distribution function can be used.

The absolute deviation function. The deviation function must be formulated in such a way that the divergence is absent when only one compound is available. In addition, G should be a function of the concentrations of the compounds in the mixture. After defining $z_i = TU_i^x(\Sigma_i^n TU_i^x)^{-1}$, the deviation from the reference can be quantified by:

$$G = a \prod_{i=1}^{n} z_i \tag{4}$$

When parameter *a* is positive antagonism is described, when *a* is negative synergism is described (table 1). Note that in the deviation function the concentrations of the individual compounds are scaled to take into account their differences in toxicity, by calculating toxic units. A convenient quantity for scaling is the *EC50*, since many functions used for describing single dose response relationships, are parameterised in such a way that the *EC50* is one of the parameters. Yet, basically any *ECx* can be used for scaling.

Table 1. Interpretation of model parameters of the extend functions, that define the functional form of the deviation pattern from the reference model.

Deviation pattern	*a*	*b*
syn/anta	**Pos:** antagonism **Neg:** synergism	
tox ratio	**Pos:** decreased toxicity related to all *w* compounds. **Neg:** increased toxicity related to all *w* compounds	**Pos:** decreased toxicity related to z_i dominance in the mixture. **Neg:** increased toxicity related to z_i dominance in the mixture.
eff. level	**Pos:** antagonism low effect levels & synergism high effect levels **Neg:** synergism low effect levels & antagonism high effect levels	***b*=1:** change at EC^{50} level* ***b*=2:** change at EC^{50} level** **0<*b*<1:** change at higher effect levels* **0<*b*<2:** change at higher effect levels** ***b*>1:** change at lower effect levels* ***b*>2:** change at lower effect levels**

* Only valid when the data is compared with the additive model.
** Only valid when the data is compared with the independent model.

Toxicant ratio dependent deviation. In order to quantify toxicant ratio dependent deviation from the reference, equation 4 may be extended to:

$$G = \left(a + \sum_{i=1}^{n-w} b_i z_i \right) \prod_{i=1}^{n} z_i \qquad ,1 \le w \le n-1 \tag{5}$$

In this equation the influence of the relative amount of each of the n - w toxicants in the mixture on the deviation pattern can be evaluated, compared to the other w. If all $b_i = 0$, there is no ratio effect, and equation 4 is obtained. See table 1 for further clarification of the parameters. Stepwise regression, forward selection or backward elimination may be helpful procedures for analysing the significance of each of the parameters.

Haas and Stirling (1994) have presented a deviation function for describing toxicant ratio dependent differences, which works very well for binary mixtures. After scaling the concentrations this deviation function is given by:

$$G = \left(a + b\left[z_1 - z_2\right]\right) z_1 z_2 \tag{6}$$

Following their approach, equation 5 should be constraint by $\Sigma_i b_i = 0$. In our experience, this constraint makes the calculation procedures for more complex mixtures unnecessarily complicated. For binary mixtures both functions may be used, although the parameter interpretation is slightly different.

Effect level dependent deviation. Effect level dependent deviation can be identified in the data after extending equation 4 with quantified isoboles. In the additive model, the *EC50* isobole is given by $\Sigma_i^n TU50_i = 1$. Isoboles may be incorporated in the deviation function as follows:

$$G = a\left(1 - b\sum_{i=1}^{n} TU\,50_i\right)\prod_{i=1}^{n} z_i \tag{7}$$

The biological interpretation of the parameters is shown in table 1. Note that parameter *b* indicates at which effect level the deviation is changing from antagonism to synergism, or the reverse. Negative values for *b* have no biological meaning.

A similar reasoning can be performed for the independent action model. However, the *EC50* isobole is defined by $\Pi_i^n h_i(c_i) = 1 - P = 0.5$, where *P* denotes the proportional toxicological effect of the mixture. Therefore, the deviation function may be written as:

$$G = a\left(1 - bP\right)\prod_{i=1}^{n} z_i \tag{8}$$

The interpretation of the parameters is similar as the additive model (table 1). The only difference is that the change occurs at the *EC50* level when $b = 2$.

Statistical inference. The identification and quantification of the deviation pattern from the reference proceeds by finding the most parsimonious model. Therefore for every model a set of parameter values have to be found that maximise the likelihood of the data given the hypothesised model. Here, the toxicity data are assumed to follow a normal distribution (see Morgan (1992) for analyzing quantal data). If it is assumed that the variance among the data for every exposure combination is equal, then minimising the sum of squared residuals (*SS*) is equivalent to maximising the likelihood of the data. Hence, the following objective function has to be minimised:

$$SS = \sum_{j=1}^{k}\sum_{l=1}^{r}(\hat{Y} - y_{jl})^2 \tag{9}$$

subject to equation 1 or equation 3, where $\hat{Y} = Y$, and y_{jl} is the measured effect in the experiment, with k combinations and r replicates.

Additional parameters in the model can be evaluated by applying the likelihood ratio test. For normally distributed data, the calculation of the test statistic can be reduced to:

$$X^2 = m \ln\left(\frac{SS_1}{SS_2}\right) \tag{10}$$

where m (= $k \cdot r$) denotes the total number of observations. Here, SS_1 and SS_2 denote respectively the sum of squared residuals of the reduced and the full model. The test statistic (X^2) obtained from the likelihood ratio test can be compared with the χ^2 distribution with df_2-df_1 degrees of freedom, where df_1 and df_2 denote the degrees of freedom of respectively the reduced and the full model. The test quantifies the chance that both models explain an equal amount of variation. If the test returns a low p-value, then the extended model describes the data significantly better. A p-value of 0.05 is generally accepted as threshold value. Note that the models for quantifying toxicant ratio deviations and effect level dependent deviations are not nested. If necessary, they may be compared with the Akaike Information Criterion (Akaike 1974).

Analysing Data

Experimental data sets. Four mixture data sets were analysed for illustrating the application of the analysis procedure. Data set 1) and 2) were used to illustrate the technical execution of the calculations, and data sets 3) and 4) were analysed to illustrate the comparison of characterized effects.

1) In our laboratory, the joint effect of Cu and Zn on population growth of the nematode *Caenorhabditis elegans* after one week of exposure in standardised soil (LUFA) was evaluated (Chapter 5). Nematode counts were recalculated relative to the control. The data are shown in the appendix. Tested range of concentrations (µg/g soil): Cu: 0-522 and Zn: 0-688.

2) The effect of simultaneous exposure to Cu, Zn, Cd and Pb on the body weight (µg per individual) of springtail (*Folsomia candida*) was studied after 4 weeks of exposure in standardised LUFA soil (Bongers and van Gestel, *in prep.*). Three replicates out of ten were randomly sampled. These were considered as a data set and analysed as such. Tested range of concentrations (nmol/g soil): Cu: 69-24763, Zn: 300-19926, Cd: 0.1-2092 and Pb: 80-27558. This extensive data set is available upon request.

3) The cytotoxic effect of 4-Hydroperoxycyclophosphamide (4-HC) and VP-16-213 (VP-16) on HL-60 cells was analysed for development of chemotherapy (Chang *et al.* 1985). The number of viable cells was counted after 1, 24, 48 and 72 hours. Tested range of concentrations (µg/ml): 4-HC: 0-29.2 and VP-16: 0-22.5.

4) The joint toxic effect of Cu and Zn on the DNA, RNA and protein content of larval fathead minnows (µg/larvae), *Pimephales promelas*, was analyzed after 4 days of exposure (Ryan *et al.* 1992). Tested range of concentrations (µg/L): Cu: 0-150 and Zn: 0-1500.

Biological response analysis. All biological response variables were non-linearly decreasing as a function of the increasing concentration of the chemicals. Also, in all data sets negative responses were odd. Hence, the log-logistic model was adopted (Haanstra *et al.* 1985) for quantifying the dose response relationships. The complement of the proportional effect was calculated by:

$$h_i(c_i) = \frac{1}{1 + (c_i / EC50_i)^{\beta_i}} \tag{11}$$

where β_i is the slope of the ith response curve. Considering equation 1 and equation 2, it can be seen that for one chemical the relationship $Y = f(c_i) = u_0 h_i(c_i)$ holds, within the context of this study. Therefore the inverse function in equation 1 could be calculated explicitly:

$$f_i^{-1}(Y) = EC50_i \left(\frac{u_0 - Y}{Y} \right)^{\beta_i^{-1}}, c_1,...,c_n > 0 \tag{12}$$

By definition, $Y = u_0$, if all $c_1,\ldots,c_n = 0$. All biological response parameters were assumed to be normally distributed. The objective function was minimised either using a program written in SAS (Nelder-Mead Simplex Method), or in a spreadsheet environment (Microsoft Excel) using the built-in solver function (Newton algorithm). Asymptotic standard errors were calculated by applying Finite-Difference approximations of the derivatives.

Results

The results of the analysis of the joint effect of Cu and Zn on population growth of the nematode *Caenorhabditis elegans* are provided in table 2. It shows how parameter estimates and the *SS* changed as a consequence of extending the reference models. See table 1 for interpretation of parameters *a* and *b* in each column. When the additive model was applied the *SS* decreased from 29352 to 27305, when the reference model was extended with additional parameter *a*, to describe synergism/antagonism. The best description of the data was obtained after incorporating a second parameter *b* to allow for an effect level dependent deviation (*SS* = 23208). Similar results were obtained when the data were analysed with the independent model. Note that introducing a parameter to allow for a toxicant ratio dependent deviation

yielded no significant reduction of the *SS*. In conclusion, compared to both references an effect level dependent deviation from the reference was identified. The functional form of the dose response relationship could be deduced from the parameter values (table 1). Parameter *a* was positive, implying antagonism at low effect levels and synergism at high effect levels. Swapping from antagonism to synergism occurred at a higher effect level than the *EC50*'s, which could be deduced from parameter *b*. For additive action $b = 0.4$, which indicated a switch from antagonism to synergism at concentrations of 1/0.4 = 2.5 times the *EC50*'s. For independent action $b = 1.3$, which indicated a switch from antagonism to synergism at 77% effect (1/1.3 = 0.77). Figure 2 illustrates that the description of the structural deviation of the mixture points from the additive reference indeed improved.

Table 2. Parameter estimates and statistics after analyzing the Cu-Zn effect on population growth of the nematode *C. elegans*. Cu is indicated by subscript *1*, and Zn by subscript *2*. *EC50*'s are given in µg/g soil. See table 1 for interpretation of parameters *a* and *b* in each column.

	additive action				independent action			
	ref	syn/anta	ratio	level	ref	syn/anta	ratio	level
u_0	99	101	101	101	97	96	96	97
β_1	1.39	1.43	1.44	1.16	1.48	1.56	1.51	1.26
β_2	1.38	1.41	1.41	1.14	1.48	1.57	1.45	1.19
$EC50_1$	100	85	84	83	103	101	102	95
$EC50_2$	152	129	130	126	160	153	158	149
a		1.97	2.00	9.64		1.38	1.21	13.94
b			0.37	0.40			0.22	1.30
SS	29352	27305	27287	23208	27892	26645	26607	23590
df	113	112	111	111	113	112	111	111
X^2		8.52	0.08	19.19		5.40	0.17	14.37
$p(X^2)$		0.0035	0.77	1E-05		0.02	0.68	0.0002

df = degrees of freedom
X^2 = Likelihood ratio
$p(X^2)$= outcome of the Likelihood ratio test

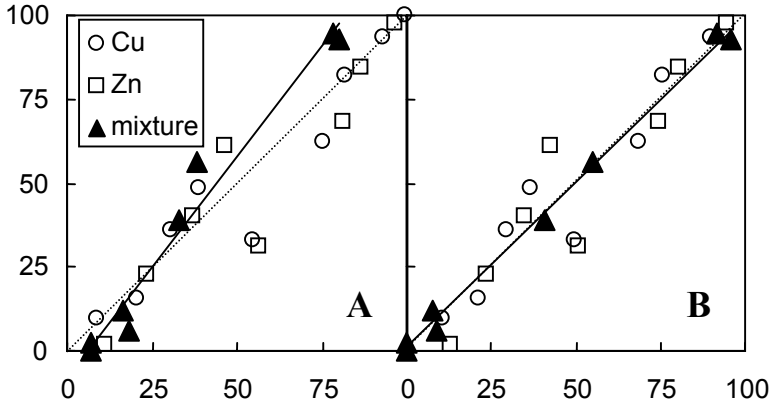

Figure 2. The modeled values in relation to observed data, for Cu, Zn, and their mixture. The dotted diagonal line indicates ideal model description. The solid line a linear regression line through the mixture data. Figure 2A depicts the results after fitting the additive reference model, figure 2B depicts the results after fitting the effect level dependent deviation from additivity. The points represent the mean of the replicates (appendix).

Figure 3A shows the analysis of the weight response of the springtail *Folsomia candida* to the four metals Cu, Zn, Cd and Pb. Using the additive model, the description improved considerably when one additional parameter for quantifying synergism/antagonism was incorporated. Forward selection (with threshold value $p(X^2) = 0.05$) revealed that none of the four substances affected the deviation pattern significantly (points in the figure overlap). Modelling effect level dependent deviation from additivity yielded the lowest value for the *SS* (374453; $p(X^2) = 0.00014$). Table 3 shows the final parameter estimates: they indicated antagonism at low effect levels and synergism at high effect levels.

Figure 3B depicts the comparison with the independent model. A significant decrease in *SS* was obtained when b_3 was allowed to enter the model, which quantified the effect on the deviation pattern associated with Cd. Adding extra parameters when b_3 was already in the model did not improve the data description significantly. There was also no indication for an effect level dependent deviation from the reference. Parameter *a* was negative (table 3), which indicated a synergistic tendency of the combined effect, but the positive value of *b* indicated a decreasing toxic effect when Cd was relatively more present in the mixture.

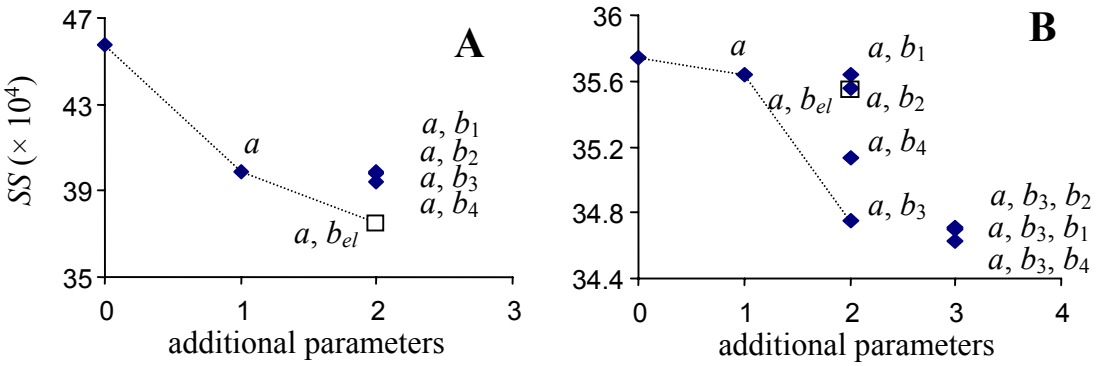

Figure 3. The results of the forward selection of parameters. The x-axis depicts the amount of additional parameters, relative to the reference model. The y-axis depicts the sums of squared errors (*SS*). The dotted line indicates the significant parameter selections. Fig. 3A shows the results of fitting the additive model, Fig. 3B shows the results of fitting the independent model. The dots are labeled to show which combination of parameters results in the indicated *SS*. The effect of Cu on the deviation function is quantified by b_1, the effect of Zn by b_2, the effect of Cd by b_3, and the effect of Pb by b_4. The open square (labeled with b_{el}) indicates the *SS* for the effect level dependent deviation from the reference.

Table 3. Parameter estimates, all significantly different from 0 (t-test; $p < 0.05$), after analyzing the effect of Cu (*1*), Zn (*2*), Cd (*3*) and Pb (*4*) on the weight of the springtail *F. candida*; *EC50*'s are given in nmol/g soil.

	Additive model effect level dep.		Independent model tox ratio: Cd	
Parameter	*estimate*	*t-test*	*estimate*	*t-test*
u_0	273	< 0.0001	276	< 0.0001
β_1	2.30	0.0002	3.27	< 0.0001
β_2	3.21	0.0004	2.72	< 0.0001
β_3	1.65	< 0.0001	2.32	< 0.0001
β_4	2.62	< 0.0001	2.68	< 0.0001
$EC50_1$	13238	< 0.0001	13164	< 0.0001
$EC50_2$	11605	< 0.0001	11318	< 0.0001
$EC50_3$	736	< 0.0001	538	< 0.0001
$EC50_4$	8445	< 0.0001	7627	< 0.0001
a	624	< 0.0001	-272	0.02
b	0.42	< 0.0001	738	0.03

Table 4 shows the comparison of characterised (cytotoxic) effects of two drugs in time. The most important aspects to note are the outcome and the value of $p(X^2)$, which quantifies the significance of the additional parameter in the model that described the result. For instance, $p(X^2)$ at t = 24h in the 5th column quantifies the significance of the additional parameter to the additive reference model to describe synergism/antagonism. If the reference was the most parsimonious model, $p(X^2)$ is not appropriate (indicated by -). The table shows that the combined effect of 4-HC and VP-16 was transient. To enable a more detailed interpretation of the dynamics some parameter estimates are discussed here. Using the additive model, after 1 h the data was best described by the reference. After 24 hours a negative estimate of additional parameter a was obtained (equation 4, a = -2.31), which implied a synergistic effect. After 48 h of exposure, a toxicant ratio dependent deviation became apparent. Parameter b was incorporated in equation 5 to examine the influence of 4-HC dominance relative to the other substances, which was in this particular case only one: VP-16. Thus, equation 5 took the form $G = (a + b_1 z_1) z_1 z_2$, where 1 and 2 indicated 4-HC and VP-16. We obtained a positive estimate for parameter $b_{4\text{-HC}}$ (b = 8.30), which implied a relatively less toxic combination when 4-HC was dominant in the mixture. On the other hand, a negative estimate for parameter a was obtained (a = -5.55), which indicated relatively increased toxic effects in case of VP-16 dominance. Finally, after 72 hours of exposure, the mixture was equally toxic as the individual components again. Similar dynamics was observed when the data was compared with the independent model (table 4). Figure 4 shows the improvement in fit after extending the independent model to allow for toxicant ratio dependent deviation (equation 5; a = -5.47; $b_{4\text{-HC}}$ = 8.84), to illustrate the analysis. Table 5 shows a comparison of characterised effects between three different response parameters of the fathead minnow *Pimephales promelas*. Note that the values of $p(X^2)$ quantify the significance of the additional parameter in the model that described the result. The joint effect of Cu and Zn depended on the response parameter measured, Cu and Zn acted additive at the DNA level, but antagonistic at the RNA and protein level.

Table 4. Results of analyzing the effect of 4-HC and VP-16 on the amount of viable HL-60 cells at various time points. The outcome, test statistic (X^2), degrees of freedom (*df*) and *p*-values ($p(X^2)$)are shown.

	Additive model				Independent model			
time	*outcome*	X^2	*df*	$p(X^2)$	*outcome*	X^2	*df*	$p(X^2)$
1	additive	-	45	-	synergism	5.48	44	0.019
24	synergism	26.51	44	2.6E-0.7	independent	-	45	-
48	tox. ratio	11.34	43	0.0008	tox. ratio	12.56	43	0.0004
72	additive	-	45	-	independent	-	44	-

- = not appropriate

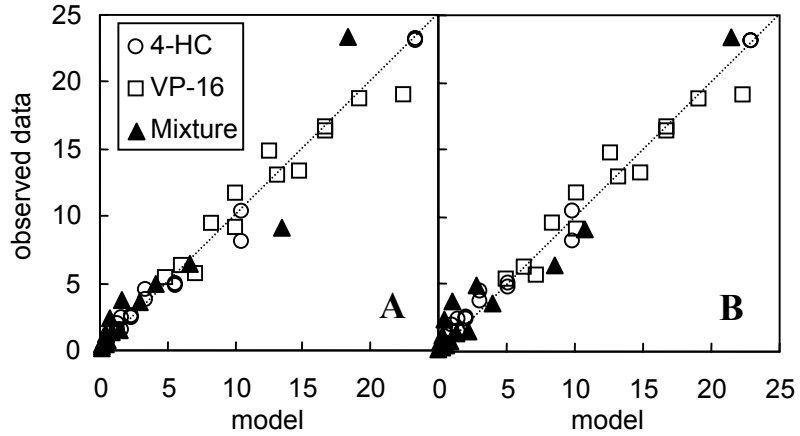

Figure 4. The modeled values in relation to observed data obtained after on the 48h exposure to 4-HC, VP-16, and their mixture. The dotted diagonal line indicates ideal model description. Figure 4A depicts the results after fitting the independent reference model, figure 4B depicts the results after fitting the toxicant ratio dependent deviation from additivity.

Table 5. Results of analyzing the effect of Cu and Zn on the DNA, RNA and protein content of *P. promelas*. The outcome, test statistic (X^2), degrees of freedom (*df*) and *p*-values ($p(X^2)$ are shown

	Additive model				Independent model			
resp.	*outcome*	X^2	*df*	$p(X^2)$	*outcome*	X^2	*df*	$p(X^2)$
DNA	additive	-	68	-	independent	-	68	-
RNA	antagonism	9.91	67	0.002	independent	-	68	-
Protein	antagonism	4.67	67	0.03	independent	-	68	-

- = not appropriate

Discussion

The generally used classification of combination mechanisms allows for the quantification of partial similarity of the joint action of chemicals (Hewlett and Plackett 1959). This has been studied in detail (Christensen and Chen 1985), but experimental data indicated that quantifying just the extent of similarity is insufficient to describe mixture effects satisfactorily (Chen and Chiou 1995). Therefore alternative approaches are required. A rigid separation between the two non-interaction models was necessary to avoid interpretation problems, when they were extended to describe deviation patterns. The quantitative approach presented above might be considered as a hybrid method using well-established mixture toxicity principles (like the mixture toxicity index) and multiple regression, combining advantages of both methods. Substituting generally used dose response curves in the mixture toxicity concepts, such as the log-logistic model (equation 12), ensured a biologically meaningful description. In addition, the nested structure of deviation parameters allowed likelihood tests.

Here, the additive model was used just to evaluate whether the mixture was relatively more/less toxic than the toxicity of the individual compounds. It was therefore not restricted to toxicants having similar modes of action only. Comparing relative toxicities is valid, irrespective of mechanistic considerations. Yet, predicting with the additive model requires toxicants with similar modes of action, because then the implicit model conditions must be satisfied. This distinction is important, since the predictive power of the models is still discussed in literature (Kortenkamp and Altenburger 1999, Lock and Janssen 2002, Silva *et al.* 2002). A similar reasoning applies to the independent model.

The choice of the suitable reference model depends on the preferences of the researcher. Here, the mathematical interpretation of the models was emphasised, which should be applicable to the response variable of interest to be useful. From that perspective the independent joint action model, which considers probabilities of response, is easier to apply to quantal data than to continuous data. In addition, deviations from the additive model are easier to interpret than deviations from the independent model, which might explain why the additive model is more often used. Yet, experiments have indicated that the independent model predicts the effect of independent acting chemicals well, also if the response is non-quantal (Backhaus *et al.* 2000), which indicates pharmacological relevance.

Over the years many techniques for data analysis have been used (Greco *et al.* 1995, Hensbergen and van Gestel 1995). Without going into details, a few remarks on these methods can be made. Many calculation techniques enable the analysis of specified mixture combinations only, like fixed equitoxic mixtures, or only certain effect levels. They lack tools to interpret all combinations simultaneously, which hampers the understanding of how the different concentration ratios of the same mixture interrelate. By means of multiple linear regression all tested combinations can be considered simultaneously (Narotsky *et al.* 1995, Nesnow *et al.* 1995, Nesnow *et al.* 1998). Yet, the biological interpretation of (higher order) interaction terms can be difficult, and effect level dependent deviations from additivity cannot be analysed.

The method presented above enables the description non-linear dose response characteristics. It relies therefore on iterative calculation procedures to estimate the model parameters. While solving the optimisation problem, one should be aware of the existence of local optima in the likelihood space. The following procedure is proposed to optimise the reliability of the analysis. First estimate the parameters values of the single dose response curves individually. Use these as starting values to analyse all parameters simultaneously. If one has to deal with a large data set containing replicates, it is advised to explore the average data first and subsequently the complete data set. Furthermore it is most helpful to depict the model values against the observed response values during the analysis process, to visualise the results. In this way, it is easier to detect odd results.

The way the additive model is quantified, and the extend functions, are equivalent to the concentration response surface model of Greco (Greco *et al.* 1995), but have more flexibility. It was shown that response surface models can be applied to various experimental designs, such as factorial design, central composite design, fixed ratio design with a single ratio, fixed ratio design with four different ratios, and D-optimal design (Greco *et al.* 1994). Similarly, also the models presented above can be used to analyse data from these designs. Factorial designs are probably most useful, because these make it possible to cover complete response surfaces.

The comparison of characterised responses puts the interaction effects in a biological perspective, as illustrated by the cell-line data and the fathead minnow data. Comparing the cell-line data with the additive model over time revealed the difference in dynamics of the cytotoxic effect. When mixed, the drugs affected cell division faster than when they were administered individually. However, cell division continued at a higher rate in the mixture than when exposed to the individual drugs. The differences in relative toxicities in the minnow data showed that the mixture was less toxic at RNA and protein level than DNA level. Thus, relatively more RNA and protein was present, which might indicate an enhanced production of (protective?) proteins when the larvae were exposed to copper and zinc simultaneously, compared to exposure to these chemicals individually. Clearly, these are just primary interpretations of the outcome of the analysis, but detailed discussions are beyond the scope of this article.

One of the future challenges is to develop new tools to predict combined effects. The classical models date back for decades and new concepts might open new research directions. Feron and Groten (2002) reviewed recent developments of mixture analysis in human risk assessment, from which environmental toxicologists might benefit also. A promising approach is physiologically based pharmacokinetic modeling (Conolly 2001). Pharmacokinetic models have been parameterised for standard test organisms for single toxicants (Thomann *et al.* 1997) and extrapolations to mixtures are possible (Haddad and Krishnan 1998, Haddad *et al.* 2000). Other recent developments include *e.g.* the use of artificial neural networks (Gagne and Blaise 1997) and multivariate techniques (Eide *et al.* 2001). Yet, the classical concepts are still most frequently used.

Elucidating all mechanisms involved in combined toxicity of environmental chemicals is virtually unfeasible. However, there is a lack of knowledge concerning the conditions and

processes that drive (and therefore may enhance) the adverse effects of chemical combinations. In our opinion, this is relevant information for risk assessment purposes and for developing predictive models. The comparison of characterised effects in various experimental designs might be helpful in pinpointing these conditions. Recognising generic patterns can lead to a better understanding of the relationship between chemical interaction, physiology and toxicity.

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Appendix

Data of the joint effect of Cu and Zn on population growth of the nematode *Caenorhabditis elegans* (table 2; figure 2).

[Cu]	[Zn]	Mean response	# replicates
0	0	100	10
14.5	0	93.7	5
32.9	0	82.1	5
44.4	0	62.2	5
86.2	0	32.7	4
136.3	0	48.4	5
178.2	0	36	4
262.3	0	15.7	4
521.9	0	9.7	4
0	13.1	97.4	5
0	39.3	84.1	5
0	51.6	68.5	5
0	125.7	31	4
0	168	61	5
0	221.4	40.4	4
0	352.9	22.9	4
0	687.9	1.9	4
10.3	39	92.7	5
31.8	11.1	94.5	5
43.6	147.9	56.2	5
128.7	54.8	39.2	5
85.2	314.7	5.7	4
249.9	103.4	12.1	4
174.3	683.7	0	4
508.1	212.6	2.7	4

Chapter 3

Combination- and dose level dependent synergistic mixture effects on life history traits of *Caenorhabditis elegans*

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Abstract

In this study sublethal effects of chronic stress of binary mixtures on life cycle events of the nematode *Caenorhabditis elegans* were studied. We investigated the exposure to mixtures of copper and cadmium, and copper and carbendazim. The mixture effects were compared to the effects of the individual mixture constituents. The life-cycle straits studied were age specific cumulative reproduction, length of the juvenile period, length of the reproductive period and growth. The cadmium-copper effect on reproduction was transient: it changed from synergistic, to a toxicant ratio dependent deviation from additivity. The mixture was relatively less toxic if relatively more cadmium was available. The effect of copper-carbendazim was synergistic at low effect levels and antagonistic at high effect levels. In general, the juvenile period was relatively non-sensitive, prolongation was observed at concentrations of 2-3 times the EC50 for reproduction, and indication for synergism was observed at the highest concentration of copper-cadmium. The length of the reproductive period was relatively more sensitive: prolongation was observed at concentrations lower than/similar to the EC50 for reproduction. In general, it should be realised that mixture toxicity may be transient and that interactions may differ among life history traits, leading to non-intuitive response patterns.

Introduction

Anthropogenic pollution often consists of mixtures of compounds rather than single toxicants only. Over the last few years much research has been performed to assess the ecotoxicological consequences of combined exposure in comparison with the effects of individual toxicants. Most bioassay studies were conducted in aquatic systems on a range of organisms like *Vibrio fischery* (Backhaus *et al.* 2000, Mowat and Bundy 2002), *Escherichia coli* and *Pseudomonas fluorescence* (Preston *et al.* 2000), duckweed (*Lemna minor*; Ince *et al.* 1999), frogs (*Xenopus lavis*; Rayburn *et al.* 1995), toads (*Bufo arenarum*; Herkovits and Helguero 1998), Sea-Urchins (*Paracentrotus lividus*; Fernandez and Beiras 2001) and zebrafish (*Danio rerio*; Roex *et al.* 2002). In addition there is an increasing interest in studying combination toxicity in soil systems, quantifying interactive effects on bioavailability and uptake by terrestrial invertebrates (Van Gestel and Hensbergen 1997).

Many organisms experience a life time exposure to chemical mixtures, which induces changes in development rate and in timing of important life cycle events. Current mixture toxicity studies often do not enable assessment of potential effects of chronic exposure, whereas changes in life cycle characteristics can have consequences at higher levels of biological organisation, such as populations (Caswell 1996). With regard to ecological risk assessment it is therefore important to obtain insight in possible consequences of combined chronic intoxication. In addition, to determine whether combined exposure leads to increased toxic risk, responses to mixtures have to be compared with the effects of the individual compounds.

Analysing life cycle responses of organisms to the toxic chemicals separately can reveal whether life history traits differ in sensitivity to the mixture constituents. Comparing mixture effects to the effects of individual chemicals reveals whether synergism or antagonism lead to non-intuitive response patterns. For studying these response patterns it was chosen to analyse the effects of binary copper-cadmium combinations and copper–carbendazim combinations on sublethal life cycle events of the nematode *Caenorhabiditis elegans*. Sublethal changes, with regard to for instance reproduction and growth, may be considered as more ecologically relevant than mortality (Barnthouse *et al.* 1986).

C. elegans has a relatively simple lifecycle, which facilitates the analysis of possible complex interactions patterns. In unexposed control conditions (16^{o}C) eggs hatch after 16 – 18 hours (Byerly *et al.* 1976). After four molts (36.5, 48, 60 and 75 hours from the time point that eggs were laid) the reproductive period starts after approximately 90 hours. The reproductive period (± 280 eggs) is continuous and ends during the 8th day. However, the post-reproductive period lasts for approximately 12 days. *C. elegans* occurs in two sexes, males and hermaphrodites, each about 1 mm in length (Wood 1988). Hermaphrodites produce oocytes as well as sperm and reproduce by self-fertilisation. Males can fertilise hermaphrodites, but usually occur in low numbers. Ecotoxicological experiments are usually performed with hermaphrodites. The nematode has been used for single toxicity experiments in liquid phase, on agar and in soils, and various endpoints have been measured (Traunspurger *et al.* 1997). *C. elegans* is a free living, soil dwelling, bacterivorous nematode. It may

therefore be considered as a model organism for studying fast reproducing opportunistic soil invertebrates. Additionally, it may represent the metazoa which are important for decomposition and nutrient cycling in soil systems (Freeman *et al.* 1999).

The toxicant combinations were chosen based on physiological and ecological interest. Copper is an essential metal, because of its specific incorporation into a large number of enzymatic and structural proteins. The role of copper in oxidation/reduction enzyme activities is a consequence of its ability to function as an electron transfer intermediate. Yet, copper intoxication can lead to direct protein damage, structural impairment of essential metal binding sites (Alt *et al.* 1990), and cellular injury due to the production of oxyradicals (Goldstein and Czapski 1986). Cadmium is a non-essential metal, it replaces zinc in various proteins (Vallee and Ulmer 1972) and causes cellular lesions such as disrupted cytosomes and shortened microvilli (Popham and Webster 1979). The combination of copper and carbendazim is relevant for field situations in agricultural areas, where organisms are exposed to mixtures of copper and fungicides. Carbendazim (MBC; methyl-2-benzimidazole carbamate) is a commonly used systemic fungicide. Benzimidazoles are known for their interference with DNA synthesis and inhibition of cellular development (Tomlin 1997). Limited information is available on the combined physiological interactions between pesticides and metals. For carbamates and copper, synergistic effects were found in the aquatic ciliate *Colpidium campylum* (Vasseur *et al.* 1988, Bonnemain and Dive 1990).

In this study we focused on single toxicity and mixture effects on age specific cumulative reproduction, length of the juvenile period, length of the reproductive period and growth of *C. elegans*, when exposed to copper and cadmium and copper and carbendazim, and their binary combinations. Effects of mixtures were compared with the effects of the individual compounds, to detect interactions and to determine whether combined exposure was transient. In this way we aimed at quantifying differences between chemicals and their combinations in sublethal effects on the life cycle of *C. elegans* when exposed chronically.

Materials and Methods

Test organism and culture

Experiments were performed with *Caenorhabditis elegans* var. Bristol, strain N2. The culture was provided by the Netherlands Cancer Institute, Amsterdam, The Netherlands. Stock cultures were kept sterile in the dark at 15^{o}C on NGM agar (Wood 1988), nematodes were transferred weekly on fresh agar plates. Agar plates were inoculated with *Escherichia coli* (strain OP50) as food source. Synchronisation of cultures was performed at 20^{o}C. Two synchronisation steps were performed. First, 10 juveniles of approximately the same age were picked from the stock culture and transferred to new agar plates. These were allowed to develop to a healthy culture, and after 5 days 10 gravid adults were transferred from this culture to a new agar plate. These adults were allowed to lay eggs for five hours, then they

were removed. In 3 days the eggs developed to adults differing maximally 5 hours in age. These were used for the experiments.

Chemicals

All chemicals were of the highest analytical grade available. Chloride salts of copper (Sigma Chemical Company, St. Louis Missouri, USA) and cadmium (Merck, Schuchardt, Germany) were used to make the stock solutions ($CuCl_2 \cdot 2H_2O$: 2mg/ml, $CdCl_2$: 1mg/ml). The solutions were made in sterilised bidestilled water. Carbendazim, 99.2 % pure, was obtained from BASF Nederland B.V., Arnhem, The Netherlands. The stock solution, (0.3 mg/ml) was prepared in ethanol. Five nominal concentrations of each tested substance were used: In Cd/Cu experiment the range of salt concentrations for Cd was 0.0; 2.0; 4.0; 6.0; 8.0; 10.0 (µg/ml agar) and for Cu 0.0; 10.0; 20.0; 30.0; 40.0; 50.0 (µg/ml agar). The concentrations in the mixtures were 1.25 + 7.0; 2.5 + 3.5; 2.5 + 7.0; 5.0 + 7.0; 2.5 + 14 (µg Cd/ml agar + µg Cu/ml agar). In Cu/carbendazim experiment the concentration range for Cu salts were the same as mentioned above and for carbendazim: 0.0; 0.3; 0.6; 1.2; 1.8; 2.4 (µg carbendazim/ml agar). The concentrations in the mixtures were 3.5 + 0.9; 7.0 + 0.45; 7.0 + 0.9; 18.6 + 1.2; 9.3 + 2.4 (µg Cu/ml agar + µg carbendazim/ml agar). A control for checking the effect of ethanol was tested additionally, containing the highest ethanol concentration applied in the experiment (1.2 ml/L). Concentration ranges were based on range finding experiments with nominal concentrations.

After autoclaving the readily prepared agar and before solidification, the test substances were mixed with the agar solution at the required concentration. Before adding carbendazim the liquid agar was cooled down to 50º C to avoid decomposition of the pesticide. For the experiments both 12-wells tissue plates and 9 cm petri dishes were used. Each well contained 1ml and each 9 cm plate 10 ml of contaminated agar. All the plates were inoculated with *E. coli*, strain OP50 (25 µl/well and 75 µl/9 cm plate) and stored at 37°C overnight to allow the bacteria to grow before experimental use. *E. coli* was relatively insensitive for the tested chemicals.

Toxicity testing

Synchronised adults were transferred from clean agar to contaminated agar plates. Three plates per treatment and 20 adults per plate were used. These adults were allowed to lay eggs for 4 hours, then they were removed, which indicated t = 0 of the experiment. The eggs, which were the nematodes under study, were kept at 15^{o}C in the dark for 2 days, to hatch and develop into juveniles. Subsequently 10 randomly picked nematodes per treatment were transferred from the agar plate to the tissue plate with corresponding toxicant combination- 1 nematode per well. The nematodes were transferred to the new wells every 24 hours, and

scored for reproductive output, survival and censoring. Pictures were taken from the remaining nematodes on the 9 cm dishes to measure their length: cadmium-copper at age = 6 d, carbendazim-copper at age = 2, 3, 4, 5, 6 and 8 d. The experiment was ended when reproduction of all individuals had stopped. Agar samples for Cd, Cu and carbendazim measurements ran simultaneously with the experiments.

Chemical analysis

It was assumed that the bioavailable fraction of the chemicals for nematodes was represented by the free (ion) concentration in the water fraction of the agar. Therefore duplicate samples from the water fraction of the agar were taken one day after starting and one day after ending the experiment. For the metal measurements, the water fraction was obtained by centrifuging 20 ml of agar for 12 min at 15000 rpm, 17540 g, in plastic tubes. The supernatant was stored in eppendorf tubes at 4^{o}C. For the carbendazim measurements, the water fraction was obtained by filtration. A stainless steel Buchner-funnel, with a 45 µm stainless steel filter, was filled with 20 ml of agar. Under-pressure created suction power that enabled the isolation of the water fraction from the agar. The water fraction was stored in glass tubes at –20^{o}C. Metal concentrations were determined by flame atomic absorption spectophotometry, using a Perkin Elmer 1100B atomic absorption spectophotometer (AAS) at a wavelength of 228.8 nm (Cd) or 324.7 nm (Cu). Carbendazim concentrations were determined by HPLC technique. Column: Waters X-terra^{TM} MSC_{18} 3.5 µm (diameter 4.6 mm, length 150 mm), provided with a guard column X-terra^{TM} MSC_{18} 3.5 µm (diameter 3.9 mm, length 20 mm). Flow: 1 ml/min., injection volume was 100 µl. The eluens was acetonitril/water (30/70;V/V), a wavelength of 285 nm was used.

Data analysis

Age specific cumulative reproduction

For analysing effects of the binary mixtures on reproduction the toxic unit model was used (Sprague 1970). Frequently the toxic unit is defined as the actual exposure concentration divided by its EC50. In this study this quantity was used to enable the comparison of mixture effects with its individual constituents, and it is indicated by TU. Under this definition, 1 TU of toxicants in the mixture should result in 50% toxic effect (reduction in number off eggs). This can be calculated by executing the following protocol: 1) fit separate dose response curves on the toxicity data of the individual chemicals to estimate EC50's, 2) divide the concentrations of the compounds in the mixture by its EC50 and calculate the sum of the TU's, 3) fit a dose response curve on the toxicity data of the mixture, subject to transformed concentrations, to evaluate whether 50% effect indeed occurs at 1 TU (Van Gestel and Hensbergen 1997). We applied this protocol for exploring the data, using a log-logistic dose

response model (Haanstra *et al.* 1985). Yet, for analysing the data in detail we applied a more general approach proposed by Haas *et al.* (1996) and in Chapter 2. Following these derivations, the binary toxic-unit mixture model can be generalised to:

$$\frac{c_1}{f_1^{-1}(Y)} + \frac{c_2}{f_2^{-1}(Y)} = \exp(G) \tag{1}$$

where c_1 and c_2 denote the concentrations of the individual chemicals in the mixture, Y indicates the biological response, and f_1^{-1} and f_2^{-1} indicate the inverse dose response functions of the individual compounds in the mixture. G denotes an extent function to quantify deviations from additivity. To enable calculations of the EC50's as well as the EC10's simultaneously, a modified log-logistic dose response model was adopted (Van Brummelen *et al.* 1996). The inverse dose response relationship can then be written as:

$$f_i^{-1}(Y) = EC50_i \exp\left(\omega_i \ln\left(\left(\frac{u_0 - Y}{Y}\right)^{\eta}\right)\right) \quad , i = 1,2 \tag{2}$$

where u_0 denotes the response in the control group, $\eta = \ln(9)^{-1}$, and $\omega_i = \ln(EC50_i/EC10_i)$. Details of the analysis procedure are outlined in Haas *et al.* (1996) and in chapter 2. In short, G enables the quantification of four distinct deviations from the additive model. 1) No deviation. 2) Synergism/antagonism in all mixture combinations (S/A). 3) Toxicant ratio dependent deviation (TR), where the deviation pattern (synergism/antagonism) depends on the mutual proportion of the toxicants in the mixture. 4) Effect level dependent deviation (EL), where synergism/antagonism depends on the dose level tested. Within this framework the synergistic/antagonistic model is an extension of the additive model with one additional parameter. Subsequently the synergistic/antagonistic model is nested in the toxicant ratio-dependent deviation model and effect level-dependent deviation model. These models have two additional parameters compared with the additive model, and they cannot be tested mutually. The biological interpretation of the additional deviation parameters, here arbitrarily named *a* and *b*, is listed in table 1. All parameters, i.e. the concentrations resulting in 10% and 50% effect ($EC10_1$, $EC10_2$, $EC50_1$, $EC50_2$), the control response (u_0) and the deviation parameters were estimated simultaneously. The most parsimonious model was found by likelihood testing, assuming a normal distribution with equal variance at all exposure combinations equivalent to multiple regression. A significance level of $p(X^2) = 0.05$ was chosen as threshold value in deciding whether an additional parameter in the model described the data significantly better. Here the X^2 indicates the test statistic, which is compared with the χ^2 distribution with df_2-df_1 degrees of freedom, and df_1 and df_2 denote the degrees of freedom of respectively the reduced and the full model. If the synergistic/antagonistic model didn't show a significant deviation, the toxicant ratio-dependent deviation and effect level-dependent deviation models were compared with the additive reference. In this way the binary effects on the cumulative reproduction was quantified for every daily age class.

Table 1. Interpretation of additional parameters in equation 1, that define the functional form of the deviation pattern from the additive model. *TU* denotes the toxic unit of a chemical, defined as the exposure concentration in the mixture divided by its EC50 (reproduction) or EC20 (length) and $z_i = TU_i(TU_1+TU_2)^{-1}$,$i = 1,2$.

Deviation pattern	*a*	*b*
synergism/antagonism (S/A) $G = az_1z_2$	**Pos:** antagonism **Neg:** synergism	
Toxicant Ratio (TR) $G = (a + b\,z_1)z_1z_2$	**Pos:** decreased toxicity related to z_1 decrease in the mixture. **Neg:** increased toxicity related to z_1 decrease in the mixture.	**Pos:** decreased toxicity related to z_1 increase in the mixture. **Neg:** increased toxicity related to z_1 increase in the mixture.
Effect Level (EL) $G = (a(1 - b[TU_1+TU_2]))z_1z_2$	**Pos:** antagonism low EL & synergism high EL **Neg:** synergism low EL & antagonism high EL	***b*=1:** change at *EC50* level **0<*b*<1:** change at higher EL ***b*>1:** change at lower EL

Reproduction events

The juvenile period (t_j) was defined as the period before reproduction started (thus after four molts, including egg stage). *C. elegans* produces approximately 280 eggs. The egg-laying rate (eggs/nematode/time) increases quickly, then rises more slowly to a maximum, after which it drops (Byerly *et al.* 1976). In this study we considered these characteristics as an age specific probability of contributing offspring to the first age class, which empirically was assumed to follow a gamma distribution. This enables the estimation of the length of the juvenile period and the length of the reproductive period. The expected total number of eggs per individual in each exposure ($\overline{M}$) was calculated as the sum over the daily age classes of the number of eggs per exposed replicate individual in that age class (censored data caused by death or disappearance were included until censoring; (Hansen *et al.* 1999)). Thus for every exposure one can calculate:

$$\overline{M} = \sum_a \frac{1}{n_a} \sum_r^n m_{r,a}$$

where $m_{r,a}$ denotes the number of eggs of replicate r in age class a in the treatment, and n_a denotes the total number of exposed individuals (replicates) during this time interval. Thus, egg-laying rate was assumed to be given by:

$$m(t,\alpha,\beta) = \overline{M}\,\frac{t^{\alpha-1}e^{-\frac{t}{\beta}}}{\beta^{\alpha}\Gamma(\alpha)}$$

where α and β are parameters of the treatment specific gamma distribution, Γ(α) is the gamma function, and t is expressed in days. In this model t = 0 was defined as the starting point of the age class in which the first eggs were found in the experiment, which was the 4th age class in both experiments. We assumed that the reproductive period started at the time point at which the cumulative probability of the gamma distribution equalled the probability of laying one egg. This probability was calculated as $p_{\text{first}} = 1/\overline{M}$. In addition, we assumed that the reproductive period ended at the time point at which the cumulative probability of the gamma distribution equalled the probability of laying the last egg. This probability was calculated as $p_{\text{last}} = 1\text{-}1/\overline{M}$. Thus, both time points can be calculated from the inverse of the cumulative gamma distribution. The juvenile period of the nematodes exposed to a certain treatment was estimated from:

$$t_j = 3 + F^{-1}\left(p_{\text{first}}, \alpha, \beta\right)$$

Here, F^{-1} indicates the inverse of the cumulative gamma distribution. The juvenile period was recalculated to hours. The length of the reproductive period was estimated from:

$$t_r = F^{-1}\left(p_{\text{last}}, \alpha, \beta\right) - F^{-1}\left(p_{\text{first}}, \alpha, \beta\right)$$

For estimating the parameters of the gamma distribution a maximum likelihood procedure was performed. The exact time points that the eggs were laid were unknown, only the time intervals in which eggs were laid were recorded. The distribution of the total number of eggs over the age classes was considered for constructing a likelihood function. The total number of eggs in each age class was calculated from $m_a^* = \Sigma_r m_{r,a}$. The total number of eggs per treatment was quantified by $M^* = \Sigma_a \Sigma_r m_{r,a}$. The likelihood of the parameters α and β given data set D resulting from a certain treatment was quantified by the multinomial distribution:

$$L\left(\alpha, \beta \mid D\right) = \frac{M^*!}{\prod_a m_a^*!} \prod_a p_a^{\,m_a^*}$$

where p_a denotes the probability of egg laying in age class a, calculated from the gamma distribution. Ignoring the constant multinomial coefficient, the negative log-likelihood function to be minimised was given by

$$\ell\left(\alpha, \beta\right) = -\sum_a m_a^* \ln\left(F\left(a, \alpha, \beta\right) - F\left(a-1, \alpha, \beta\right)\right)$$

where the difference between the cumulative gamma distribution $F(a,\alpha,\beta)$ and $F(a\text{-}1,\alpha,\beta)$ quantifies the probability of egg laying during the age class a.

Bootstrap methods were used to construct 95% confidence intervals around t_j and t_r. The confidence intervals were based on 2000 resamples and corrected for bias (Efron and Tibshirani 1993). While interpreting the results, non-overlapping confidence intervals were generally used as a simple test of equality of two estimates. This is however a conservative approach, and a randomisation test was performed in case of doubt, based on a random sample of 2000 permutations (Manly 1991).

Bodysize

The perimeter of the nematodes were measured with the Image-Pro Express computer program (Express, 2000), and recalculated to micrometers (µm). A minimum of 9 replicates was analysed, except for day 8, where 3 replicates were analysed. The perimeter was halved to estimate the length.

Also the mixture effect on body length was analysed with equation 1. Yet, the dose response was not sigmoïdal. Within the concentration range tested the data could reasonably be described with an exponential function, which was substituted in equation 1:

$$f_i^{-1}(Y) = -\frac{1}{\tau_i}\ln\left(\frac{Y}{u_0}\right) \quad ,i = 1,2$$

where τ indicates the slope of the curve. Obviously, the length data did not decrease to 0, which meant that the EC50 was not suitable for describing effect level dependent deviations from addititvity (table 1). EC20's were calculated instead, because 20% effect was a suitable bisectional effect level in the response range observed. Therefore, TU in table 1 was defined as $c_i/EC20_i$ for compound i, in case growth data was analysed.

Results

Chemical analysis

The cadmium concentrations measured in the water fraction of the agar ranged from 0 to 3.18 µg/ml, the copper concentrations from 0 to 20.56 µg/ml and the carbendazim concentrations from 0 to 772 ng/ml. The exact values are shown in the appendix. The metal measurements at the beginning of the experiment and at the end were very similar, indicating no increase (or decrease) in binding of compounds in the agar during the experimental period. Therefore the values in the appendix show the means and standard deviations of the measurements at both time points. The results of the carbendazim measurements were less satisfactory: the analysis was hampered by experimental error and only the measurements at the end of the experiment could be used for dose response analysis. However, carbendazim was present at the end of the experimental period assuring continuous exposure.

Age specific cumulative reproduction

In both experiments, reproduction lasted roughly from the age of 4 days until the age of 12 days, where the controls finished reproducing somewhere between the 8th and the 9th age class. Before detailed data analysis, the mixture data was first explored by evaluating whether the mixture induced a 50% reduction of the cumulative reproduction at 1 TU. For example, at the age of 8 days the EC50 for cadmium was 0.58 (0.47-0.69) µg/ml and for copper 3.12 (2.49-3.76) µg/ml. Analysing the response to the mixture, subject to concentrations divided by the above-mentioned EC50's, yielded an EC50 value of 0.71 (0.59-0.82) TU. The differences in dose response curves are depicted in figure 1A. The 50% effect was obtained at lower concentrations than 1 TU, indicating a synergistic deviation from the additive model at the 50% effect level. The carbendazim-copper exposure yielded an EC50 for carbendazim of 661 (621-702) ng/ml and for copper of 10.43 (6.49-14.37) µg/ml at the age of 8 days. It resulted in an EC50 of the mixture of 0.42 (0.19-0.64) TU, also indicating synergism at the EC50 level. The differences in dose response curves were substantial, as shown in figure 1B.

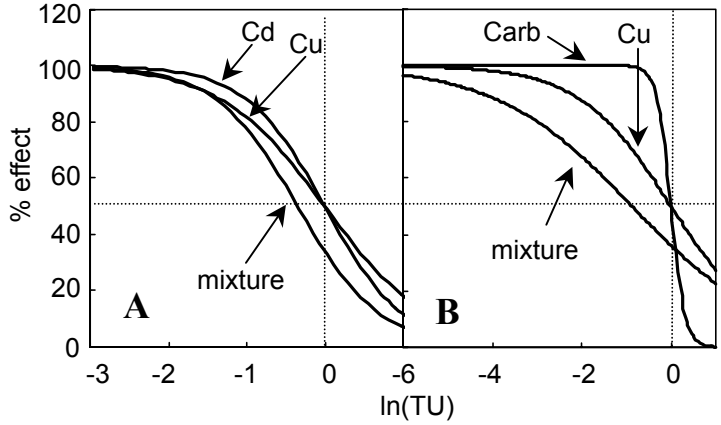


Figure 1. Dose response curves quantifying the effect of copper, cadmium and the mixture (1A), and the effect of copper, carbendazim and the mixture (1B) on the cumulative reproduction on day 8. Concentrations are expressed as toxic units.

To investigate the joint action of the chemicals in detail, for every age class the nested modelling analysis was applied. Figure 2A and 2B show the effect of cadmium and copper individually on reproduction at every time point of the reproductive period. The dose response curves shown were estimated using the most parsimonious binary mixture model (equation 1), and are thus the marginals of the response surface. It can be seen that in all cases the response to the single toxicants could be described well. Figure 3A and 3B show the effect of carbendazim and copper individually in time. Here, equation 1 was also able to describe the observed trends in the single toxicity data. It appeared that copper was less toxic in this experiment than in the cadmium-copper experiment, which can also be seen in table 2 and 3, where EC10's and EC50's are shown. The EC10's and EC50's of each binary mixture were estimated simultaneously using the mixture model. Note that the EC50's at the age of 8 days are very similar to those mentioned above, estimated with a single dose response equation. The EC values in table 2 and 3 indicate that the compounds become relatively less toxic as

exposure time proceeds. Only the EC10's of copper in table 2 decrease over time, indicating a decreasing slope of the dose response curve due to the delayed reproduction peak as a consequence of copper exposure.

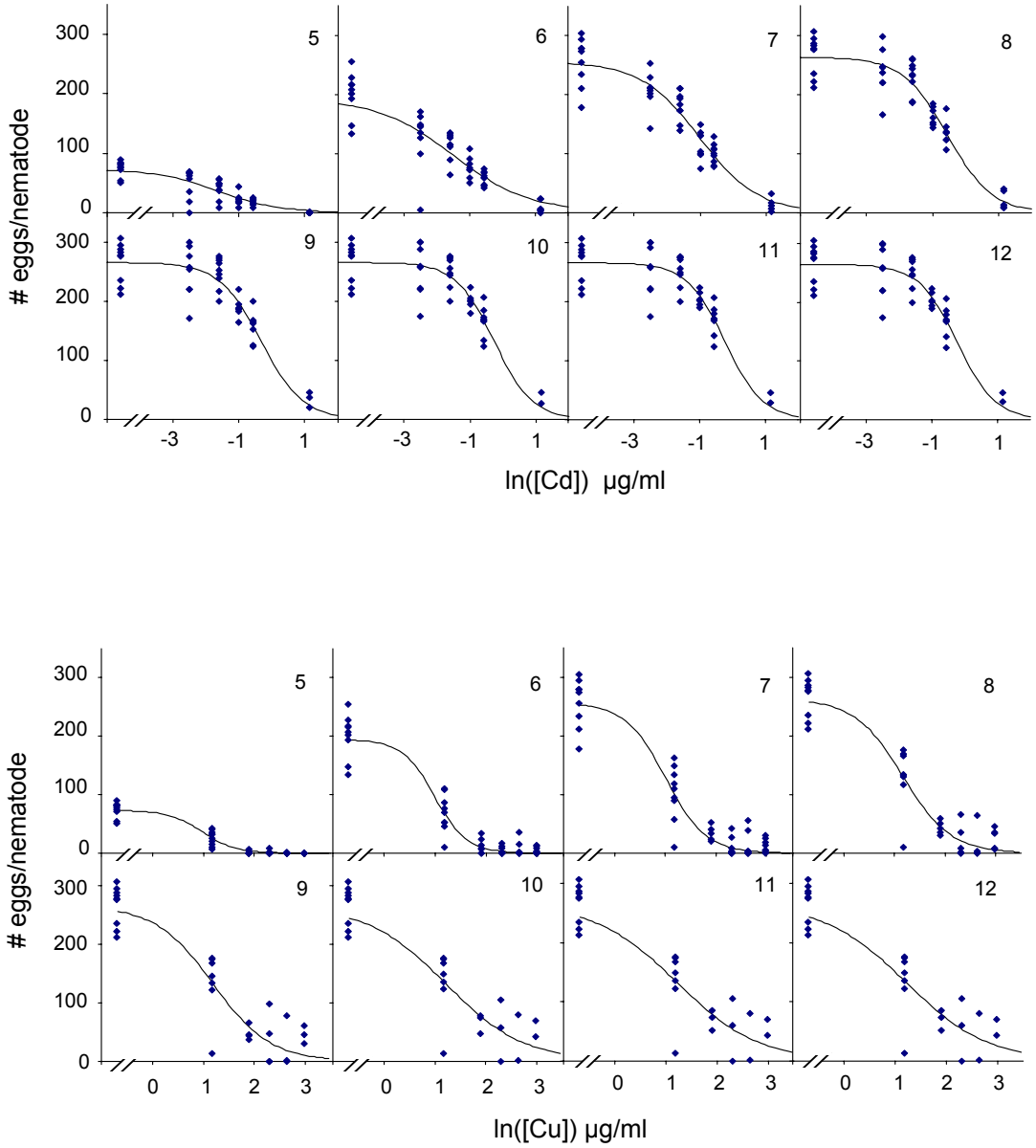


Figure. 2. Effect of cadmium (2A) and copper (2B) on the cumulative reproduction. The curves indicate the marginals of the age specific dose response surface. The numbers in the upper-right corner of the plots denote the age class in days.

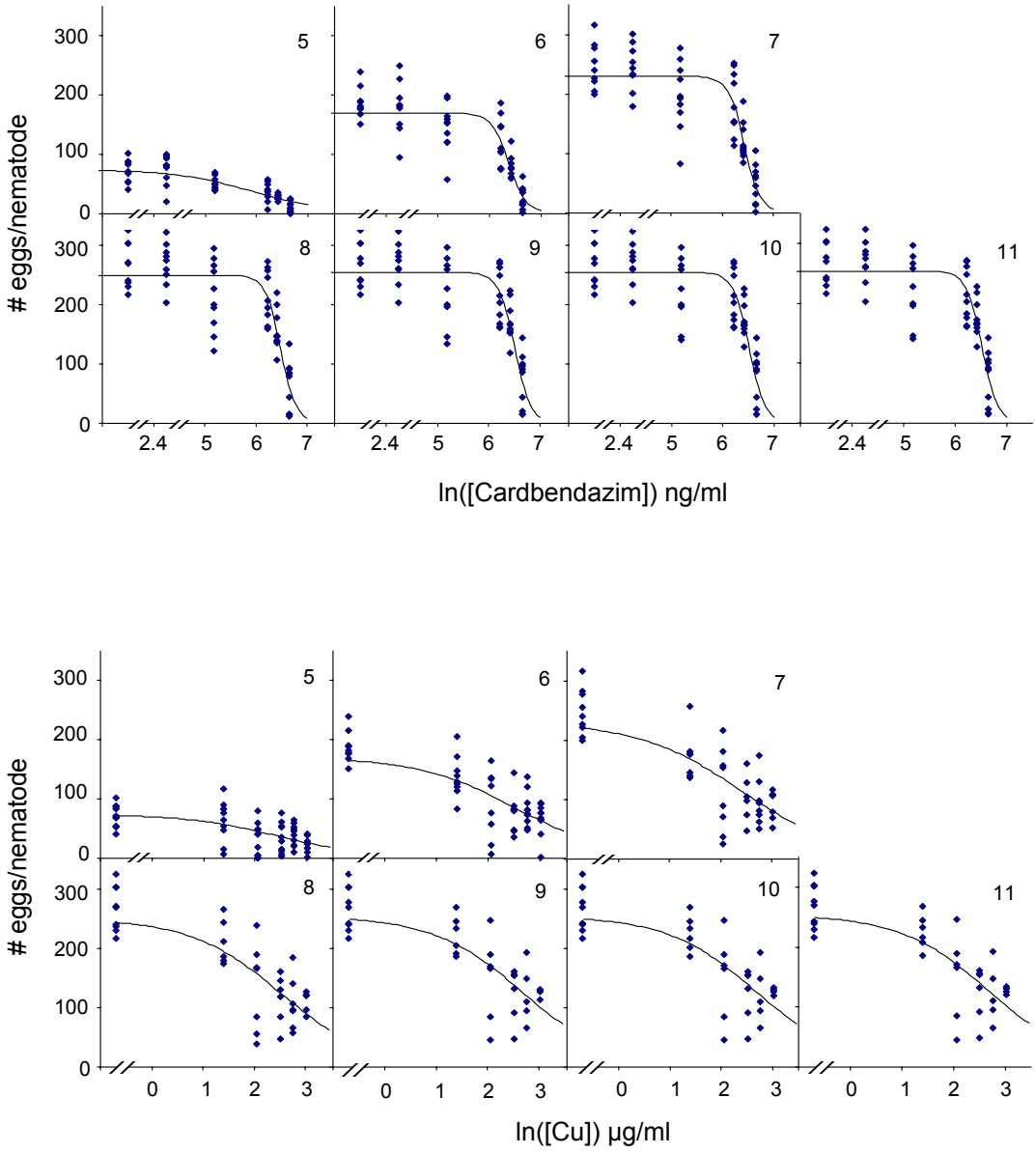


Figure. 3. Effect of carbendazim (3A) and copper (3B) on the cumulative reproduction. The curves indicate the marginals of the age specific dose response surface. The numbers in the upper-right corner of the plots denote the age class in days. The reproduction of the ETOH control was: day5: 71.9 (25.4), day6: 198.6 (23.8), day7: 264.6 (14.0), day8: 278 (30.0), day9: 278.1 (30.0), day10: 278.1 (30.0), day11: 278.1 (30.0).

Table 2. Age specific EC10 and EC50 values (with 95% confidence intervals) of cadmium and copper.

	cadmium		copper	
Age (d)	EC10 µg/ml	EC50 µg/ml	EC10 µg/ml	EC50 µg/ml
5	0.025 (0.006 – 0.04)	0.19 (0.14 – 0.25)	1.30 (0.55 – 2.05)	2.66 (2.14 – 3.17)
6	0.015 (0.003 – 0.03)	0.21 (0.16 – 0.27)	1.32 (0.70 – 1.94)	2.65 (2.22 – 3.08)
7	0.056 (0.027 – 0.085)	0.37 (0.30 - 0.44)	1.04 (0.61 – 1.47)	2.75 (2.33 - 3.18)
8	0.13 (0.07 – 0.19)	0.58 (0.47 - 0.69)	1.14 (0.70 – 1.58)	3.21 (2.72 - 3.70)
9	0.18 (0.09 – 0.27)	0.73 (0.55 - 0.90)	0.95 (0.44 – 1.45)	3.28 (2.60 - 3.95)
10	0.21 (0.10 – 0.33)	0.76 (0.56 - 0.97)	0.62 (0.13 – 1.11)	3.36 (2.42 - 4.29)
11	0.22 (0.10 – 0.34)	0.79 (0.56 - 1.02)	0.59 (0.09 – 1.08)	3.37 (2.37 - 4.36)
12	0.22 (0.10 – 0.34)	0.79 (0.56 - 1.02)	0.59 (0.09 – 1.08)	3.37 (2.37 - 4.36)

Table 3. Age specific EC10 and EC20 values (with 95% confidence intervals) of carbendazim and copper.

	carbendazim		copper	
Age (d)	EC10 ng/ml	EC50 ng/ml	EC10 µg/ml	EC50 µg/ml
5	72 (-1 – 146)	382 (241 – 523)	1.63 (-0.36 – 3.63)	11.72 (7.32 – 16.12)
6	406 (329 – 484)	591 (549 – 633)	1.62 (0.01 – 3.23)	12.53 (8.96 – 16.10)
7	439 (370 – 509)	624 (584 – 664)	1.23 (-0.07 – 2.54)	10.99 (7.63 – 14.36)
8	470 (402 – 538)	660 (620 – 700)	1.81 (0.21 – 3.41)	12.29 (8.88 – 15.71)
9	481 (412 – 550)	675 (635 – 716)	2.17 (0.30 – 4.03)	14.34 (10.10 – 18.59)
10	487 (418 – 556)	680 (640 – 721)	2.21 (0.30 – 4.13)	14.54 (10.27 – 18.81)
11	487 (418 – 556)	681 (640 – 721)	2.24 (0.36 – 4.12)	14.55 (10.32 – 18.78)

The results of the joint exposure to copper and cadmium are summarised in figure 4A. Effect level dependent deviations from additivity were not detected. Therefore figure 4A shows the parameter estimates of the additional parameters in equation 1 to quantify synergism/antagonism (one parameter: open circles), and to quantify toxicant ratio dependent deviations from additivity (two parameters: black squares and black triangles). Negative parameter values denote an enhanced toxicity, and positive values indicate a reduced toxicity of the mixture. The numbers indicate the significance of the additional parameter in the

toxicant ratio model, compared with the model that describes synergism/antagonism. Analysing the data only with the synergism/antagonism model, revealed a synergistic effect when copper and cadmium were combined, which is in agreement with the TU analysis. The synergistic effect is gradually decreasing: it is significant until the age of 9 days. Significance values were p_{day5} = 2.1E-7, p_{day6} = 2.4E-6, p_{day7} = 8.8E-7, p_{day8} = 0.0002, p_{day9} = 0.06, p_{day10} = 0.55, p_{day11} = 0.89, p_{day12} = 0.93. Yet, in figure 4A it can be seen that from age class 7 the model for quantifying a toxicant ratio dependent deviation from additivity described the data significantly better (p = 0.025). The additional parameter was incorporated to quantify the effect of copper (parameter b in the TR model; table1). This parameter was negative indicating that the mixture was relatively more toxic if relatively more copper was available (all compounds are rescaled to Toxic Units). On the other hand, the other parameter (indirectly associated with cadmium; table 1) was positive, indicating that the mixture was relatively less toxic if relatively more cadmium was available.

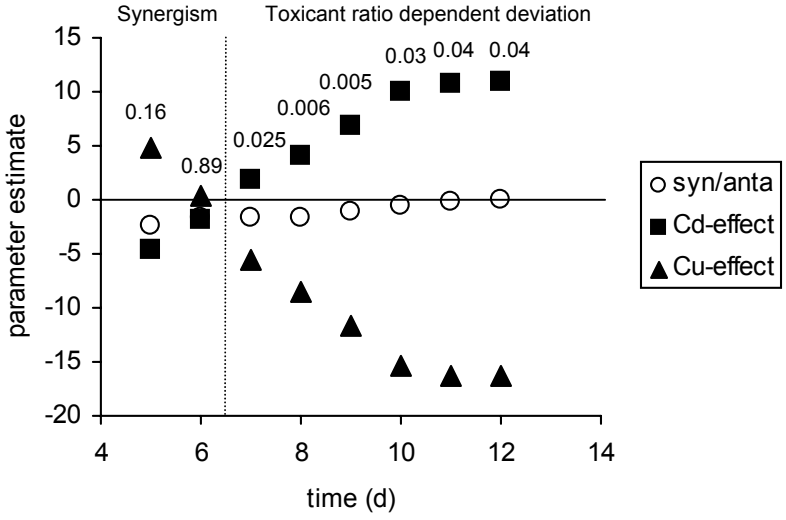


Figure. 4A. The parameter estimates of the model quantifying synergism/antagonism (one parameter: open circles), and for quantifying toxicant ratio dependent deviations from additivity (two parameters: black squares and black triangles) describing the copper-cadmium effect in time. Negative parameter estimates indicate enhanced toxicity and positive parameter estimates reduced toxicity. The numbers are the significance p-values of the additional parameter in the toxicant ratio model, compared with the model that describes synergism/antagonism. See text for detailed explanation.

The same procedure was performed to quantify the effect of copper and carbendazim. It revealed that maximum parsimony could not be obtained with the model describing toxicant ratio dependent deviations. Therefore figure 4B shows the parameter estimates of the additional parameters in equation 1 quantifying synergism/antagonism (one parameter: open circles), and quantifying effect level dependent deviations from additivity (two parameters: black squares and black triangles). Again, the negative estimates of the single additional parameter indicate synergism. Significance values were p_{day5} = 4.38E-7, p_{day6} = 8.31E-6, p_{day7}

= 7.77E-5, p_{day8} = 4.69E-4, p_{day9} = 4.84E-4, p_{day10} = 6.59E-4, p_{day11} = 6.40E-4, in accordance with the TU analysis, where synergism at the EC50 level at the age of 8 days was found. From age class 6 until the end of the reproductive period a significant effect level dependent deviation from additivity was detected. The interpretation of the additional parameters is somewhat different than for the TR model. The negative estimates for alpha indicate synergism at low effect levels and antagonism at high effect levels (chapter 2). Beta quantifies the effect concentration (or dose level) where the swapping from synergism to antagonism actually occurred. It can be calculated from 1/beta * EC50, therefore in figure 4B the parameter estimates for 1/beta are shown. For instance, at age class 8 beta = 0.82, thus swapping from synergism at low effect levels to antagonism at high effect levels occurred at concentrations of 1.22 * the EC50 values of the individual chemicals.

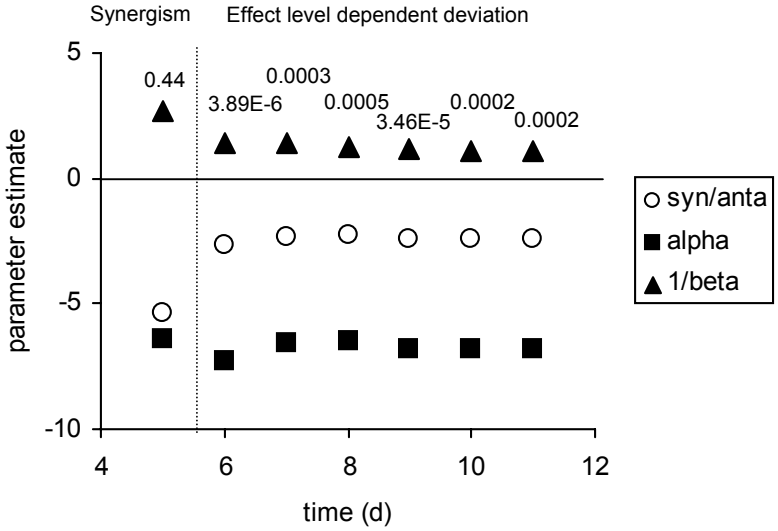


Figure 4B. The parameter estimates of the model quantifying synergism/antagonism (one parameter: open circles), and for quantifying effect level dependent deviations from additivity (two parameters: black squares and black triangles) describing the copper-carbendazim effect in time. Negative parameter estimates in the syn/anta model indicate synergism. Negative values for alpha indicate synergism at low effect levels and antagonism at high effect levels, where swapping occurs at dose levels of 1/beta * EC50. The numbers are the significance *p*-values of the additional parameter in the effect level model, compared with the model that describes synergism/antagonism. See text for detailed explanation.

Reproduction events

Figure 5 shows three examples of assuming a gamma distribution of the age specific reproduction. For this presentation the distribution was made discrete, to allow for an easy comparison with the data. It illustrates that the reproductive rate in the control was well described, but that some divergence was observed when the nematodes were intoxicated. Yet, the onset and ending of the reproductive period were described well, which were of primary interest in this study.

Figure 6 shows the effects of copper and cadmium, and copper and carbendazim on the juvenile period of *C. elegans*. In these plots all concentrations are scaled relative to the EC50's for cumulative reproduction determined at the age of 5 days, the first age class that an EC50 could be estimated (figure 2; table 2; table 3). The rescaling enables the comparison of the responses to the different compounds and their mixtures with respect to a toxic strength, specific for young adults. The effect of copper and cadmium on the juvenile period is shown in figure 6A. The length of the juvenile period of the control was estimated as 94.5 (92.6 – 95.9) h. It can be seen that the juvenile period was substantially lengthened at concentrations higher than approximately ln(TU) = 1, which is at concentrations of about 2.7 times the EC50 with regard to the effect on cumulative reproduction. In addition, the prolongation is accompanied by an increase in the uncertainty around the estimate. The mixture effect follows the same trend, and does not deviate much from the effect of the single toxicants. Only the juvenile period of the nematodes exposed to the highest mixture concentrations was considerably increased, which might indicate a certain "synergism".

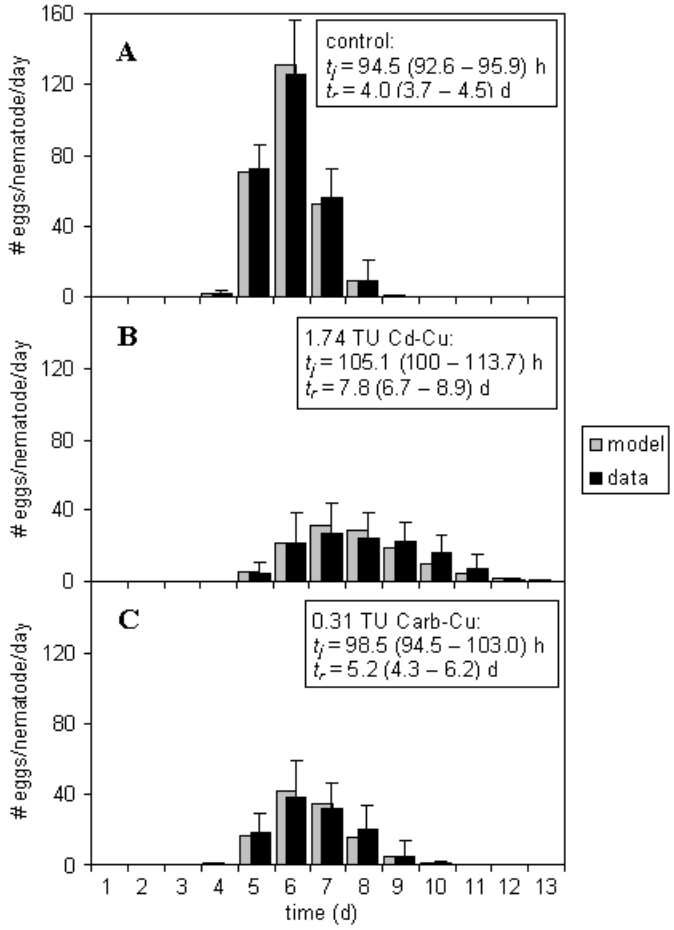


Figure. 5. Egg laying rate (number of eggs/nematode/day) of the control (5A), the 0.93 TU copper-cadmium exposed (5B), and the 0.21 TU carbendazim-copper exposed (5C) nematodes. A comparison between a discrete gamma distribution and the actual egg counts is shown.

Figure 6B shows the effect on the juvenile period of copper and carbendazim. The upper black dot denotes the ethanol control. Note the different scale compared to figure 6A. Only a minor increase in juvenile period was observed. Yet, the highest concentrations tested was ln(TU) = 1, whereas figure 6A indicates that higher concentrations were required for substantial effects on the juvenile period. Still, the juvenile period was prolonged from 92.9 (90.9 – 95.1) h in the control until 102.3 (98.3 – 108.8) h in the highest carbendazim exposure. It can be seen that at low concentrations the juvenile period was estimated somewhat shorter than in the control, which may indicate an hormesis effect.

Figure 7 shows the effects of copper and cadmium, and copper and carbendazim on the length of the reproductive period of *C. elegans*. In these plots all concentrations are scaled relative to the EC50's for cumulative reproduction determined at the age of 5 days. Thus, responses are scaled relative to the same toxic strength as in figure 6. It was observed that, when exposed to copper and cadmium, the nematodes prolonged their reproductive period at lower concentrations, whereas it was shortened at the higher toxicant concentrations (fig 7A). Again an increase in uncertainty around the estimate was observed at higher exposure levels. The response to copper and carbendazim was less pronounced (fig. 7B). Some prolongation was observed, for instance, the copper treatment at ln(TU) = -1.06 was significantly different from the control (randomisation test: p = 0.0025). However, since the higher copper treatments are not different from the control a clear pattern is absent. In addition, only the highest carbendazim concentrations influenced the length of the reproductive period of *C. elegans*. In both figures 6 and 7, the response to the mixture was similar as the response to the individual chemicals.

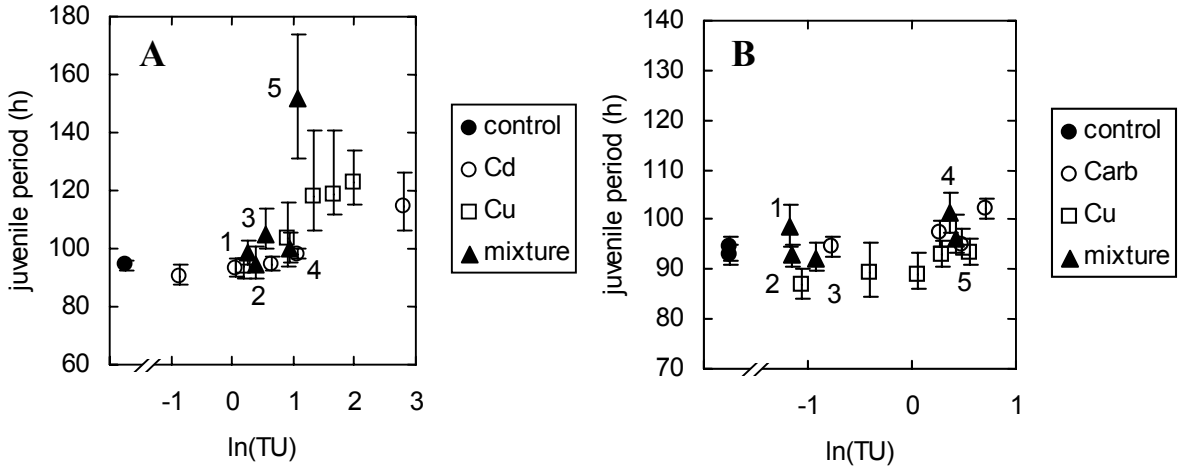


Figure 6. Effect of cadmium, copper, and combinations (6A), and carbendazim, copper and combinations (6B) on the length of the juvenile period (hours) with 95% confidence intervals. Concentrations of the toxicants are scaled relative to the EC50's of cumulative reproduction on day 5. Numbers indicate the mixture points with TU-ratio's: 6A (Cd/Cu): 1=0.27/0.73, 2=0.56/0.44, 3=0.47/0.53, 4=0.63/0.37, 5=0.33/0.67. 6B (Carb/Cu): 1=0.55/0.45, 2=0.20/0.80, 3=0.64/0.36, 4=0.78/0.22, 5=0.60/0.40. In 6B the upper black dot denotes the ETOH control.

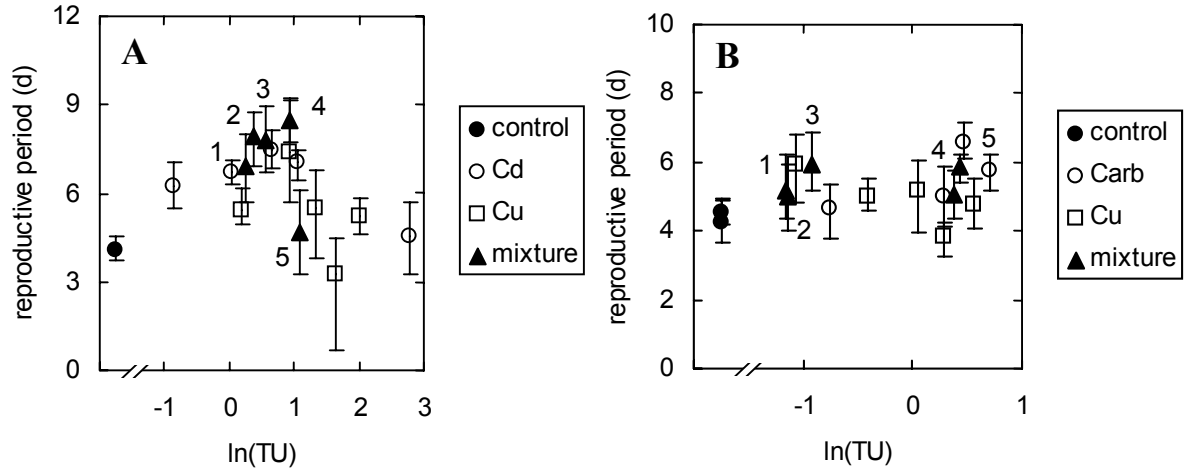


Figure 7. Effect of cadmium, copper, and combinations (7A), and carbendazim, copper and combinations (7B) on the length of the reproductive period (days) with 95% confidence intervals. Concentrations of the toxicants are scaled relative to the EC50's of cumulative reproduction on day 5. Numbers indicate the mixture points with the TU-ratio's that coincide with figure 6. In 7B the lower black dot denotes the ETOH control.

Body size

Figure 8 depicts the effect on growth of 613 ng/ml carbendazim (8A), and 21 µg/ml copper (8B) compared to the control (ethanol didn't alter growth). It shows that the growth curves of exposed and control nematodes started to diverge between the third and the fourth age class, representing the young adults. Therefore the dose response of carbendazim, copper and their combinations were analysed at day 4, 5, 6 and 8. The results are shown in table 4. The second, third, and fourth column show the significance values of the additional parameters in the model to describe synergism/antagonism (S/A), toxicant ratio dependent deviation, (TR) and effect level dependent deviation (EL) from additivity. One additional parameter yielded a significant result at all ages except at the age of 8 days. The additional parameters were negative (a_{day4} = -2.88, a_{day5} = -5.09, a_{day6} = -4.97), indicating synergism. Also the additional parameter obtained on day 8 was negative (a_{day8} = -1.40), indicating a synergistic trend. Incorporating a second additional parameter to allow for an effect level dependent deviation from additivity was significant at all the ages analysed. The values of the additional parameters indicate the same as in figure 4B: synergism at low effect levels, and antagonism at higher effect levels, where the dose level of inflection is higher than the EC20's of the individual mixture constituents.

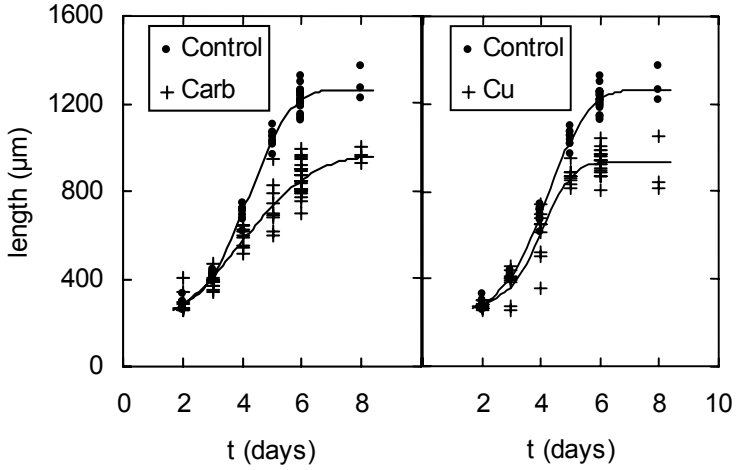


Figure 8. Effect of 613 ng/ml carbendazim (8A) and 21 µg/ml copper (8B) on length.

To illustrate the analysis, dose response relationships at the age of 6 days are shown in figure 9 and 10. The dose response curves are the estimates from the most parsimonious (EL) model, thus the marginals of the dose response surface. The mixture exposed nematodes were generally smaller than the nematodes exposed to the individual chemicals, which was quantified by the significant additional S/A parameter. It can also be seen that the synergism decreased at higher TU-levels, i.e. the body size did not decrease further at higher mixture dose levels, hence the dose level dependency. The improvement in model fit is shown in figure 10. The picture is largely self-explanatory. The diagonal line indicates ideal model description.

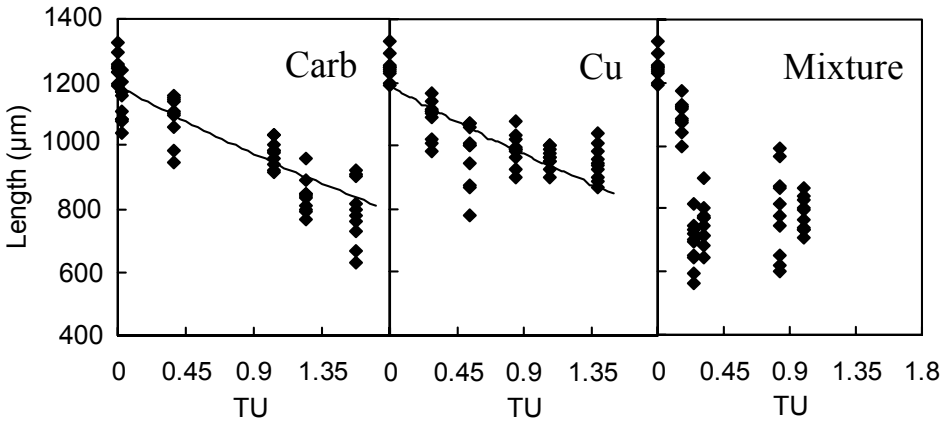


Figure. 9. Dose response relationship for carbendazim, copper, and their combination on nematode length at the age of 6 days. The curves are quantified with the most parsimonious model, and indicate the marginals of the dose response surface. TU is the exposure concentration divided by its EC20 for length.

The last row in table 4 shows the analysis of the effect of copper and cadmium at the age of 6 days. One additional parameter yielded a significant improvement in data description. The parameter was negative (a_{day6} = -4.44), indicating synergism. After addition of a second parameter a toxicant ratio dependent deviation from additivity was detected. Similarly as in figure 4A, parameter *b* was added to quantify the effect of copper (table 1). The negative value indicated that the mixture was relatively more toxic if relatively more copper was available. Parameter *a* was positive, indicating that the mixture was relatively less toxic if relatively more cadmium was available.

Table 4. Quantification of the effect of carbendazim-copper, and cadmium-copper (lower row) on age specific length. First three columns indicate the significance of the deviation from the additive model: S/A = synergism/antagonism, TR = toxicant ratio dependent deviation, EL = effect level dependent deviation. Control, slope are the parameters of the individual dose response curves, EC20 was estimated from the inverse dose response curves, and *a* and *b* are the deviation parameters.

Age (d)	S/A $p(X^2)$	TR $p(X^2)$	EL $p(X^2)$	Control (µm)	slope	EC20*	*a*	*b*
carbendazim - copper								
4	0.002	0.38	5.57E-7	704	0.0003[a] 0.017[b]	763[a] 16.2[b]	-8.66	0.95
5	2.78E-7	0.15	1.01E-17	1069	0.0005[a] 0.015[b]	460[a] 15.3[b]	-10.13	0.65
6	1.48E-8	0.33	7.98E-26	1189	0.0005[a] 0.015[b]	490[a] 14.7[b]	-10.32	0.68
8	0.06	0.15	0.0006	1281	0.0005[a] 0.022[b]	415[a] 9.98[b]	-5.57	0.59
cadmium - copper								
6	1.02E-7	0.007	0.81	1276	0.108[c] 0.026[b]	2.16[c] 8.79[b]	6.59	-15.5

a = carbendazim
b = copper
c = cadmium
* EC20 in ng/ml for carbendazim, and µg/ml for copper and cadmium.

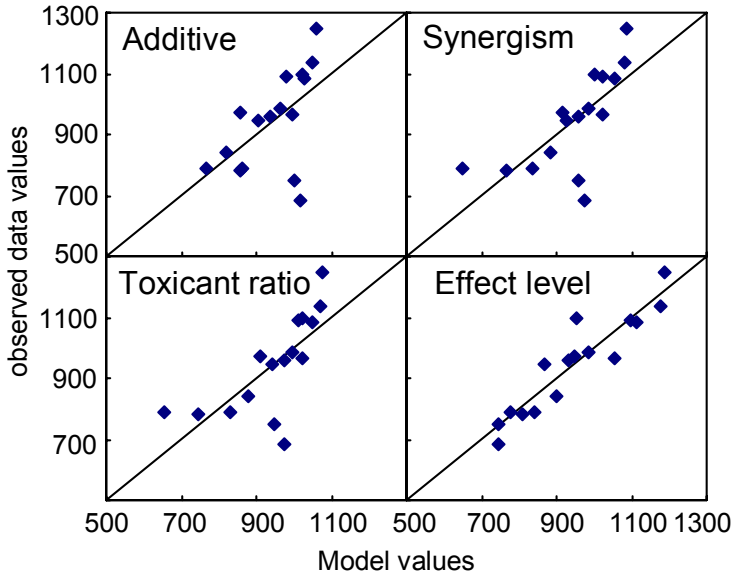


Figure. 10. Comparison of the model description with the data, after analysing the effect of carbendazim and copper on growth at the age of 6 days. The diagonal lines indicate ideal model description. The points are the means of 10 replicates. Comparisons between data and four deviation patterns are shown: no deviation, synergistic/antagonistic deviation, toxicant ratio dependent deviation and effect level dependent deviation.

Discussion

When analysing chronic exposure to mixtures one has to deal with the possibility that the toxicity of the mixture relative to the toxicity of the individual components can vary over time. In literature there are various indications for transient mixture effects. For instance, the synergistic cytotoxic effect on tumour cells of 5-Fluorouracil and 2α-Methyldihydrotestosterone observed in Sprague-Dawley-rats, diminished after 4 weeks of exposure despite daily treatment (Teller *et al.* 1982). A reanalysis of the cytotoxic effects of 4-Hydroperoxycyclophosphamide (4-HC) and VP-16-213 (VP-16) on human HL-60 cells (Chang *et al.* 1985) revealed that the synergistic effect observed after 24 hours exposure, changed into a ratio dependent deviation from additivity after 48 hours. After 72 hours of exposure, synergism was completely dissipated (chapter 2). Similar effects were reported by Aschele *et al*. (Aschele *et al.* 1998) for other cytotoxic chemicals. Van Gestel and Hensbergen (Van Gestel and Hensbergen 1997) observed that effects of combinations of cadmium and zinc on reproduction, dry weight and wet weight of *Folsomia candida* were time dependent. But also stable patterns have been reported. For instance the effects of four Chlorinated Dibenzo-*p*-dioxins on growth, survival, hematologic effects and EROD induction of rats, was additive irrespective of acute or (sub) chronic exposure (Viluksela *et al.* 1998). Data concerning chronic exposure to mixtures of compounds are nevertheless scarce.

In this study, the effect of copper and cadmium changed from synergism to a toxicant ratio dependent deviation from the additive model after the age of 6 days, and was still primarily synergistic until age class 9. It was determined that a relative increase in cadmium coincided with a relative decrease in toxicity of the mixture. It is however difficult to connect toxicity patterns with underlying physiological mechanisms. Some interactions have been reported that may help interpreting the results. For instance, in Enchytraeds sublethal concentrations of cadmium were found to induce a gene encoding a non-metallothionein, cysteine-rich protein (CRP), important in metal detoxification (Willuhn *et al.* 1994). Copper alone induced the CRP-gene at minimum levels only. Yet, (pre) induction of the CRP-gene by cadmium reduced the toxicity of copper (Willuhn *et al.* 1996). These kind of physiological processes might also play a role in the observed cadmium-related reduced toxicity in *C. elegans*.

The effect of copper and carbendazim showed synergism at low effect levels and antagonism at high effect levels, where swapping from synergism to antagonism occurred at concentrations of ±1.22 times the EC50 values of the individual mixture constituents. Note however that the toxic strength of the mixture constituents changed over time because of the dependence on the toxicity of the individual chemicals (table 3). Consequently the highest relative mixture concentration was 1.53 TU at age is 5 days. (figure 6B), and 1 TU at age class 11. (figure 7B). Thus, at age class 11 antagonism was actually not measured, but extrapolation of the mixture effect would result in antagonism. With respect to the actual data it is therefore more accurate to state that synergism decreased as the dose level increased, similarly as shown in figure 9 for growth.

In this study the same concentration range of copper was analysed twice. Although the extracted concentrations were practically the same the toxicity differed 3-4 orders of magnitude. These differences are hard to explain, since the experimental design was very controlled. Yet, it is a known phenomenon in ecotoxicity studies (Van der Geest *et al.* 2000) and it emphasises the importance of testing the individual chemicals and their mixtures simultaneously to obtain reliable insight in combined action (Van Gestel and Hensbergen 1997).

The juvenile period is known to be a demographically important life cycle parameter (Kooijman and Metz 1984, Caswell 1996, Kammenga *et al.* 1996), and prolonged time to reproduction as a consequence of toxic stress has been found in various analyses (Levin *et al.* 1996, Van Straalen and Kammenga 1998, Hansen *et al.* 1999). Considering the relevance of this trait it is interesting to know whether the observed synergistic effects on cumulative reproduction at the age of 5 days had to be attributed to a deteriorated prolongation of the juvenile period or to a diminished reproductive output of the young adults. Indication for additional adverse mixture effects on time to maturity was only found in the highest copper-cadmium treatment (fig 6). This suggests that the observed synergistic effects on cumulative reproduction had little to do with changes in reproductive timing, but rather with a decreased number of eggs. In general, prolonged time to maturity was observed at concentrations of 2 – 3 times the EC50 for cumulative reproduction. Therefore the juvenile period may be considered as relatively non-sensitive to the toxicants tested. This is in agreement with

analyses of copper effects on the juvenile period of other nematode species (Kammenga and Riksen 1996).

The increased uncertainty around the estimated length of the juvenile period when toxicant concentrations increased seemed to hamper conclusions about toxic effects, in terms of significant differences. However, it should be considered as an effect in itself. It was also found in former experiments, when the influence of cadmium on the juvenile period of *C. elegans* was measured (thus not estimated by modelling; Kammenga *et al.* 2000). When measuring the juvenile period, basically the time until the moment of laying the first egg is determined. This is time to event data, which should be analysed with the proportional hazards model (Cox 1972). In short, with the proportional hazards model one quantifies the time to event by determining an instantaneous failure rate, where failure denotes the occurrence of the event (thus, equivalent to a "first egg laying rate" in this context). For example, in the control many nematodes laid their first egg in a small time frame, therefore a fast rate would have been quantified. The increased uncertainty observed in the data actually represented a decreased instantaneous failure rate, indicating that the failure rate was affected by toxicant exposure. A similar reasoning applies to the measurements of the length of the reproductive period.

When *C. elegans* was exposed to copper and cadmium, the length of the reproductive period showed a pattern of increased length at lower-, and decreased length at higher concentrations. A similar pattern was observed when *Plectus acuminatus* was exposed to copper (Kammenga and Riksen 1996). Spreading reproduction out over a greater range has been quantified to increase fitness (Caswell 2001), and it may represent the phenotypic plasticity in response to toxic stress (Kammenga *et al.* 1997). The decrease may be interpreted as a physiological consequence of the high intoxication. The data from the copper-cadmium treatment indicated that the length of the reproductive period was more sensitive to the toxicants than the length of the juvenile period. Both the lowest concentration of cadmium ($\ln(TU) = -0.87$) and copper ($\ln(TU) = 0.20$) were found to enhance the length of the reproductive period (fig 7A), whereas the juvenile period was affected only in the highest concentrations (fig 6A). The data from the copper-carbendazim data was less clear. Copper had an effect at $\ln(TU) = -1.06$ (fig 7B; randomisation test: $p = 0.0025$), carbendazim at $\ln(TU) = 0.47$ and $\ln(TU) = 0.7$ (non-overlapping confidence intervals), and the mixture at $\ln(TU) = -0.92$ and $\ln(TU) = 0.42$ (non-overlapping confidence intervals). On the other hand, the juvenile period was influenced only by two treatments of the highest concentrations (mixture: $\ln(TU) = 0.37$; non-overlapping confidence intervals, and carbendazim: $\ln(TU) = 0.7$; non-overlapping confidence intervals; fig 6B). Although this observation may suggest a lower sensitivity of the juvenile period, the differences are to small for drawing conclusions.

Despite the clear synergistic effect of copper-carbendazim on growth, mixture effects on time to reproduction and length of the reproductive period were not found (fig. 6B, 7B, 9). Thus, the data indicated that in these experiments the timing of life cycle events was not correlated directly with body size. On the other hand, the growth response to both mixtures agreed remarkably well with the effect on reproduction, suggesting some relationship between these traits. Yet, the decreased reproductive output can be interpreted in two ways. Direct

effect: mixtures may interact synergistically on the mechanisms important for reproduction. Indirect effect: via assimilation, growth and maintenance due to mixture induced changes in energy allocation (Kooijman and Bedaux 1996). Kooijman (Kooijman and Bedaux 1996) argues that indirect effects alter time to reproduction. From this point of view direct effects may be more important in explaining the synergistic effects on reproduction, despite the correlation with the effect on growth.

Changes in life cycle traits such as discussed above may be considered as toxicant induced shifts in energy allocation to reproduction, growth and development, storage and survival (Kozlowski 1991). These shifts may have consequences at the population level (Calow and Sibly 1990). It was shown that mixture effects, in comparison with effects of its individual constituents, can lead to complex response patterns. In this study, cumulative reproduction was relatively more decreased when toxicants were combined, whereas effects on the reproductive period were similar to effects of the individual chemicals. Transient mixture effects on *C. elegans* were observed, but only for the reproductive response to cadmium-copper exposure. Thus, compared to exposure to individual toxicants, combined exposure may lead to differences in energy allocation patterns, either due to direct or due to indirect interactions, but transient mixture effects complicate the data interpretation. The ecological consequences of these interrelationships are difficult to assess and generally ignored in ecotoxicological studies on combined exposure.

Acknowledgements

We thank Kees van Gestel, Marina Bongers, Claus Svendsen, Jason Weeks, Mia Sjögren, Minze Leistra and Arrienne Matser for fruitful discussions and cooperation in the project. In addition we thank Michiel Kraak and Mirjam Bekker for their help and contributions. This work was funded by the EU (ENV4-CT97-0507).

Appendix

Measured concentrations in the water fraction of the agar of cadmium and copper, and carbendazim and copper and their standard deviations.

cadmium - copper exposure		carbendazim - copper exposure	
Water soluble Cd µg/ml	Water soluble Cu µg/ml	Water soluble Carb ng/ml	Water soluble Cu µg/ml
bdl	< 0.1	< 7	< 0.3
bdl	3.24 (0.551)	< 7	4.04 (0.216)
bdl	6.67 (0.662)	< 7	7.78 (0.168)
bdl	10.02 (0.569)	< 7	12.30 (0.441)
bdl	13.91 (0.720)	< 7	15.75 (0.340)
bdl	19.62 (0.164)	< 7	20.56 (0.375)
0.08 (0.021)	< 0.1	14.63 (5.95)	< 0.3
0.203 (0.026)	< 0.1	178.60 (10.10)	< 0.3
0.37 (0.012)	< 0.1	504.65 (29.72)	< 0.3
0.56 (0.137)	< 0.1	613.25 (-)*	< 0.3
3.18 (0.35)	< 0.1	772.33 (320.27)	< 0.3
0.08 (0.007)	2.86 (0.187)	24.84 (7.62)	2.97 (0.120)
0.16 (0.038)	2.46 (0.131)	65.31 (6.63)	1.65 (0.052)
0.19 (0.034)	5.22 (0.240)	97.55 (10.20)	1.70 (0.032)
0.14 (0.022)	1.48 (0.131)	431.48 (145.15)	3.76 (0.032)
0.31 (0.058)	2.49 (0.400)	353.33 (24.17)	7.08 (0.246)

bdl = below detection limit
* = 1 replicate

Chapter 4

Demographic analysis of toxic mixture effects

Martijs J. Jonker, Jan E. Kammenga

Abstract

The joint toxic effect of chemical combinations is frequently analysed at the individual level: organisms are exposed to mixtures of compounds and combined effects are compared to the effects of the individual chemicals in terms of synergism or antagonism. The population level consequences of the observed interactions are difficult to assess. In this study the translation of combined effects at the individual level to the population level were analysed. Equivalent to regular mixture toxicity analysis, the effect of the mixture, compared to the effect of its individual constituents was of interest. Firstly, combined effects on a hypothetical life history were discussed, and the consequences of different response patterns of life history parameters for population growth rate (λ) were evaluated. Different response scenarios illustrated that the combined effect on λ, compared to the effect of the individual mixture constituents depended on three factors: i) the sensitivity of each life history trait to each of the toxicants, ii) the combination effect of the toxicants on each life history trait, iii) the sensitivity of λ to changes in each life history trait. A detailed analysis of mixture effects on the life history of nematode *Caenorhabditis elegans* showed that synergistic effects on reproduction were transferred to the population level. Toxicant exposure changed the elasticity to the stage-classified model parameters, where combined exposure induced the changes at lower dose levels. The application of structured population models in mixture toxicity analysis puts interactive effects in an ecological perspective.

Introduction

Mixture effects are analysed with toxicological models. Therefore they judge the consequences of effects on the level of cellular biochemistry for individuals, and not consequences at the levels of population, community, and ecosystem, which are ecologically more relevant (Newman and Jagoe 1996). One of the aims of ecotoxicological research is to link physiological effects of chemicals on individuals with the biological levels above (Moriarty 1988), and structured population models have been shown to be helpful tools herein (Kooijman and Metz 1984, Kammenga *et al.* 1996b). In this contribution we discuss the population consequences of combination toxicity. This may be a rather confusing subject. Therefore it is useful to explore the possibilities of responses that might be expected when a population of organisms is exposed to a toxic combination of chemicals, within a simple example. Subsequently an analysis will be performed on life cycle responses of *C. elegans*. The main interest is analysing the effect of the mixture, in comparison with the effect of its individual constituents. To keep this comparison simple, only responses to binary mixtures will be considered.

For this theoretical exercise a simple life history of a hypothetical organism was adopted that was used in conceptual population discussions before (Calow and Sibly 1990, Calow *et al.* 1997). The life cycle consists of two stages, a juvenile stage and an adult stage, as illustrated in figure 1. In this model the symbols S_j and S_a represent the survivorship of the juveniles and adults, t_j is the juvenile period until first breeding, t_a is the time between breeding events, and n is the number of female offspring produced at each breeding. In the text that follows the life history is used similar to Calow *et al.* (1997) in their discussion on risk assessment.

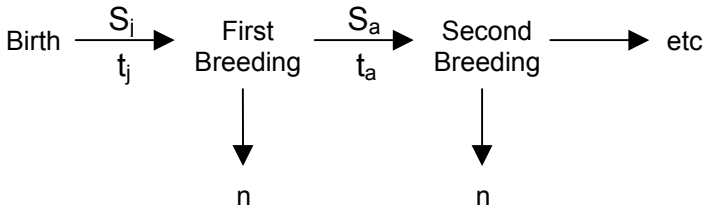


Figure 1. The two-stage life cycle in which S_j and S_a represent the survivorship of the juveniles and adults, t_j is the juvenile period until first breeding, t_a is the time between breeding events, and n is the number of female offspring produced at each breeding.

The performance of a population of organisms with a life history such as presented in figure 1 can be summarised in one quantity: the population growth rate, denoted by λ. If the population doubles, λ = 2, and if it halves, λ = ½. The unit of time in which the population doubles (or halves), as well as the dimensions of t_j and t_a depend on the modelling context, and can be expressed in days, weeks or years, but the time units have to be consistent in the model. Suppose that S_j is independent of t_j, and S_a is independent of t_a, then the population growth rate (dimension: t^{-1}) can be calculated from the Euler-Lotka equation, which can be written as:

$$1 = nS_j\lambda^{-t_j} + S_a\lambda^{-t_a} \quad (1)$$

Quantifying the implications of exposure of individuals to toxic mixtures for their population growth rate comprises two aspects: i) the effect of the mixture on the life history parameter values indicated by S_j, t_j, S_a, t_a and n, and ii) the effect of the changes in these parameter values on population growth rate. Clearly, further simplification is required to explore how these aspects may interact. Two scenarios will be discussed.

The first scenario is defined by $t_j = t_a = 1$ (e.g. 1 year). It reduces equation 1 to

$$\lambda = nS_j + S_a \quad (2)$$

with the advantage that λ can be calculated directly from nS_j and S_a. The results are shown in figure 2, where λ is depicted as a (linear) function of two life history traits: a combination of n and S_j, and S_a separately (life history traits and life history parameters are the same in this text). In this form the life history model is relatively easy to interpret. Three life history types with λ = 1, can be recognised in figure 2: (A) an iteroparous population where adult survival is high (S_a = 0.9), (B) an iteroparous population where S_a = 0.5, and (C) a semelparous population, where adults die after reproduction (S_a = 0). The second scenario under discussion is defined by $t_j \neq t_a$, but will be worked out later. First the mixture toxicity response patterns are introduced.

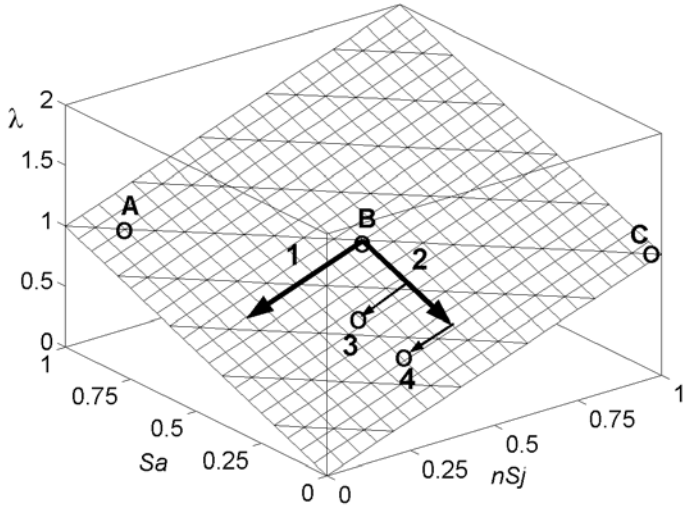


Figure 2. The relationship between population growth rate (λ), adult survivorship (S_a), and the juvenile survivorship times reproduction ($S_j n$), $t_j = t_a = 1$. Type A = an iteroparous population where adult survival is high (S_a = 0.9), type B = an iteroparous population where S_a = 0.5, and type C = a semelparous population, where adults die after reproduction (S_a = 0). The arrows indicate toxicant effects according to the 4 response patterns (see text).

The easiest way to explore combined effects is to define extreme classifications on a continuous biological scale. Consider two traits (nS_j and S_a), and two toxicants. Four extreme response patterns may be defined:

1. Trait 1 is sensitive to both toxicants and trait 2 isn't sensitive at all.
2. Trait 2 is sensitive to both toxicants and trait 1 isn't sensitive at all.
3. Trait 1 is sensitive to toxicant 1, and trait 2 is not. Trait 2 is sensitive to toxicant 2 and trait 1 is not.
4. Trait 1 is sensitive to toxicant 1, and trait 2 is sensitive to both toxicants.

One more simplification concerns the dose response relationship of the traits to the toxicants. These are assumed to be linear, with similar slopes. In the examples that follow, one toxicant is assumed to reduce the trait by 35%. Thus, pattern 1 and 2 cause a 70% reduction of the sensitive life history trait. Given these simplifications it can be asked what happens if an iteroparous population, with $S_a = 0.5$ and $\lambda = 1$, is exposed to these toxicants.

Scenario I: $t_j = t_a = 1$.

In case of response pattern 1, nS_j decreases with 70%, as indicated by arrow 1 in figure 2. Obviously, λ decreases as nS_j is lowered. Similarly, response pattern 2 causes a decrease of 70% of S_a, indicated by arrow 2, with negative consequences for the population growth rate. In case of response pattern 3, both traits decrease with 35%, and the new parameter values result in a population growth rate indicated by circle 3 on the response plain. Close inspection of these new λ-values accompanied by the three new life history types, shows that in this linear model all λ-values are the same (compare the λ-values with the iso-effect lines). Thus, although the new life history types are very different, population growth rate decreased to the same extent in all three cases. Response pattern 4, indicated by circle 4 on the response plain, causes the largest adverse effect on λ. It should be noted that synergism or antagonism, on either one or both traits, can lead to a larger, or smaller effect on λ.

Scenario II: $t_j \neq t_a$

First we explore the consequences of combined exposure if t_j is smaller than t_a. Figure 3 shows the population growth rate as a function of the two life history traits nS_j and S_a, with $t_j = 0.2$ and $t_a = 1$. It shows that the effect of nS_j on population growth rate is curved. The iteroparous population is still located in the middle of the line $\lambda = 1$, while it is exposed to a mixture of two toxicants. Considering the four extreme cases, similar responses are observed in figure 3 as in figure 2. Again, in case of response pattern 1, nS_j decreases with 70% as indicated by arrow 1, and response pattern 2 causes a 70% decrease of S_a depicted by arrow 2. Similarly, response pattern 3 causes both traits to decrease with 35%, and the new values of

the parameters result in population growth rate 3 on the response plain. Although the dose responses of the traits are similar to the first scenario, the consequences for the population growth rate are different. Close inspection of the obtained λ-values reveals that the additive response of S_a causes a larger adverse effect on λ than the additive response of nS_j. If the individual chemicals alter each trait separately, an intermediate adverse effect on population growth rate is obtained. Again, response pattern 4 causes the most deteriorated effect.

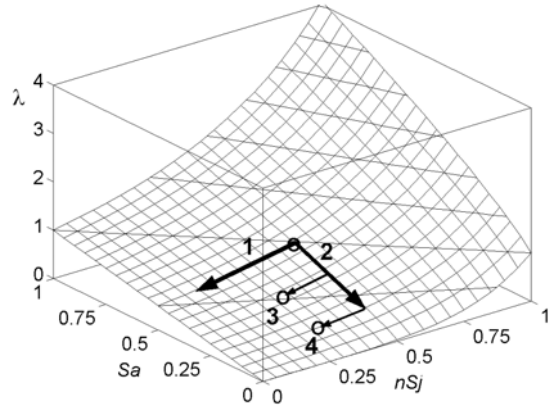


Figure 3. The relationship between population growth rate (λ), adult survivorship (S_a), and the juvenile survivorship times reproduction (S_jn), when t_j = 0.2 and t_a = 1. The arrows indicate toxicant effects according to the 4 response patterns (see text).

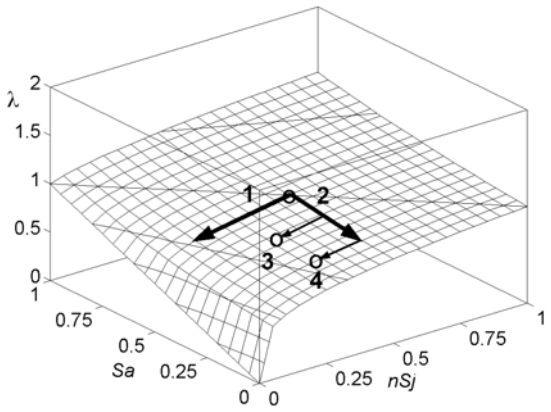


Figure 4. The relationship between population growth rate (λ), adult survivorship (S_a), and the juvenile survivorship times reproduction (S_jn), when t_j = 5 and t_a = 1. The arrows indicate toxicant effects according to the 4 response patterns (see text).

Figure 4 shows the population growth rate as a function of the two life history traits with t_j = 5 and t_a = 1. Here, the additive response of S_a causes a smaller adverse effect on λ than the additive response of nS_j. Again, an intermediate effect is obtained if each trait is altered by a separate toxicant. Yet, response pattern 4 yields a similar effect as response pattern 1. On the contrary, when trait 2 is sensitive to toxicant 1, and trait 1 is sensitive to both toxicants (the reverse as response pattern 4) a more adverse effect is obtained. Both in figure 3 and 4 synergistic or antagonistic effects, either on one or both traits, can lead to a larger or smaller effects on λ. It is obvious that the impact of synergistic and antagonistic effects depend on the functional relationship between the life history parameter of concern and population growth rate, i.e. the sensitivity of λ to changes in this trait. If a certain trait is synergistically affected, and λ is relatively sensitive to changes in this life history parameter, then the adverse effect on lambda can be larger than expected intuitively.

Toxicant induced changes in parameter values can increase the sensitivity of λ to additional changes in this life history trait, but also to changes in others than the ones affected. This can for instance be seen in figure 4. The additive effect on nS_j enhances the sensitivity of λ to a minor additional decrease of this trait: the response plain of λ is decreasing sharply, *i.e.* $d\lambda/dnS_j$ is enlarged. The ecological relevance of this phenomenon is that the population may

become more vulnerable to other environmentally induced life-cycle changes on nS_j (Kammenga *et al.* 2001), for instance shortage of food. Combination effects of toxicants can lead to unexpected changes in sensitivity of λ to potential changes in a life history trait. An example can be seen in figure 4, where a 10% synergistic effect on nS_j enhances the sensitivity of λ to potential changes in S_a ($d\lambda/dS_a$ is enhanced).

Incorporating more biological realism into these response models leads to complex toxicity patterns. For instance, a chemical might have an additive effect on one trait and a small effect on another, leading to response patterns with "partially sensitivity", compared to the extreme cases mentioned above. Thus a 70% effect on trait 1 can for example be accompanied by a 10% effect on trait 2. In addition, relaxing the assumption of equal linear dose response functions entails non-additivity of the responses. In this case, the calculation of toxic units can be used to take non-linearity and relative differences in toxicity into account. The example above considers only effects on two life history traits, but the chemicals may alter S_j, t_j, S_a, t_a and n differently, leading to a 5 dimensional response plain of λ. However, the examples illustrate that the combined effect on population growth, compared to the effect of the individual mixture constituents depends on three factors:

1. The sensitivity of each life history trait to each of the toxicants.
2. The combination effect of the toxicants on each life history trait.
3. The sensitivity of λ to changes in each life history trait.

With these examples in mind we can start analysing if and how these interactions take place in real biological data. Therefore we considered the results of data from experiments that examined sublethal effects of cadmium-copper and carbendazim-copper on various life history traits of the free living, soil dwelling, bacterivorous nematode *Caenorhabditis elegans*. The nematodes were exposed to 5 mixture combinations, and 5 dose levels of the individual mixture components (and a control) aiming at comparing combined effects with the effects of the individual chemicals. The life history traits under study were age specific reproduction, length of the juvenile period, length of the reproductive period, and growth. These data were used for a demographic analysis. Both experiments showed a similar result: a synergistic effect on reproduction whereas the mixture effect on other life history traits was similar as the effect of the individual compounds. Therefore it was decided to address the following questions:

1. Are synergistic effects on reproduction relevant at the population level?
2. Do synergistic effects on reproduction alter the contribution of other traits to λ?

Model and Parametrisation

Assuming discrete time steps, and assuming that the life cycle can be described as discrete stages, then the Euler-Lotka equation can be written in matrix form:

$$\mathbf{n}(t+1) = \mathbf{A}\mathbf{n}(t) \tag{3}$$

The state of the population at time t is given by the vector $\mathbf{n}(t)$, whos entries $n_i(t)$ give the abundance of each stage. The matrix $\mathbf{A}$ is the projection matrix. Its $(i,j)^{th}$ entry is denoted by a_{ij} and gives the number of stage i individuals at $t + 1$ per stage j individuals at time t. Thus, a_{ij} are the (time invariant) vital rates or life-cycle parameters, that describe the rates at which individuals move among life cycle stages by survival, growth, maturation, reproduction etc. The discrete time step or projection interval is defined as 1 day for the *C. elegans* model. The asymptotic rate of population increase is represented by the dominant eigenvalue of the projection matrix and is denoted by λ, as in the examples above. The sensitivity of λ to changes the matrix elements a_{ij} can be calculated from

$$\frac{\partial \lambda}{\partial a_{ij}} = \frac{v_i w_i}{\mathbf{v} * \mathbf{w}} \tag{4}$$

where $\mathbf{v}$ and $\mathbf{w}$ are the left and right eigenvectors, v_j and w_j are the i^{th} and j^{th} element of $\mathbf{v}$ and $\mathbf{w}$, and * denotes the complex conjugate transpose.

Two types of perturbation analysis can be performed by means of life history modelling: prospective and retrospective perturbation analysis. Prospective analysis is usually performed by calculating elasticity values, which are defined as proportional contributions of matrix elements a_{ij} to λ (De Kroon *et al.* 1986):

$$e_{ij} = \frac{a_{ij}}{\lambda}\frac{\partial \lambda}{\partial a_{ij}} \tag{5}$$

Elasticity values measure the effect of a proportional change in a_{ij}. In other words: if all a_{ij} 's were changed by the same proportion than the parameter with the largest elasticity has the strongest impact on population growth rate.

Retrospective perturbation analyses are usually performed by means of decomposition analysis. When individuals are experimentally treated and the effects on λ between control and treatment need to be analysed, then the contributions of differences in each of the matrix entries to the observed differences in λ can be approximated by (Caswell 1996b):

$$\lambda^{(k)} \approx \lambda^{(co)} + \sum_{ij} (a_{ij}^{(k)} - a_{ij}^{(co)}) \left.\frac{\partial \lambda}{\partial a_{ij}}\right|_{\bar{\mathbf{A}}} \tag{6}$$

where $\lambda^{(k)}$ and $\lambda^{(co)}$ denote the population growth rate of the toxicant treated and control nematodes, and $\bar{\mathbf{A}} = (\mathbf{A}^{(k)} + \mathbf{A}^{(co)})/2$. To explore the effects of the individual toxicants and their combinations, both analyses were used. Elasticity values were interpreted as toxicant induced changes in sensitivity to stress (Kammenga *et al.* 2001), and decomposition analysis enables

the interpretation of interactions between i) the sensitivity of a trait to a toxicant (combination) and ii) the sensitivity of population growth to changes in this trait.

An age-classified model makes maximum use of the demographic information available. It was used to quantify the consequences for λ of toxicant induced changes in reproduction and survival. Maternity, i.e. the number of offspring per individual aged *x* per day, was calculated directly from the data, as illustrated by figure 5 in chapter 3. Juvenile mortality was estimated from other experimental data, and was only observed after exposure to highest copper concentrations, and to relatively high cadmium concentrations. Since the experiments were designed to analyse sublethal effects, information on adult survival was limited. The following approach was found to yield the best description possible. The mortality data observed in the experiments was used to fit a logistic survivor function ($l(t,c)$) with a time-dependent *LC50*, using a maximum likelihood estimation procedure (Kooijman 1981), for the individual compounds and the mixture separately.

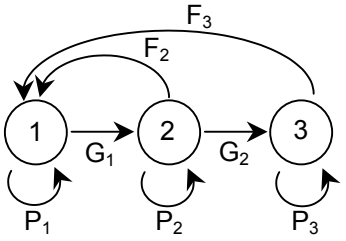
$$l(t,c) = \frac{1}{1 + \exp\left(\beta\left(\ln c - \ln\left(LC50(t)\right)\right)\right)} \quad \text{, where} \quad \ln\left(LC50(t)\right) = \frac{\kappa}{t} + \tau \tag{7}$$

where c denotes the concentration of the chemical (combination) of interest. Thus, the survival $l(t,c)$ depends on time (t) and the chemical concentration (c) via the parameters β (i.e. slope), κ and τ. For the combined exposure it was assumed that mortality depended on the joint toxic strength of the chemicals, using the toxic unit (*TU*) approach (Sprague 1970). Here, the toxic unit was defined as $TU = c/EC20$ (*EC20* for population growth rate), and the toxic units of the single compounds were summed to calculate the toxic strength of the mixture. The survivor function was used to estimate the age class specific survival. If juvenile mortality was observed it was incorporated by assuming $S_a = S_j l(t,c)$ (Kammenga *et al.* 1996a). Carbendazim exposure did not induce any mortality in these experiments. Other experimental single toxicity data indicated that mortality occurred at concentrations much higher than the ones tested here. The survival probabilities (P_i) and the fertilities (F_i) in the age-classified matrix were calculated using the birth flow model (appendix).

For the age-classified model, 95% confidence intervals were calculated from 1000 bootstrap estimates using the percentile method. Confidence intervals were corrected for bias. The bootstrap design consisted of an exact duplication of the experimental design and parameter estimation procedures.

Evaluating effects on age at maturity and length of the reproductive period, as studied in chapter 3, can be more biological meaningful than age specific survival probabilities and fertilities only. To incorporate these traits explicitly in the population model a reparameterisation of the projection matrix was required to develop a stage-classified model. It was decided to adopt the parameterisation that Brault and Caswell used (Brault and Caswell 1993) to analyse killer whale populations, shown in figure 5. The model considers three stages: “daylings”, a stage that lasts one day only, juveniles and reproductive adults. Post-reproductive stages don’t contribute to population growth rate and are therefore omitted here. The P_i gives the probabilities of surviving and remaining in the same stage, G_i gives the

probabilities of surviving and moving to the next stage, and F_i denote the stage specific fertilities. Clearly, P_i, G_i and F_i depend on survival probabilities and growth rates. Therefore they were calculated from the parameters σ_i, γ_i, and m, which are the stage specific survival- and growth probabilities and the mean reproduction. These calculations are shown in the appendix. The parameters σ_i, γ_i and m are usually called the underlying vital rates (Caswell 2001). Stage specific survival probabilities were calculated from the survivor function $l(t,c)$ and stage specific growth probabilities were calculated as the reciprocals of the estimated stage durations, where $\gamma_2 = 1/(t_j\text{-}1)$ to correct for the daylings. The mean reproduction was calculated as the ratio of the average total number of eggs per individual, to the length of the reproductive period. The probability of remaining in the first stage, P_1 is zero since this stage lasts for one day by definition. Generally, the individuals reach maturity within a projection interval, for instance, the juvenile period of control nematodes lasted a little less than 4 days. The nonzero value of F_2 represents the reproduction of the juveniles that mature and reproduce somewhere halfway a projection interval.



$$\begin{bmatrix} 0 & F_2 & F_3 \\ G_1 & P_2 & 0 \\ 0 & G_2 & P_3 \end{bmatrix}$$

Figure 5. The life-cycle graph and the corresponding stage classified population projection matrix. Stage1: daylings, stage2: juveniles, stage 3: reproductive adults. Because the duration of the dayling stage is the same as the projection interval, $P_1 = 0$. Right: the resulting projection matrix.

Results

For illustrations on the number of offspring per individual aged x per day we refer to figure 5 in chapter 3. It can be seen that the age specific reproduction was decreasing and the reproductive period lasted longer when the nematodes were intoxicated. Substantial prolongation of the time to reproduction was only found at high dose levels (2-3 times the EC50 for reproduction). Adult survival generally started to decrease after day 6, a pattern that was reasonably described by the survivor function. Two examples of the estimated survivor functions are shown in figure 6. Figure 6A shows the survival to copper. The 5 curves are survival curves for the 5 dose levels tested: 0, 4.04, 7.78, 12.3, 15.75 and 20.56 µg/ml. Figure 6B shows the survival to copper and cadmium. The tested TU levels were 0, 0.701, 0.946, 1.01, 1.348 and 1.836 TU.

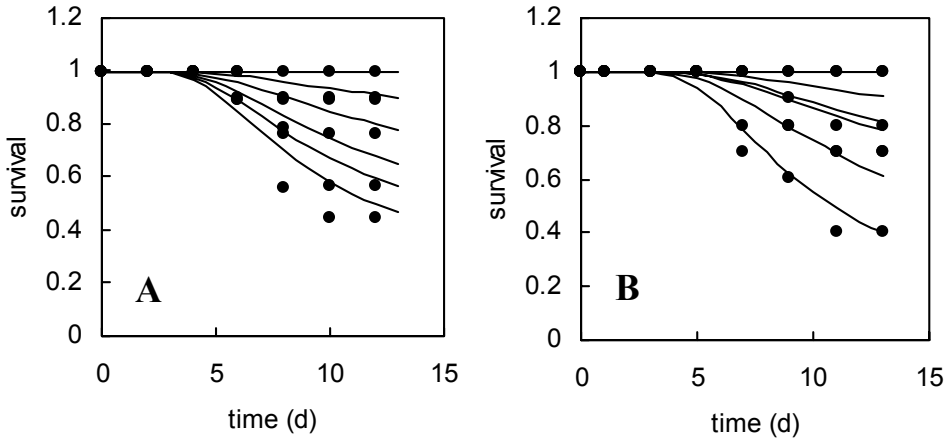


Figure 6. Survivorship functions of the mixture treated nematodes: copper (figure 6A; control and 5 dose levels) and copper-cadmium (figure 6B; control and 5 dose levels). The curve is a logistic function fitted according Kooijman (1981).

Population growth decreased when the nematodes were exposed to increased concentrations of copper and cadmium (figure 7A and 7B). The population growth rate of the control was 3.02 (2.96 – 3.07) d^{-1}, and decreased until 1.60 (1.45 – 1.74) d^{-1} due to the cadmium treatment and until 1.54 (1.44 – 1.63) d^{-1} due to the copper treatment. The pattern of the data points is noteworthy: λ decreased approximately linearly until ± 1.55 d^{-1}, which seemed to be a certain bottom line. Therefore the dose response takes an exponential functional form. Linear regression on the first five λ-values of the cadmium treatment and the first four λ-values of the cadmium treatment enabled the estimation of EC20 values. For cadmium the EC20 was 0.47 µg/ml, and for copper 3.66 µg/ml. The EC20's could be used to calculate *TU'*s (*TU = c/EC20*), enabling the comparison of mixture data with copper and cadmium individually. All mixture data were located in the linear part of the dose response. Figure 7C shows the λ-values of the mixture treatments and copper and cadmium treatments related to total *TU'*s. The λ-values of the mixture treatments spread out a little, but they are generally located below the λ-values that resulted from individual copper and cadmium treatment, thus synergistic patterns were observed.

The same analysis was performed on the copper-carbendazim treated nematodes. The population growth rate of the control nematodes was 2.98 (2.92 – 3.05) d^{-1}. The toxicant treatment did not reduce λ as much as in the copper-cadmium experiment. The highest copper treatment resulted in a population growth rate of 2.29 (2.17 – 2.39) d^{-1}, and the highest carbendazim treatment in a rate of 2.09 (1.99 – 2.19) d^{-1}. The EC20 for copper was 17.79 µg/ml, and for carbendazim 634 ng/ml. Both the decreased effect on λ and the higher EC20 for copper compared to the copper-cadmium exposure are the result of the lower copper toxicity as discussed in chapter 3. Figure 7D shows the λ-values of the mixture treatments and copper and carbendazim treatments related to total *TU'*s. Again, the λ-values of the mixture treated nematodes were generally more decreased than the λ-values that resulted from individual copper and carbendazim treatments.

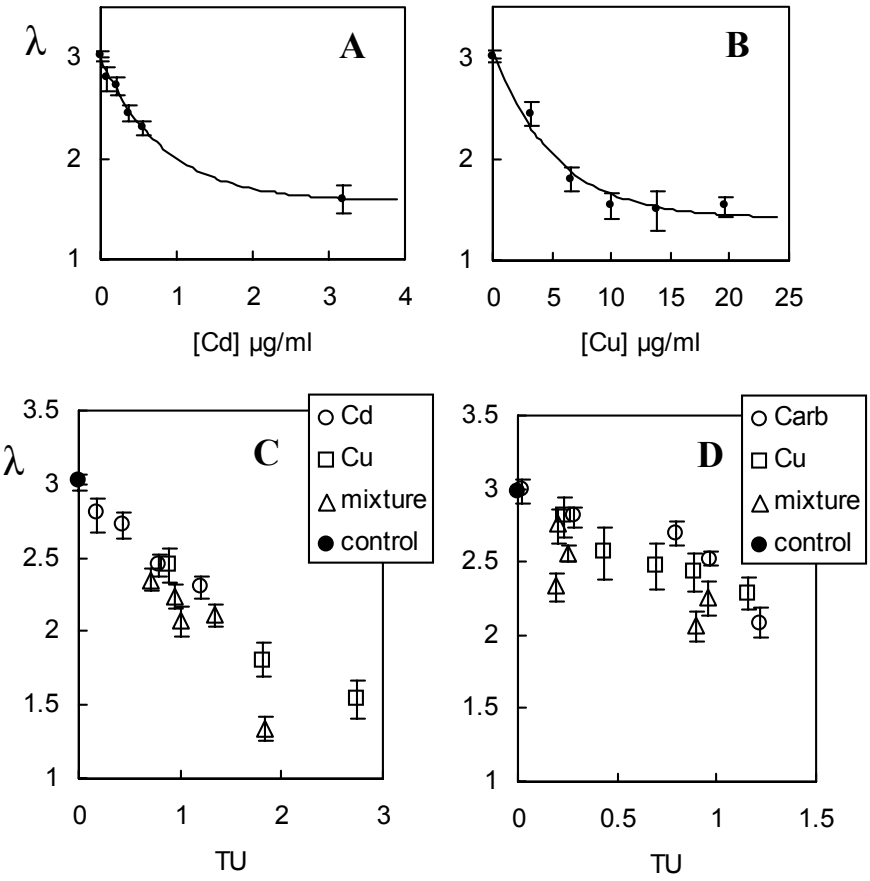


Figure 7. The relationship between population growth rate (λ) and cadmium (7A), copper (7B), copper-cadmium (7C) and copper-carbendazim (7D) calculated from the age-classified model, where *TU=c/EC20*. The error bars indicate 95% confidence intervals.

To illustrate the impact of the intoxication on the life history of *C. elegans*, the sensitivity of λ to changes in age specific fertility (F_i) and survival probability (P_i) of the control and the highest copper treated nematodes (19.62 µg/ml, data from the copper-cadmium experiment) are depicted in figure 8. Obviously, in unexposed conditions λ is very sensitive to changes in survival probability in the beginning of life. This sensitivity is decreasing due to intoxication. However, exposure to toxicants led to a higher sensitivity of λ to the survival probabilities of age class 5 and higher. Toxicant exposure also led to a higher sensitivity of λ to changes in fertility. The observed changes in sensitivity were also reflected in the toxicant induced changes in the elasticity pattern (figure 8). It can be seen that the elasticity of λ to changes in fertility is lower in the copper treatment than in the control, whereas the sensitivity is higher (figure 8). Elasticity includes information on reproductive output (equation 5), and the decrease in reproduction that resulted from copper exposure caused a decrease in elasticity.

To obtain more insight in the demographic mechanisms that mediated the results in figure 7, decomposition analysis was performed. In figure 9, the results of the lowest and the highest copper, cadmium and mixture treatment are shown. Pcon denotes the contributions of the age specific survival probabilities and Fcon denotes the contributions of the age specific fertilities.

These graphs are representative of the patterns generally observed in all exposures. Note the differences in scale. The toxic units correspond with the ones in figure 7C (although the highest copper and highest cadmium treatment are off scale). In general, decreased fertility contributed much more to the decreased population growth rate than the decreased survival probability. The large confidence intervals reflect the uncertainty in mortality rates, as described in "model and parametrisation". However, also the contribution of the largest uncertainty in mortality was still 10 orders of magnitude smaller than the contribution of fertility. The lowest single metal exposures show some "hormesis" effect in fertility in the third age class. It was more pronounced in the cadmium treatment than in the copper treatment, which was probably due to the lower relative concentration tested. Comparing the mixture effects with the effects of the individual constituents indicates that the 0.7 TU mixture treatment induced more pronounced negative contributions to population growth rate via fertility than the 0.89 *TU* Cu treatment. In addition, the highest mixture treatment was tested at a relatively lower toxic strength then the individual components (1.84 *TU* vs 5.37 and 6.79 *TU*), yet the contributions of fertility were of similar magnitude.

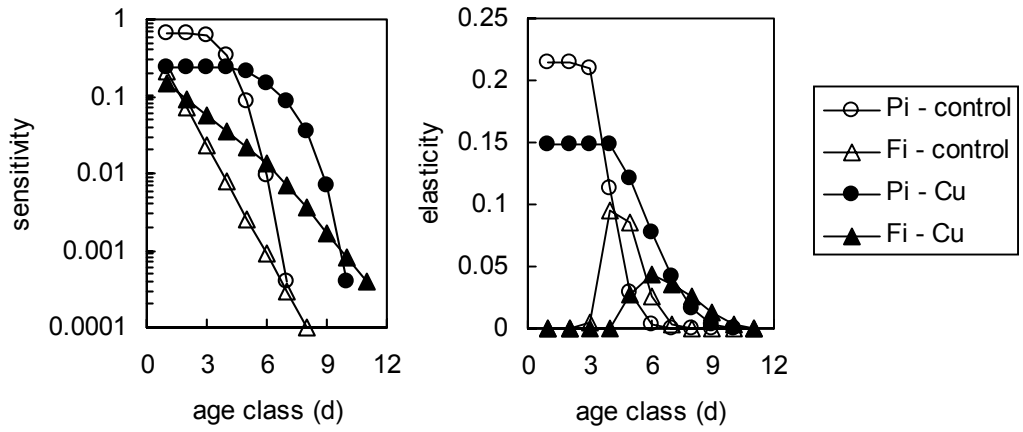


Figure 8. The sensitivity (left) and elasticity (right) of λ to changes in age specific fertility (F_i) and survival probability (P_i) for control and copper exposed (19.62 µg/ml) nematodes.

The stage-classified model enabled the analysis of the toxic effects on the stage structure of the life cycle. For brevity, only the analyses on copper-cadmium exposure are discussed here, but the copper-carbendazim treatment yielded similar results. The parameter values obtained from the data are shown in table 1. Decreased γ_2 and γ_3 values compared to the control indicate prolonged time to maturity and prolonged reproductive period.

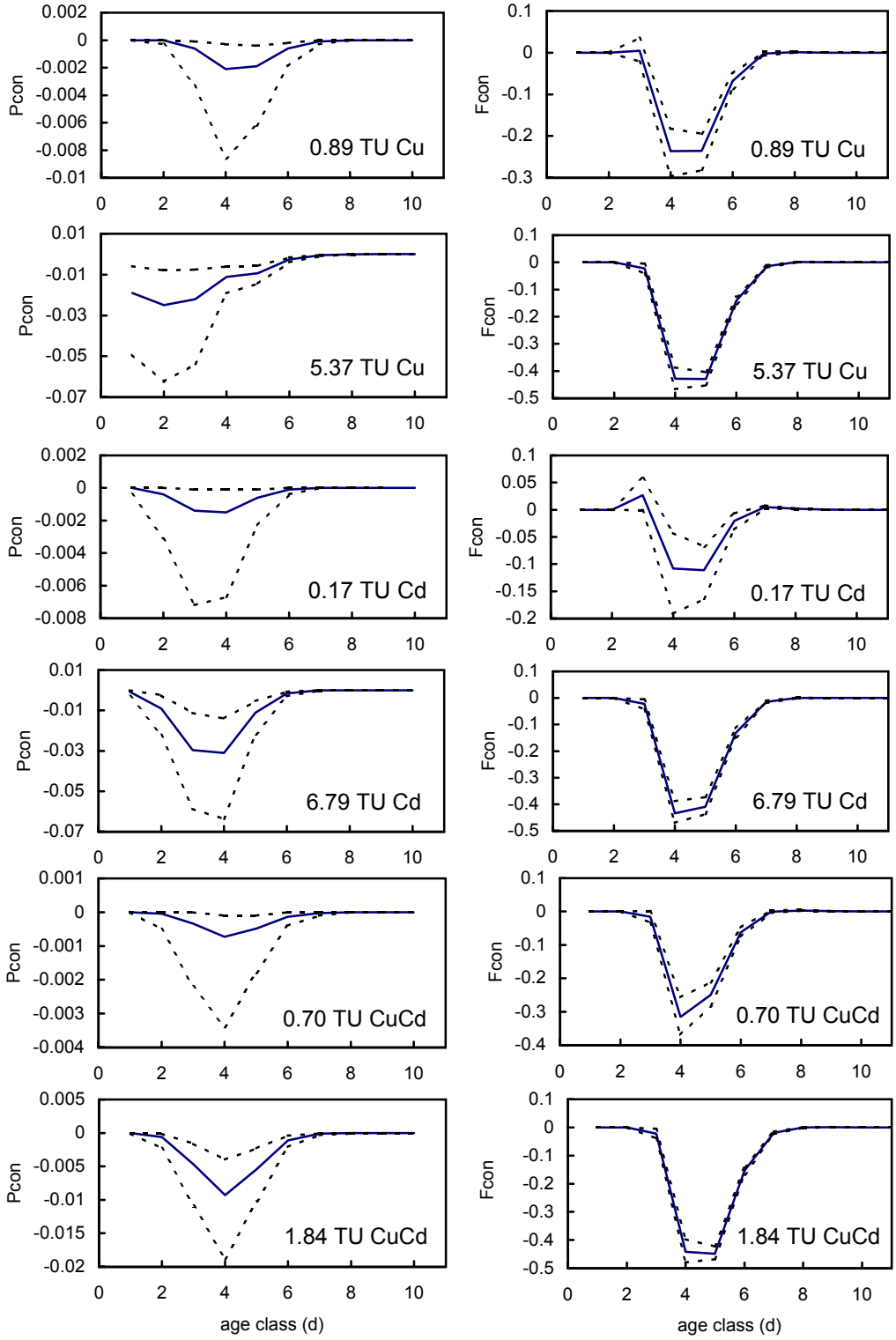

Pcon
Fcon
0.89 TU Cu
5.37 TU Cu
0.17 TU Cd
6.79 TU Cd
0.70 TU CuCd
1.84 TU CuCd
age class (d)

Figure 9

Figure 9 (former page). Decomposition analyses of cadmium, copper and combined treatment effects on λ. The graphs show the contributions of age specific survival probability (Pcon) and age specific fertility (Fcon) to reductions in λ. The dashed lines indicate 95% confidence intervals.

Figure 10 shows the population growth rates calculated from this model. It can be seen that because of giving up the information on reproductive timing (only an average maturity was used) the population growth rates differed from the age-classified model. In the control λ was 4.41 d^{-1}, and decreased until 1.63 d^{-1} due to the cadmium treatment and until 1.68 d^{-1} due to the copper treatment. The highest mixture treatment induced a reduction until 1.32 d^{-1}. Thus, population growth rates in the low exposure conditions were estimated higher whereas the growth rates in the high exposures were similar. However, the pattern of the data points is very similar to figure 7, therefore it may be stated that by reparameterising the model the "scale" between the highest and the lowest population growth rate was enlarged, without changing the character of the model.

Table 1. Values of the lower level parameters in the stage classified models. The concentrations are shown in µg/ml, $TU = c/EC20$ hence dimensionless, m is the average reproduction in eggs/day, σ_i and γ_i are probabilities.

[Cd]	[Cu]	*TU*	*m*	γ_2	γ_3	σ_1	σ_2	σ_3
0	0	0	65.63	0.340	0.247	1	1	1
0.081	-	0.173	38.03	0.363	0.160	1	0.997	0.956
0.203	-	0.433	37.50	0.348	0.149	1	0.991	0.895
0.370	-	0.790	26.55	0.340	0.134	0.985	0.957	0.780
0.561	-	1.198	24.13	0.324	0.142	0.966	0.900	0.686
3.182	-	6.788	7.38	0.265	0.219	1	0.794	0.542
-	3.239	0.886	23.41	0.345	0.183	1	0.998	0.730
-	6.668	1.823	8.99	0.302	0.136	1	0.992	0.389
-	10.02	2.739	5.82	0.255	0.183	1	0.967	0.396
-	13.91	3.803	6.35	0.253	0.310	0.957	0.875	0.492
-	19.62	5.366	9.50	0.243	0.192	0.955	0.870	0.236
0.139	1.476	0.701	23.04	0.321	0.145	1	0.999	0.935
0.080	2.858	0.946	14.92	0.342	0.127	1	0.998	0.840
0.158	2.461	1.010	15.85	0.296	0.128	1	0.994	0.807
0.312	2.492	1.348	14.77	0.317	0.119	1	0.991	0.635
0.191	5.223	1.836	3.94	0.188	0.215	1	0.843	0.582

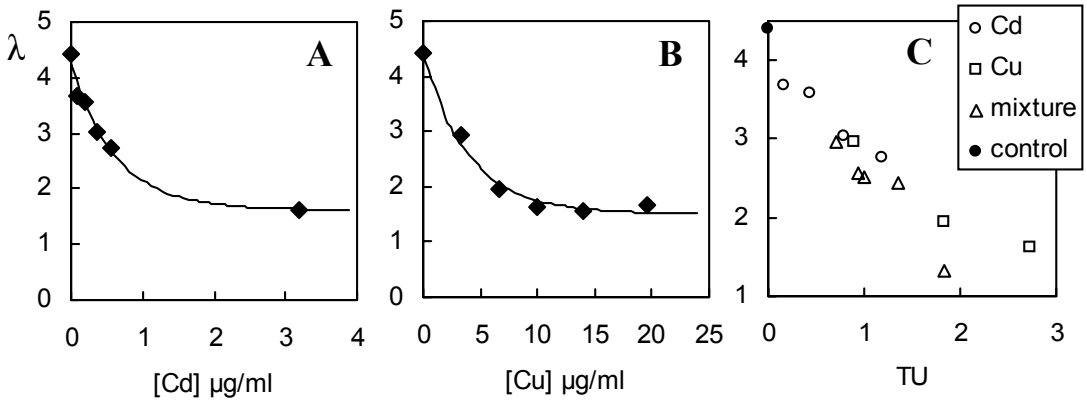


Figure 10. The relationship between population growth rate (λ) and cadmium (figure 10A), copper (figure 10B) and the mixture (figure 10C), calculated from the stage-classified model ($TU_i=c/EC20$).

Figure 11 shows the elasticity of λ to the model- and the lower level parameters. While interpreting the graphs it is important to realise that the model parameters are composed of the lower level parameters (appendix). Most interesting are patterns where lines cross, it indicates that the order of relative importance of the traits for λ changed due to toxicant exposure. For instance, the elasticity of λ to F_2 decreased when the nematodes were exposed to cadmium. As a result, it changed from the second important trait in the control to the fifth important trait in the highest cadmium treatment (note that G_2 and F_3 overlap). Recall that F_2 is the fertility of the second stage, i.e. reproduction in the early phase of life. Comparing it with reproduction in general, it can be seen that the elasticity of λ to m was only decreasing a little, and that the order of relative importance remained unchanged (figure 11). This appeared to be a general pattern when the nematodes were exposed to toxicants. Similarly, the elasticity of population growth rate to surviving and staying in the second stage, P_2, increased and became very important when exposed to cadmium, copper and their combination. However, the elasticity to σ_2 increased a little, and to γ_2 decreased a little, but the order of relative importance of the lower level parameters remained constant. The graphs show that the elasticity to surviving and remaining as reproductive adult (P_3), as well as to the length of the reproductive period (γ_3) was generally low. Combined exposure did not induce different patterns than the individual chemicals. Yet, the toxicity patterns were induced at a lower relative toxicity, as indicated by the different TU scales.

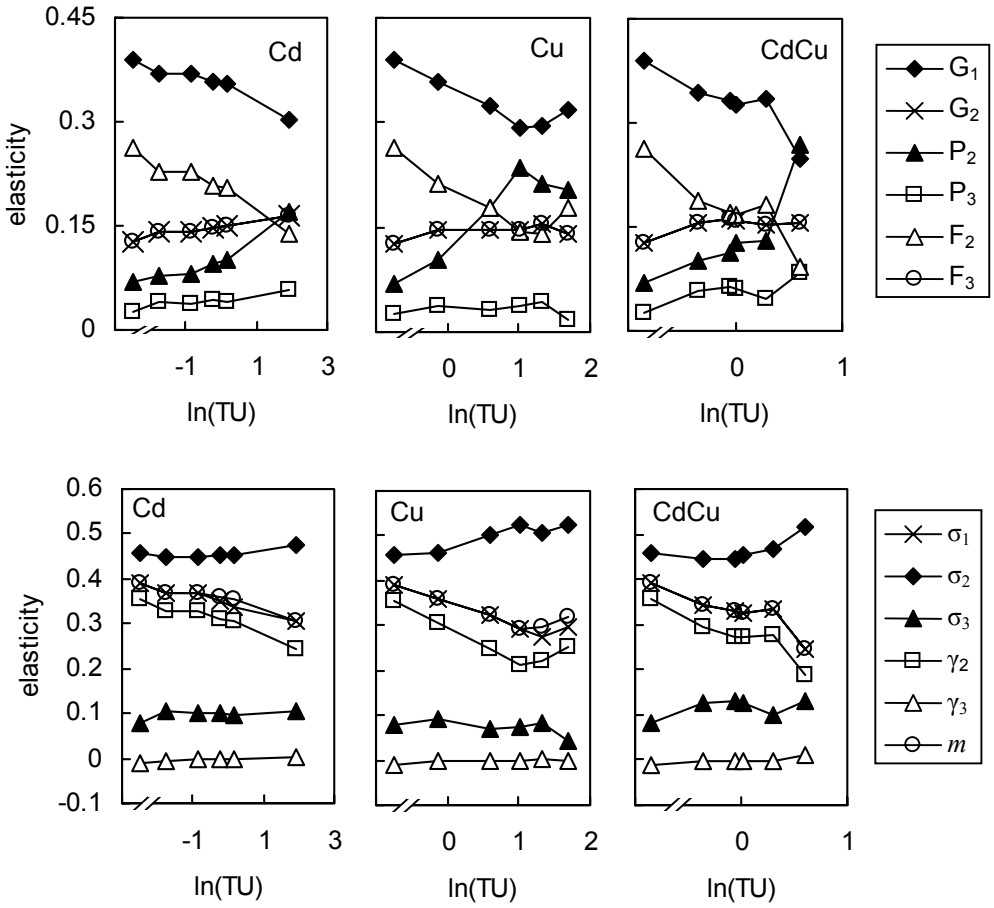


Figure 11. The relationship between elasticity of λ to changes in model- and lower level parameters and cadmium, copper, copper-cadmium treatment.

To help interpreting the elasticity patterns of figure 11, some representative sensitivity patterns are shown in figure 12. It can be seen that λ was very sensitive to changes in time to maturity (represented by γ_2). This is a general phenomenon in demography, and it is therefore generally considered as an important demographic parameter (Kooijman and Metz 1984, Kammenga *et al.* 1996a). From that perspective it is noteworthy that its elasticity, the relative importance, was not particularly high (figure 11). It can also be seen that the sensitivity to changes in reproduction, F_2, F_3 as well as m, were low. Thus, the high elasticity values were a consequence of the enormous reproduction of the nematode (± 280 eggs/individual).

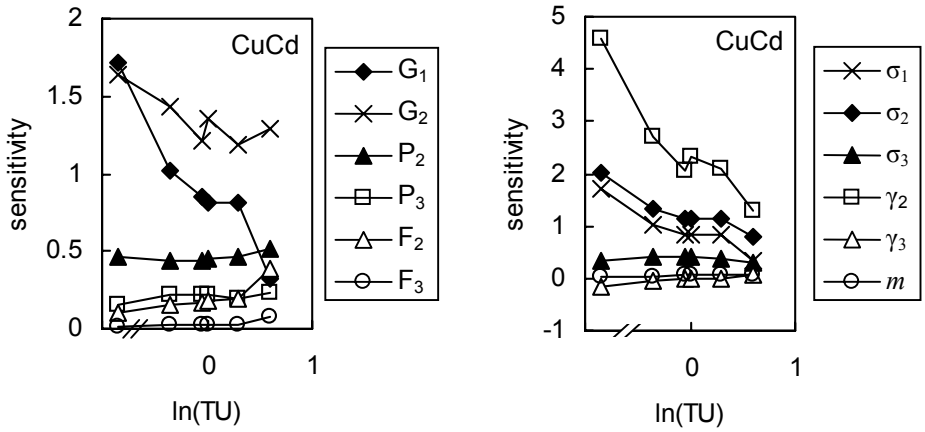


Figure 12. The relationship between sensitivity of λ to changes in model- and lower level parameters and the copper-cadmium treatment.

Discussion

We started this chapter with an introductory discussion on how several combined exposure response patterns could affect population growth rate. It was illustrated that synergistic effects on life history parameters could lead to either more adverse or less adverse effects than intuitively expected. For the nematode *C. elegans*, the analysis showed that synergistic effects on reproduction were transferred directly to the population level. The direct relationship is a consequence of the differences in sensitivity to the toxicants between the different traits. Figure 9 shows that the most important adverse effects on λ occurred in the fourth and fifth age class. These are the age classes that follow directly after the time at maturity. Reproduction decreased from 66 eggs per nematode per day to 7.4 (cadmium), 9.5 (copper) and 3.94 (mixture) in the highest exposure conditions, which are reductions of ± 90% (table 1). On the other hand, survival at the time of maturity decreased from from 1 (control) to 0.79 (cadmium), 0.87 (copper) and 0.84 (mixture). The importance of reproduction for population growth rate depended only on its magnitude (figure 11, figure 12). Since reproduction was relatively sensitive to toxicants, its decrease induced a decrease in λ at concentrations much lower than the other traits were affected, such as survival (at relevant early ages) or time to reproduction. Yet, the translation of synergistic effects to the population level was somewhat hampered due to the low sensitivity of λ to changes in reproduction (figure 8A, figure 12). Figure 7D shows that not all combined exposures resulted in a synergistic effect on λ, whereas synergism on reproduction was detected in all treatments.

The large difference in sensitivity between the different traits also explained the typical exponential functional form of the dose response (figure 7A, 7B, 10A, 10B). The decrease in fitness at lower toxicant concentrations was induced by the decrease in reproduction, but this effect was levelling off: as long as the nematodes managed to stay alive until maturity, λ would probably not decrease any further. Only higher juvenile mortality would probably

reduce population growth rate more. Yet, the juveniles appeared to be relatively tolerant to intoxication. Studies on toxicant effects on juveniles conducted in our laboratory (from which these σ_1 and σ_2-values were derived) indicated that substantial juvenile mortality (± 40% effect) was induced at 35 µg/ml Cu, which is above the concentration levels tested here. It should however be realised that toxicity tests were performed with healthy nematodes, and therefore healthy juveniles. If a population is exposed chronically juveniles might be less tolerant due to toxicant exposure in the eggs via the mother. These effects may alter the population significantly due to the high sensitivity of λ to σ_1 and σ_2.

The stage-classified model did not exactly reproduce the results of the age-classified model. The discrepancy is explained from the calculation of an average maturity, by which we gave up the information on differences in reproductive output early- and later during the life cycle. This error was the largest when reproduction was high, such as in the control. However, since the pattern of the λ-values in figure 7 and figure 10 are similar, we feel that the results are comparable. A different stage-classified model was proposed by Levin *et al.* (1996) that approximated the age-classified model much better. However, in this model time to maturity was quantified in discrete time steps of the projection interval, which was not suitable for the *C. elegans* data.

In chapter 3 it was shown that exposure to toxicants induced a prolongation of the reproductive period. Intuitively, this might be considered as a strategy to increase λ by reproducing as much as possible, given the experienced stress. However, in figure 9 it can be seen that there is virtually no positive contribution of prolonged reproduction. Also the elasticity and sensitivity of λ to σ_3 was very low (elasticity even negative). Prolonging the reproductive period had clearly no advantageous effects for the nematode and is better explained as a direct physiological consequence of toxicant stress.

The application of population models in mixture toxicity research puts interactive effects in an ecological perspective. It incorporates time dependency of mixture effects (chapter 3). In addition, the importance of synergistic and antagonistic effects on different response parameters can be weighted according to their contribution to population growth rate. For instance, for organisms with similar life histories to *C. elegans*, synergistic effects on juvenile survival can have important population consequences that most probably can hardly be compensated by antagonistic effects on adult survival. In the analyses of the effects on *C. elegans* interactions on demographic mechanisms were limited, because reproduction was much more sensitive to toxicants than all other life history traits. Considering the introductory examples it would be worthwhile for ecological effect assessment, to study the combined effects on other life histories where the sensitivities of the various life cycle traits to toxicants are at similar concentration levels.

Appendix

The age-classified model

The birth-flow model is a parameterisation of the age-classified model for populations in which births occur continuously over the projection interval (Caswell 2001). It approximates the continuous characteristics of the life cycle, which was given up after diving in artificial age classes. The age specific survival probability of age class i can be calculated from the survivor function $l(x)$, by taking the average over the interval $i\text{-}1 \leq x \leq i$:

$$P_i = \frac{l(i) + l(i+1)}{l(i-1) + l(i)}$$

And the age specific fertility was calculated from

$$F_i = \frac{l(0) + l(1)^{1/2}}{2}\left(m_i + P_i m_{i+1}\right)$$

The stage-classified model

Stage specific survival probabilities were calculated from the survivor function and stage specific growth probabilities were calculated as the reciprocals of the estimated stage durations, where $\gamma_2 = 1/(t_j\text{-}1)$ to correct for the daylings. The parameters for the stage-classified model were calculated from (Caswell 1996a):

$$\begin{aligned}
G_1 &= \sqrt{\sigma_1} \\
P_1 &= 0 \\
G_2 &= \gamma_2 \sigma_2 \\
P_2 &= (1-\gamma_2)\sigma_2 \\
P_3 &= (1-\gamma_3)\sigma_3 \\
F_2 &= \frac{\sigma_1^{1/2} G_2 m}{2} \\
F_3 &= \frac{\sigma_1^{1/2}(1+P_3)m}{2}
\end{aligned}$$

The sensitivities of λ to changes in σ_i, γ_i and m are given by:

$$\frac{\partial \lambda}{\partial \sigma_1} = \frac{\partial \lambda}{\partial G_1}\left(\frac{1}{2\sigma_1^{1/2}}\right) + \frac{\partial \lambda}{\partial F_2}\left(\frac{F_2}{2\sigma_1}\right) + \frac{\partial \lambda}{\partial F_3}\left(\frac{F_3}{2\sigma_1}\right)$$

$$\frac{\partial \lambda}{\partial \sigma_2} = \frac{\partial \lambda}{\partial G_2}\gamma_2 + \frac{\partial \lambda}{\partial P_2}(1-\gamma_2) + \frac{\partial \lambda}{\partial F_2}\left(\frac{F_2}{\sigma_2}\right)$$

$$\frac{\partial \lambda}{\partial \sigma_3} = \frac{\partial \lambda}{\partial G_3}\gamma_3 + \frac{\partial \lambda}{\partial P_3}(1-\gamma_3) + \frac{\partial \lambda}{\partial F_3}\left(\frac{\sigma_1^{1/2}(1-\gamma_3)m}{2}\right)$$

$$\frac{\partial \lambda}{\partial \gamma_2} = \frac{\partial \lambda}{\partial G_2}\sigma_2 - \frac{\partial \lambda}{\partial P_2}\sigma_2 + \frac{\partial \lambda}{\partial F_2}\left(\frac{F_2}{\gamma_2}\right)$$

$$\frac{\partial \lambda}{\partial \gamma_3} = \frac{\partial \lambda}{\partial P_3}\sigma_3 - \frac{\partial \lambda}{\partial F_3}\left(\frac{\sigma_1^{1/2}\sigma_3 m}{2}\right)$$

$$\frac{\partial \lambda}{\partial m} = \frac{\partial \lambda}{\partial F_2}\left(\frac{F_2}{m}\right) + \frac{\partial \lambda}{\partial F_3}\left(\frac{F_3}{m}\right)$$

Elasticities of λ to changes in σ_i, γ_i and m were calculated from equation 5.

Chapter 5

Toxicity of simple mixtures to the nematode *Caenorhabditis elegans* in relation to soil sorption

Martijs J. Jonker, Roland A.J.C. Sweijen, Jan E. Kammenga

Abstract

Single and combined toxicity of copper-zinc, copper-cadmium, cadmium-lead, copper-carbendazim and copper-carbendazim-iprodione to the nematode *Caenorhabditis elegans* were studied. The aim was to compare interactive effects on soil sorption to joint toxicity. Nematodes were exposed in LUFA soil, and the one-week population increase was estimated as toxicity endpoint. Joint toxicity patterns were quantified by comparing mixture effects to the effect of its individual constituents, and were related to total concentrations in the soil, water-soluble concentrations and 0.01 M $CaCl_2$ extractable concentrations. Differences in partition coefficients were found between the metals when applied individually, and the metal with the highest partition coefficient affected the sorption of metal with the lowest partition coefficient when both were combined. Consequently, both the ratio and the relative toxicity of individual mixture constituents depend on assumptions about exposure conditions, which was taken into account in the data quantification procedure applied. Both the additive and the independent model were generally inadequate to describe metal mixture effects. Compared to the additive model, synergism was observed at dose levels higher than the EC50's of the individual compounds. Compared with the independent model antagonism was mostly detected. Analysing the interactive sorption effects on joint toxicity yielded no general pattern.

Introduction

Contaminated soils usually contain mixtures of chemicals rather than just single compounds. Therefore, ecotoxicologists have increasingly focused on the development of a firm scientific framework to address joint toxic effects in soils. So far, a few detailed studies have been performed on soil invertebrates. For instance, studies on the effect of copper and zinc on *Enchytraeus crypticus* (Posthuma *et al.* 1997), the effect of cadmium and zinc on *Folsomia candida* (Van Gestel and Hensbergen 1997), and effects of various mixtures on earthworms (*e.g.* Khalil *et al.* 1996a, Khalil *et al.* 1996b, Weltje 1998). More recently, (Lock and Janssen (2002) investigated the combined effect of 4 metals on *Enchytraeus albidus*. Yet, compared to aquatic biota, toxic mixture data concerning soil invertebrates is still scarce. In this study, mixture toxicity experiments were performed with the nematode *Caenorhabditis elegans* in soil to investigate adverse interactive effects of several metal and fungicide combinations. *C. elegans* is a free living, soil dwelling, bacterivorous nematode, and it is being considered as a model organism for studying fast reproducing opportunistic soil invertebrates (Freeman *et al.* 1999). Additionally, it may represent the metazoa which are important for decomposition and nutrient cycling in soil systems.

The toxicity of the binary metal mixtures cadmium and lead, copper and cadmium and copper and zinc were compared with the toxicity of the individual compounds. The combinations consist of two non-essential metals, an essential and a non-essential metal and two essential metals. A mixture of copper, carbendazim and iprodione was investigated additionally. This combination is relevant for the field situation in agricultural areas, where many organisms are exposed to mixtures of copper and fungicides. Carbendazim is a benzimidazole, and a commonly used systemic fungicide. Benzimidazoles are known for their interference with DNA synthesis and inhibition of cellular development (Tomlin 1997). Iprodione belongs to the group of dicarboximides and is also a systemic fungicide. It inhibits germination of spores and growth of fungal mycelium (Tomlin 1997). The biochemical mode of action is not known. At high dosage, uncoupling of mitochondrial electron transport has been recorded, but this is not thought to be the primary mode of action. Limited information is available on the combined physiological interactions between pesticides and heavy metals. For carbamates and copper, synergistic effects were found in the aquatic ciliate *Colpidium campylum* (Vasseur *et al.* 1988, Bonnemain and Dive 1990).

Combined toxicity in soil systems encompasses several levels of interaction (Calamari and Alabaster 1980). First, the combined effect is subject to chemical interactions in the soil matrix, influencing sorption and hence bioavailability. Second, physiological interactions during the uptake processes by the organism can affect the joint effect of the chemicals. In addition, interactions at the intoxication processes, at the receptors and target sites can occur, which affect toxicodynamics and therefore the combined effect. It has been shown that when the effects of toxicant combinations are considered, insight in the relative importance of these interaction levels is of great value (Posthuma *et al.* 1997, Van Gestel and Hensbergen 1997, Jason and Lanno 2000). Therefore, the aim of the study was to compare interactive effects of the chemicals on soil sorption to joint toxicity patterns.

Materials and methods

Test organism and culture

Experiments were performed with *Caenorhabditis elegans* var. Bristol, strain N2. The culture was provided by the Netherlands Cancer Institute, Amsterdam, The Netherlands. Stock cultures were kept sterile in the dark at 15°C on NGM agar (Wood 1988), nematodes were transferred every week on fresh agar plates. Agar plates were inoculated with *Escherichia Coli* (strain OP50) as food source. Experiments and synchronisation of cultures were performed at 20°C in the dark. To obtain synchronised cultures for the experiments, two synchronisation steps were performed. First, 10 juveniles of approximately the same age were picked from the stock culture and transferred to new agar plates. These were allowed to develop to a healthy culture, and after 5 days 10 gravid adults were transferred from this culture to a new agar plate. These adults were allowed to lay eggs for five hours, then they were removed. In 3 days the eggs developed to adults differing maximally 5 hours in age. These were used for the experiments.

Soil treatment

LUFA 2.2 soil, a well-characterised loamy sand, was used for toxicity testing. It was ordered at the Bezirks Verband Pfalz Landwirtschaftiche Untersuchungs- und Forschungsanstalt Speyer, Speyer, Germany. Two batches of 20 kg were used. The organic carbon content was respectively 2.27 ± 0.28 % and 2.19 ± 0.08 %. The soil consisted for 13.80 ± 1.0 and 14.3 ± 1.9 of particles < 0.02 mm. The pH values (0.01 M CaCl2) were 6.1 ± 0.2 and 5.8 ± 0.2. Cation exchange capacity was 9 ± 2 and 11 ± 2 mval/100g. LUFA 2.2 is a natural soil, and contained therefore nematodes and other microfauna that could interfere with *C. elegans* in the test system. The indigenous organisms were eradicated by microwave heating (3 min., maximum power) before experimental use of the soil.

All chemicals used were of the highest analytical grade. The water, used for the experiments, contained a defined mixture of minerals, resembling those found in interstitial water of sandy forest soils (Schouten and Van der Brugge 1989). Copper chloride ($CuCl_2 \cdot 2 H_2O$) and zinc chloride ($ZnCl_2$) were obtained from Sigma Chemical Company, St. Louis Missouri, USA, cadmium chloride ($CdCl_2$) and lead chloride ($PbCl_2$) were obtained from Merck, Schuchardt, Germany. Carbendazim and iprodione, 99.2 and 97.3 % pure, were obtained from BASF Nederland B.V., Arnhem, The Netherlands. The metals were added as aqueous solutions. When the solubility was exceeded, the solution was warmed to avoid precipitation (*e.g.* with $ZnCl_2$ and $PbCl_2$). The fungicides were dissolved in acetone and added to 10% of the soil. The acetone was allowed to evaporate, and the spiked soil was added to the other 90% and thoroughly mixed on the roller bank for at least three hours. Then, water (in case of mixture: with copper) was added (0.1 ml/gr. dry soil). Before experimental use, the moist soils were left for two weeks to allow for equilibration. During the experiment, the soil

moisture content was 80% of the WHC. The effect of the acetone treatment was tested separately.

Toxicity testing

The short life cycle of *C. elegans* enables the investigation of population growth, an ecologically relevant toxicological endpoint. In this study age synchronised gravid young adults were transferred to spiked soils at the start of the experiment and after 1 week of exposure the nematodes of next (overlapping) generations were extracted and counted. This can be considered as a measure of population growth performance. Moreover, it is a combined response parameter, integrating effects on growth, survival, (avoidance) behaviour, reproduction etc.

The experimental design for the mixture analysis was based on the toxic unit concept (TU), where the toxic unit of a chemical was defined as the exposure concentration in the mixture divided by its EC50. Concentration ranges were based on nominal doses, and nominal EC50's were obtained from range finding experiments. To cover different combinations and different effect levels, nominal concentrations of the mixtures were based on expected toxic strengths of 0.25, 1, 2 and 4 TU, where each toxic unit was tested twice: as a combination of ¼ of toxicant 1 and ¾ of toxicant 2, and as a combination of ¾ of toxicant 1 and ¼ of toxicant 2. Individual compounds were tested simultaneously, at the same nominal concentrations. Thus, each binary mixture experiment consisted of 24 treatments and a control.

When the nematodes were extracted, the soil was lost (see below). Therefore, identically treated soil for chemical analysis ran simultaneously with the toxicity test in separate jars. The testing design consisted of 5 replicates per treatment for toxicity testing (5 gram dry soil each, in glass petridishes (Ø 6 cm)), two replicates per treatment for chemical analysis at the beginning of the experiment (40 gram dry soil each), and two replicates per treatment for chemical analysis at the end of the experiment (40 gram dry soil each). Since the experiments were too large to handle at once, it was decided to split the experiment, and to test the individual chemicals and the combinations of the low toxic units simultaneously, and the individual chemicals and the combinations of the high toxic units simultaneously.

The experiment started when 20 young gravid adults were introduced into each test container, with *Escherichia coli* (strain OP50) as food source (0.1 ml bacterial suspension/gr. dry weight soil). After 1 week nematodes were extracted from the soil. For nematode extraction, the soil from the test containers was suspended in 50 ml $MgSO_4$ solution, with a density of 1.22. In this suspension the organic material (with nematodes) floats, and the inorganic material sinks (Persmark *et al.* 1992). This process is quickened by centrifuging the samples (centrifuge: Centaur 2; 3 min., 1800g). A small amount of kaolien clay was added to obtain a stable pellet. The supernatant was sieved twice: with a mesh size of 550 μm to remove large particles, subsequently with a mesh size of 10 μm to catch the nematodes. The nematodes were flushed from the sieve, shortly heated (50^{o}C) and fixed in a 37%

formaldehyde / 4% fuchsine-acid ($C_{20}H_{17}N_3Na_2O_9S_3$, colouring the nematodes) solution. The next day, red coloured nematodes could be counted.

Chemical analysis

Soil samples were dried (40°C, overnight) and digested with nitric acid and hydrochloric acid to determine metal concentrations. Metal concentrations were measured by ICP-MS. For obtaining water-soluble and calcium extractable metal concentrations samples of 5 gram of dried soil were suspended in 50 ml of deionized water, or in 0.01 M $CaCl_2$. After 2 h of shaking, the suspension was allowed to settle, and the pH was measured in the supernatant. Then, the supernatant was filtered (0.45 µm membrane filter, Schleicher & Schuell, Germany) and the filtrate was stored at 4°C. In the filtrate metal concentrations were determined by atomic absorption spectophotometry, using a Perkin Elmer 1100B atomic absorption spectophotometer (AAS). Concentrations were measured either by flame atomic absorption spectophotometry, or by furnace atomic absorption spectophotometry, dependent on the concentration level, at a wavelength of 228.8 nm (Cd), 324.7 nm (Cu), 213.9 nm (Zn) or 283.3 nm (Pb).

For fungicide measurements, 5 gram of soil was suspended in 10 ml HPLC-water, and 25 ml destilled ethylacetate. After 1 h of shaking, the suspension was allowed to settle, and the ethylacetate extract was drawn off. The ethylacetate extracts were stored at 4°C. The fungicides were determined by HPLC technique. Column: Waters X-terra™ MSC_{18} 3.5 µm (diameter 4.6 mm, length 150 mm), provided with a guard column X-terra™ MSC_{18} 3.5 µm (diameter 3.9 mm, length 20 mm). Flow: 1 ml/min., injectation volume was 100 µl. For carbendazim, the eluens was acetonitril/water (30/70;V/V), and it was measured at a wavelength of 285 nm, for iprodione, the eluens was acetonitril/water (60/40;V/V), and it was measured at a wavelength of 210 nm.

Data analysis

Using the measured water-soluble and $CaCl_2$ extractable concentrations, sorption of the metals was quantified using the Freundlich isotherm (Travis and Etnier 1981). The parameters in the Freundlich isotherm were quantified by means of linear regression:

$$\log(C_s) = \log(K_f) + \frac{1}{n}\log(C_w)$$

where C_s is the total concentration in soil (µg/g dry wt), K_f is the Freundlich adsorption constant (L/kg), C_w is the concentration in water or in 0.01 M $CaCl_2$ (mg/L), and n is the shape parameter. In addition, fitting the following model on the single and combined sorption

data of a certain metal enabled the analysis of the significance of the differences in Freundlich isotherm parameters between the individual metal and this metal in a mixture:

$$y = \beta_0 + \beta_1 x + \beta_2 \tau + \beta_3 x\tau$$

where y is equivalent to $\log(C_s)$ of the individual compound, or this compound in the mixture, β_0 to $\log(K_f)$ of the individual toxicant, x to $\log(C_w)$ of the individual compound, or this compound in the mixture, β_1 to $1/n$ of the individual toxicant, β_2 to the difference in $\log(K_f)$ between the individual compound and this compound in the mixture, β_3 to the difference in $1/n$ between the individual compound and this compound in the mixture, and τ is an indicator variable, indicating the mixture data. The deviations of β_2 and β_3 from 0 were evaluated with a t-test.

Mixture effects were analysed applying the computational framework proposed by Haas *et al.* (1996) and in chapter 2. Following their derivations, the binary toxic-unit mixture model can be generalised to:

$$\frac{c_1}{f_1^{-1}(Y)} + \frac{c_2}{f_2^{-1}(Y)} = \exp(G) \tag{1}$$

where c_1 and c_2 denote the concentrations of the individual chemicals in the mixture, Y indicates the biological response, and f_1^{-1} and f_2^{-1} indicate the inverse dose response functions of the individual compounds in the mixture, here quantified by a log-logistic model (Haanstra *et al.* 1985). G denotes an extent function to quantify deviations from additivity. The data was also compared with the independent model, assuming no correlation of sensitivities. To allow for deviations from the independent model, it can be written as:

$$Y = u_0 \Phi\left(\Phi^{-1}[h_1(c_1)h_2(c_2)] + G\right) \tag{2}$$

where u_0 denotes the response in the control group, h_i indicates the complement of the proportional effect of c_i (here quantified by a log-logistic model (Haanstra *et al.* 1985)), which are transformed by means of the standard cumulative normal distribution function (Φ). The reference models are defined by $G = 0$ in eq (1) and (2). Details of the model applications are outlined in Haas *et al.* (Haas *et al.* 1996) and in chapter 2. In short, G enables the quantification of four distinct deviations from the reference model. 1) No deviation. 2) Synergism/antagonism in all mixture combinations (S/A). 3) Toxicant ratio dependent deviation (TR), where the deviation pattern (synergism/antagonism) depends on the mutual proportion of the toxicants in the mixture. 4) Effect level dependent deviation (EL), where the deviation (synergism/antagonism) depends on the dose level tested. Within this framework the synergistic/antagonistic model is an extension of the reference model with one additional parameter. Subsequently the synergistic/antagonistic model is nested in the toxicant ratio-dependent deviation model and effect level-dependent deviation model. These models have

two additional parameters compared with the reference model, and they cannot be tested mutually. The biological interpretation of the additional deviation parameters, here arbitrarily named *a* and *b*, is listed in table 1. For brevity, only the equations and interpretations in comparison with the additive model are given. Yet, similar equations with the same parameter interpretations are used for the independent model. All parameters, *i.e.* slopes of the dose response curves, the concentrations resulting in 50% effect ($EC50_1$, $EC50_2$), the control response (u_0) and the deviation parameters were estimated simultaneously. The most parsimonious model was found by likelihood testing, assuming a normal distribution with equal variance at all exposure combinations, equivalent to multiple regression. A significance level of $p(X^2) = 0.05$ was chosen as threshold value in deciding whether an additional parameter in the model described the data significantly better. Here the X^2 indicates the test statistic, which is compared with the χ^2 distribution with df_2-df_1 degrees of freedom, and df_1 and df_2 denote the degrees of freedom of respectively the reduced and the full model. If the synergistic/antagonistic model didn't show a significant deviation, the toxicant ratio-dependent deviation and effect level-dependent deviation models were compared with the reference.

Table 1. Interpretation of additional parameters in equation 1, that define the functional form of the deviation pattern from the additive model. *TU* denotes the toxic unit of a chemical, defined as the exposure concentration in the mixture divided by its EC50, and $z_i = TU_i(TU_1+TU_2)^{-1}$,i = 1,2.

Deviation pattern	*a*	*b*
synergism/antagonism (S/A) $G = az_1z_2$	**Pos:** antagonism **Neg:** synergism	
Toxicant Ratio (TR) $G = (a + b\,z_1)z_1z_2$	**Pos:** decreased toxicity related to z_1 decrease in the mixture. **Neg:** increased toxicity related to z_1 decrease in the mixture.	**Pos:** decreased toxicity related to z_1 increase in the mixture. **Neg:** increased toxicity related to z_1 increase in the mixture.
Effect Level (EL) $G = (a(1 - b[TU_1+TU_2]))z_1z_2$	**Pos:** antagonism low EL & synergism high EL **Neg:** synergism low EL & antagonism high EL	***b*=1:** change at *EC50* level **0<*b*<1:** change at higher EL ***b*>1:** change at lower EL

Results

pH and metal concentrations

The pH values (0.01 M $CaCl_2$) in the controls at the start of each experiment showed some differences. For the copper-zinc experiment the pH in the controls was (value (sd)) 6.28 (0.04), for the copper-cadmium experiment 6.29 (0.14), for the cadmium-lead experiment 6.11 (0.05) and for the copper-fungicide experiment 5.91 (0.04). No clear pattern was observed between the pH values at the beginning and the end of the experiment. In most

control samples a modest decrease of approximately 0.1 was observed, indicating stable experimental conditions. In the copper-fungicide experiment no pH change over time was observed. Yet, metal addition induced pH changes. The pH was most affected by copper. The highest copper treatments resulted in a decrease in pH until approximately 5.5, and in the copper-fungicide experiment even until 5.06 (0.04). Smaller effects on pH were observed due to zinc addition (highest Zn treatment: pH = 5.66 (0.06)) and lead addition (highest Pb treatment: pH = 5.92 (0.03)). Iprodione, carbendazim and cadmium did not alter soil pH. The pH values measured in water showed similar patterns.

Table 2, 3, 4 and 5 show the estimated parameter values of the Freundlich isotherms quantifying sorption for the different metals solely and in the mixture, and the significance of these parameter differences. Clearly, the water-soluble and $CaCl_2$ extractable concentrations of cadmium and zinc were influenced by the presence of copper (table 2 and 3). All parameters in the Freundlich isotherm were significantly affected, except the slope parameter for $CaCl_2$ extracted concentrations of cadmium. On the other hand the water-soluble and $CaCl_2$ extractable concentrations of copper were hardly affected by the presence of the other metals. Only a zinc related change in K_f of copper for the $CaCl_2$ extractable concentrations was observed. Illustratively, the water-soluble concentrations of zinc and copper are depicted in figure 1. It shows that the sorption of copper was not altered by the presence of zinc, whereas sorption of zinc decreased due to the presence of copper.

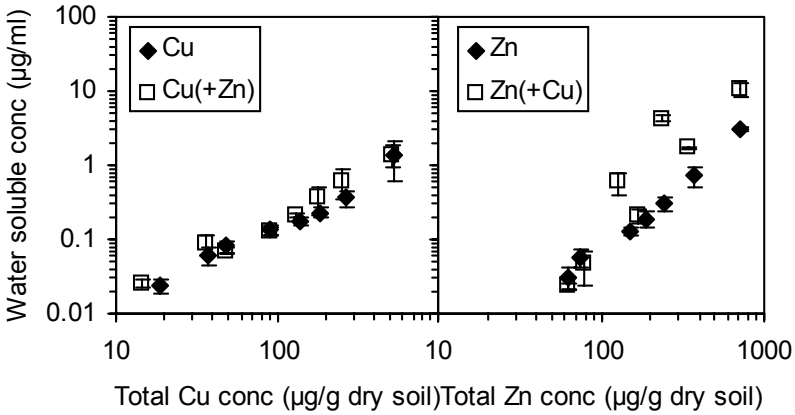


Figure 1. Water soluble concentrations of Cu (left) and Zn (right) in the LUFA soil (error bars = standard deviation).

Table 2. Parameter estimates and 95% confidence intervals of the Freundlich isotherms quantifying sorption of copper solely and combined with zinc, and zinc solely and combined with copper. The asterisk indicates a significant difference ($p < 0.05$) between parameter estimates for the single metal and the metal combined.

Metal	extraction	K_f	95% conf.	n	95% conf.	r^2
Cu	water	528	436 - 639	1.15	1.05 – 1.28	0.93
Cu(+Zn)	water	419	354 - 498	1.17	1.07 – 1.29	0.93
Zn	water	418	384 - 454	1.94	1.83 – 2.07	0.97
Zn(+Cu)	water	220*	193 - 250	2.99*	2.56 – 3.59	0.85
Cu	CaCl2	467	384 - 567	1.48	1.34 – 1.66	0.92
Cu(+Zn)	CaCl2	326*	2.81 - 376	1.63	1.50 – 1.79	0.94
Zn	CaCl2	206	197 - 217	2.26	2.15 – 2.38	0.98
Zn(+Cu)	CaCl2	136*	121 - 152	2.86*	2.51 – 3.34	0.88

Table 3. Parameter estimates and 95% confidence intervals of the Freundlich isotherms quantifying sorption of copper solely and combined with cadmium, and cadmium solely and combined with copper. The asterisk indicates a significant difference ($p < 0.05$) between parameter estimates for the single metal and the metal combined.

Metal	extraction	K_f	95% conf.	n	95% conf.	r^2
Cu	water	715	595 - 860	0.85	0.78 – 0.92	0.95
Cu(+Cd)	water	755	643 - 887	0.84	0.79 – 0.91	0.96
Cd	water	433	352 - 534	1.28	1.20 – 1.37	0.96
Cd(+Cu)	water	192*	151 - 243	1.59*	1.44 – 1.77	0.92
Cu	CaCl2	557	466 - 665	1.63	1.49 – 1.79	0.94
Cu(+Cd)	CaCl2	478	393 - 580	1.73	1.56 – 1.94	0.92
Cd	CaCl2	84	78 - 91	1.65	1.57 – 1.73	0.98
Cd(+Cu)	CaCl2	49*	43 - 56	1.71	1.55 – 1.90	0.93

It also appeared that cadmium sorption was affected by the presence of lead in the soil (table 4). The presence of lead changed the values of K_f and n for the water-soluble cadmium concentrations, and only the K_f for the $CaCl_2$ extractable cadmium concentrations. On the other hand, the presence of cadmium did not significantly alter the water-soluble and $CaCl_2$ extractable concentrations of lead. It was also checked whether the presence of fungicides altered the Freundlich isotherm parameter estimates for copper. In table 5 it can be seen that both the water-soluble and $CaCl_2$ extractable concentrations of copper were not affected: no significant differences were observed between the various copper isotherms.

The consequence of these interactions is that the constitution of a mixture depends on how the exposure conditions are measured. Ratios of the individual components in a mixture based on total concentrations can differ substantially from ratios based on water-soluble and $CaCl_2$ extractable concentrations. This is illustrated in figure 2, where the concentrations of copper and cadmium are expressed in toxic units. The relative differences in mixture constitutions were taken into account by analysing a complete dose response plain by means of equation 1 and 2.

Table 4. Parameter estimates and 95% confidence intervals of the Freundlich isotherms quantifying sorption of cadmium solely and combined with lead, and lead solely and combined with cadmium. The asterisk indicates a significant difference ($p < 0.05$) between parameter estimates for the single metal and the metal combined.

Metal	extraction	K_f	95% conf.	n	95% conf.	r^2
Cd	water	782	490 - 1249	1.20	1.07 – 1.35	0.91
Cd(+Pb)	water	206*	149 - 284	1.79*	1.58 – 2.05	0.90
Pb	water	2533	1899 - 3378	1.87	1.63 – 2.20	0.86
Pb(Cd)	water	2308	1799 - 2963	1.76	1.55 – 2.04	0.87
Cd	CaCl2	88	76 - 101	1.58	1.47 – 1.71	0.96
Cd(+Pb)	CaCl2	50*	43 - 58	1.72	1.58 – 1.89	0.94
Pb	CaCl2	2462	2190 - 2768	2.09	1.90 – 2.33	0.96
Pb(+Cd)	CaCl2	2056	1787 - 2365	2.29	2.04 – 2.60	0.93

Table 5. Parameter estimates and 95% confidence intervals of the Freundlich isotherms quantifying sorption of copper solely and combined with carbendazim and iprodion. The asterisk indicates a significant difference ($p < 0.05$) between parameter estimates for the copper single and copper combined.

Metal	extraction	K_f	95% conf.	n	95% conf.	r^2
Cu	water	327	306 - 350	0.93	0.89 – 0.97	0.99
Cu(+Carb)	water	306	252 - 371	0.98	0.87 – 1.12	0.89
Cu(+Carb+Ipro)	water	329	305 - 357	0.90	0.86 – 0.94	0.98
Cu	CaCl2	321	290 - 356	1.39	1.31 – 1.49	0.97
Cu(+Carb)	CaCl2	338	291 - 393	1.26	1.16 – 1.39	0.94
Cu(+Carb+Ipro)	CaCl2	323	286 - 366	1.34	1.25 – 1.45	0.96

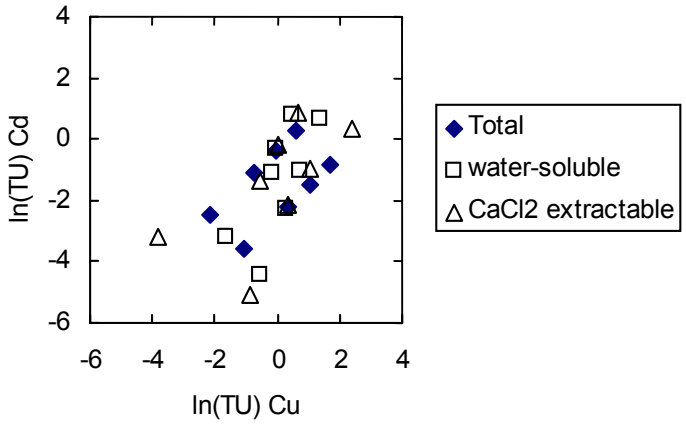


Figure 2. The proportions of copper and cadmium in the mixture, expressed as log-transformed toxic units.

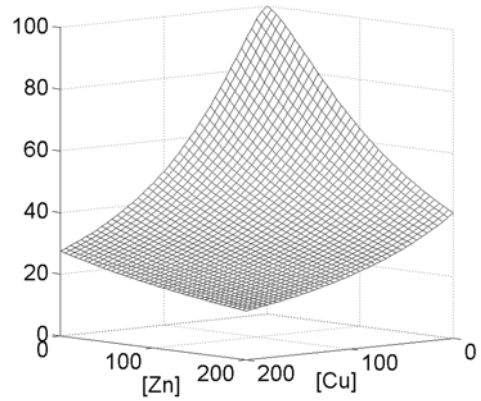
Toxicity

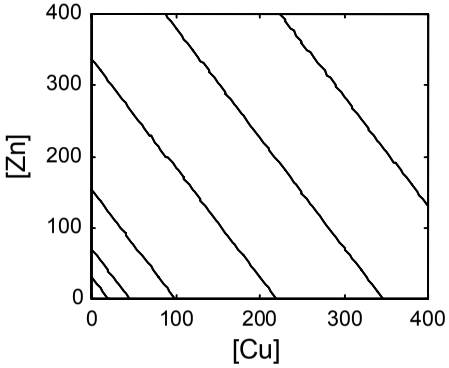
In order to compare the toxicity of each mixture with the toxicity of its individual constituents the nested modelling analysis was applied (as described in materials and methods), in the course of which exposure conditions were assumed to be represented by the total, water-soluble or $CaCl_2$ extractable concentrations of metals. The results for copper-zinc exposure are shown in table 6. It appeared that when the effect on the extracted number of nematodes was related to total and water-soluble concentrations, copper and zinc were approximately equally toxic at the EC50 level. However, when the toxicity was related to $CaCl_2$ extractable concentrations copper appeared to be more toxic than zinc.

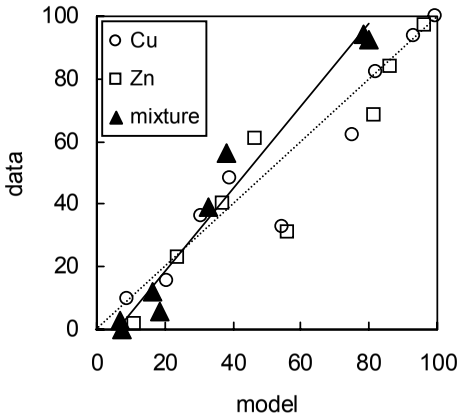
To illustrate the data analysis procedure, the calculations on the effect of total concentrations of copper and zinc is presented in detail. Three comparisons of the data with the toxic unit model (equation 1) are depicted in figure 3. It shows the additive model, the S/A model and the EL model. The upper row shows the response plain, the middle the isobolic representation of the models and the lower shows comparisons of the model description with the data. The diagonal dotted lines in these graphs indicate ideal model description. The solid lines show a regression analysis over the mixture data. The smaller the discrepancy between the dotted and the solid line, the better the model describes the mixture data. Each point is an average of 5 replicates.

Next page Figure 3. The additive model (equation 1), with two deviation patterns: the S/A model (= synergistic/antagonistic) and the EL model (= effect level dependent deviation). The upper row shows the response plain representing the proportional number of extracted nematodes (vertical axis) as a function of copper and zinc concentrations (µg/g dry soil). The middle row shows the isobolic representation of the models; the isoboles indicate (from left to right): 10%, 25%, 50%, 75%, 85% and 90% effect, copper and zinc concentrations are depicted in µg/g dry soil. The lower row shows comparisons of the model description with the data. The diagonal dotted lines in these graphs indicate ideal model description. The solid lines show a regression analysis over the mixture data. Each point is an average of 5 replicates.

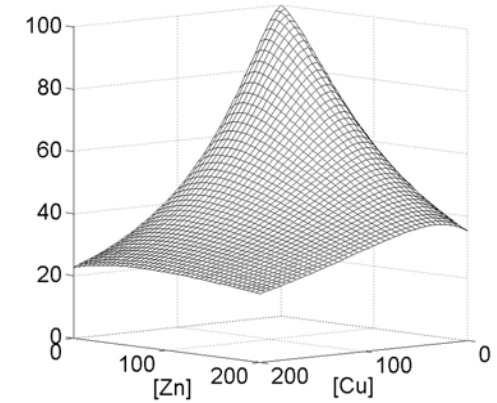
Additive

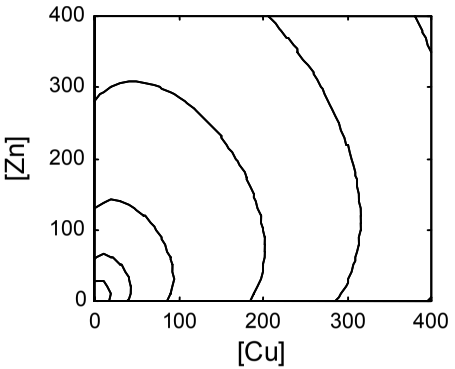


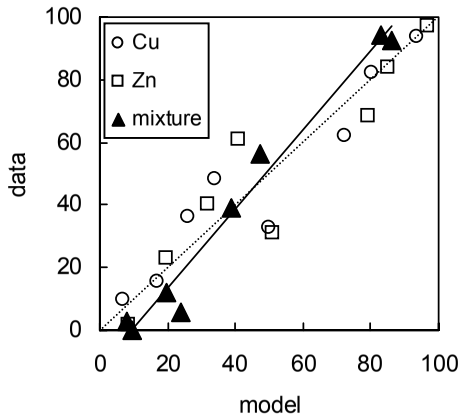




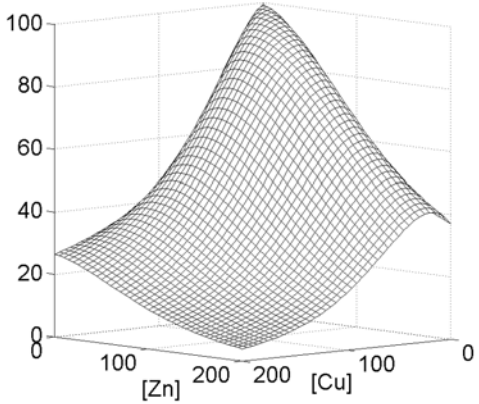
Synergism/Antagonism

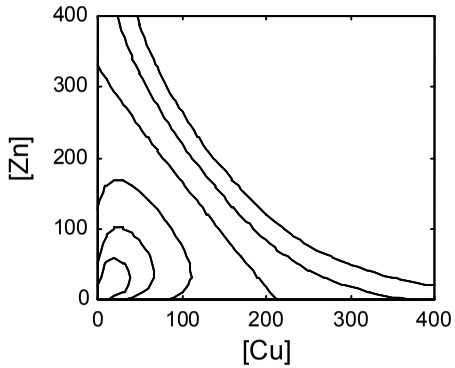






Effect level dependent deviation





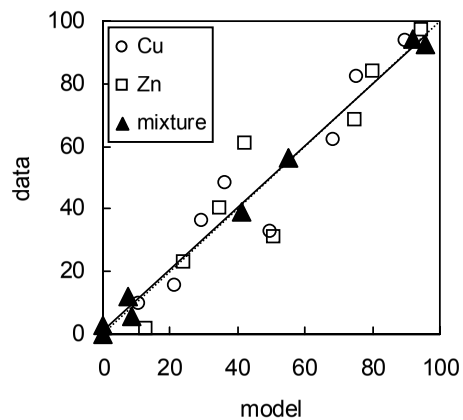


Table 6. Parameter values and 95% confidence intervals of individual the dose response curves of copper and zinc, estimated using the most parsimonious binary mixture model (equation 1), where the toxicity was related to total, water-soluble and CaCl2 extractable concentrations. The $p(X^2)$-values indicate the significance the additional deviation parameters in equation 1 to determine the functional form of the dose response plain: S/A = synergism/antagonism, TR = toxicant ratio dependent deviation, EL = effect level dependent deviation. The conclusion describes the functional form of the dose response plain in comparison with the additive model.

Measurement	metal	slope	EC50 (µg/g soil)	Control (%)	S/A $p(X^2)$	TR $p(X^2)$	EL $p(X^2)$	conclusion
Total	Cu	1.16 (0.84-1.48)	83 (62-103)	102 (94-109)	0.003	0.77	1E-05	Low EL => anta Hight EL => syn
	Zn	1.14 (0.80-1.49)	126 (94-158)					
water	Cu	1.77 (1.19-2.34)	0.92 (0.71-1.13)	106 (95-118)	0.002	0.013	0.61	Cu > Zn => syn Cu < Zn => anta
	Zn	0.79 (0.54-1.04)	0.64 (0.39-0.90)					
CaCl2	Cu	1.22 (0.84-1.60)	0.44 (0.30-0.57)	105 (94-117)	0.0002	0.07	0.35	Antagonism
	Zn	0.70 (0.47-0.93)	3.60 (1.86-5.34)					

In table 6 it can be seen that adding one additional deviation parameter in equation 1 to describe synergism or antagonism resulted in a significant improvement in data description. The estimated value was 1.97, which is positive, indicating antagonism. This is depicted in the second column of graphs in figure 3. The lowest graph in the second column shows that the description the mixture data points at the lower effect levels was somewhat improved, but the model systematically underestimated the effect at the higher effect levels. Adding an extra deviation parameter in equation 1 to allow for a toxicant ratio dependent deviation from additivity did not improve the description of the data (tabel 5), however, adding an extra deviation parameter to allow for an effect level dependent deviation enabled proper data description. The estimated deviation parameter values were a_{Total} = 9.64 and b_{Total} = 0.40, indicating antagonism at low effect levels and synergism at high effect levels as illustrated by the third column of graphs in figure 3. The lowest graph shows that the mixture data points were described adequately. The parameter values enable deduction of the dose level where the swapping from synergism to antagonism actually occurred. It can be calculated from 1/beta * EC50, thus for the copper-zinc example swapping from synergism to antagonism occurred at concentration levels of 1/0.4 * EC50 = 2.5 * EC50. This can be seen in the isobolic representation of the EL model.

When the exposure conditions were represented by water-soluble or $CaCl_2$ extractable concentrations of copper-zinc different results were obtained. For instance, when the water-soluble concentrations were considered a toxicant ratio dependent deviation from additivity was detected. The values of the additional parameters in the TR model were a_{H2O} = 3.08 and b_{H2O} = -5.29. From these values it can be concluded that the deviation from additivity was mainly antagonistic, but the negative *b* value indicated that a relative increase in copper coincided with a relative increase in toxicity of the mixture (table 1). When the exposure conditions were represented by the $CaCl_2$ extracted concentrations an antagonistic deviation

from additivity was determined (the estimated value of the additional deviation parameter was 4.17).

The analysis of the other mixtures proceeded in the same way. The results of exposing the nematodes to combinations of copper and cadmium are summarised in table 7. It appeared that cadmium and copper where equally toxic when the toxicity was related to total metal concentrations. Based on the water-soluble concentrations similar results were obtained, whereas copper appeared to be more toxic than cadmium when the $CaCl_2$ extractable concentrations were considered. Also the characterisation of the combined effects differ. No deviations from additivity were detected when the toxicity was compared with total concentrations of metals. Relating the toxicity to water-soluble and $CaCl_2$ extractable concentrations yielded an effect level dependent deviation, although this conclusion was not that strong for the $CaCl_2$ extractable concentrations (p = 0.04). The additional parameter values in the EL model were a_{H2O} = 6.9 and b_{H2O} = 0.4, and a_{CaCl2} = 4.7 and b_{CaCl2} = 0.34, indicating antagonism at low effect levels and synergism at high effect levels, were the antagonism changes into synergism at effect levels higher than the 50%.

Table 7. Parameter values and 95% confidence intervals of individual the dose response curves of copper and cadmium, estimated using the most parsimonious binary mixture model (equation 1), where the toxicity was related to total, water-soluble and CaCl2 extractable concentrations. See text and table 6 for detailed explanations.

Measure ment	metal	slope	EC50 (µg/g soil)	Control (%)	S/A $p(X^2)$	TR $p(X^2)$	EL $p(X^2)$	conclusion
Total	Cu	0.82 (0.62-1.03)	97 (66-128)	101 (93-110)	0.88	0.52	0.25	Additive
	Cd	0.55 (0.33-0.77)	110 (48-171)					
water	Cu	1.00 (0.61-1.39)	0.89 (0.56-1.22)	105 (92-118)	0.43	0.73	0.002	Low EL => anta Hight EL => syn
	Cd	0.27 (0.14-0.41)	0.83 (0.09-1.56)					
CaCl2	Cu	0.62 (0.43-0.82)	0.38 (0.23-0.53)	101 (93-108)	0.43	0.09	0.04	Low EL => anta Hight EL => syn
	Cd	0.34 (0.20-0.48)	7.14 (0.79-13.5)					

Table 8 shows the results of analysing the effect of cadmium and lead on the number of extracted nematodes. From the EC50 values it can be concluded that cadmium was more toxic than lead when the toxicity was related to total concentrations. Based on the water-soluble concentrations both metals appeared to be similarly toxic. Based on $CaCl_2$ extractable concentrations however, lead was more toxic than cadmium at the EC50 level. All assumed exposure conditions yield an effect level dependent deviation from additivity. In all cases the first additional parameter was positive (a_{Total} = 9.5, a_{H2O} = 39.5, a_{CaCl2} = 20.8) indicating antagonism at low effect levels and synergism at high effect levels. In addition, in all cases the second additional parameter was smaller than 1 (b_{Total} = 0.51, b_{H2O} = 0.78, b_{CaCl2} = 0.24),

which implies that the toxic effect switches from antagonism to synergism at higher effect levels than the EC50.

Table 8. Parameter values and 95% confidence intervals of individual the dose response curves of cadmium and lead, estimated using the most parsimonious binary mixture model (equation 1), where the toxicity was related to total, water-soluble and CaCl2 extractable concentrations. See text and table 6 for detailed explanations.

Measurement	metal	slope	EC50 (µg/g soil)	Control (%)	S/A $p(X^2)$	TR $p(X^2)$	EL $p(X^2)$	conclusion
Total	Cd	0.30 (0.21-0.40)	180 (62-299)	93 (88-99)	0.48	0.69	2E-06	Low EL => anta Hight EL => syn
	Pb	1.40 (1.07-1.72)	870 (725-1015)					
water	Cd	0.27 (0.18-0.36)	3.48 (0.99-5.98)	86 (80-91)	0.27	0.33	3E-05	Low EL => anta Hight EL => syn
	Pb	2.70 (1.70-3.69)	1.07 (0.95-1.19)					
CaCl2	Cd	0.36 (0.27-0.45)	4.06 (2.52-5.59)	97 (93-101)	0.19	0.31	5E-10	Low EL => anta Hight EL => syn
	Pb	0.85 (0.64-1.07)	0.64 (0.47-0.81)					

The effect of the fungicide solvent in the copper-fungicide experiment on the extracted number of nematodes appeared to be negligible. In the acetone treated soil a non-significant increase from 100 ± 16.9 % to 115 ± 22.1 % (t-test, $p = 0.28$) was observed. From the range finding experiments it appeared that iprodione was not toxic for the nematodes. A relatively high dose of 75 ± 18 µg/g was therefore added to the copper-carbendazim mixture to investigate whether iprodione would influence the combined toxicity. These results are shown in figure 4. It shows the log-logistic dose response curves relative to the total amount of toxic units of copper and carbendazim in the mixture. The parameter estimates of the dose response curves (with 95% confidence intervals) without iprodione were: control: 98 (85-112), slope: 1.53 (0.86-2.23) and EC50: 1.39 (0.95-1.83). The parameter estimates with iprodione were: control: 101 (87-114), slope: 1.02 (0.69-1.51) and EC50: 1.36 (0.84-1.88). Clearly, iprodione did not alter the copper-carbendazim toxicity. In this experiment it was observed that addition of iprodione alone even enhanced the amount of extracted nematodes from the soil, indicating a stimulating effect on population growth rate. Relative to the control (100 ± 20.8 %), the extracted nematodes increased to 169 ± 36.1 %, which is significant at the 5 % level (t-test, $p = 0.016$). A similar effect was found for the lowest non-toxic dose of carbendazim. Table 9 lists the results after analysing the response to copper-carbendazim exposure. The carbendazim concentrations in the pore water were not measured. Therefore the toxicity of the binomial combination of copper and carbendazim was only evaluated using total concentrations. It appeared that the effect of copper and carbendazim in soil could be well described by the additive model.

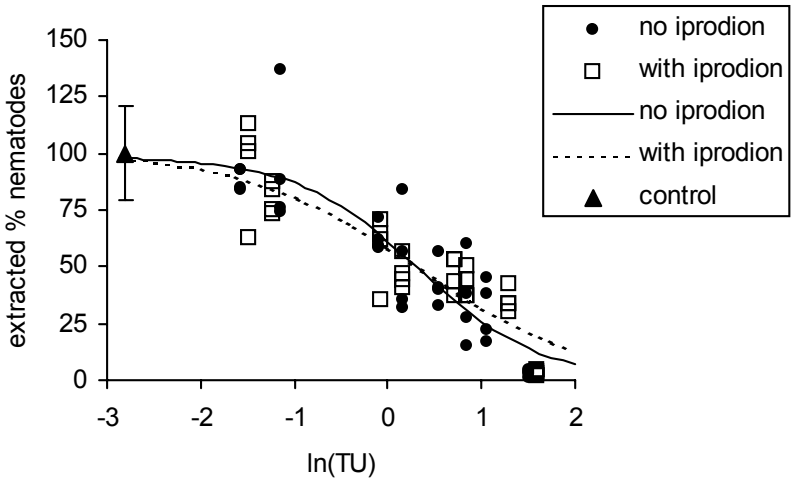


Figure 4. The effect of iprodion on the toxicity of a combination of copper and carbendazim. The log-logistic dose response curves are presented as a function of the total amount of toxic units of copper and carbendazim in the mixture. The error bar around the control indicates the standard deviation.

Table 9. Parameter values and 95% confidence intervals of individual the dose response curves of copper and carbendazim, estimated using the most parsimonious binary mixture model (equation 1), where the toxicity was related to total concentrations. See text and table 6 for detailed explanations.

Measure-ment	metal	slope	EC50 (µg/g soil)	Control (%)	S/A $p(X^2)$	TR $p(X^2)$	EL $p(X^2)$	conclusion
Total	Cu	1.58 (0.88-2.28)	138 (94-182)	104 (90-117)	0.12	0.24	0.42	additive
	Carb	0.98 (0.46-1.50)	16.3 (8.92-23.6)					

All mixture data sets were also compared with the independent model. The significance levels of the deviation models, and the conclusions are listed in table 10. Compared to the independent reference, most combinations acted antagonistically on *C. elegans*. Synergism was found when the nematodes were exposed to cadmium and lead, with regard to the total and $CaCl_2$ extracted concentrations, and when the response to copper and zinc was compared with the total concentrations.

Table 10. Significance of additional deviation parameters in the independent model (equation 2): S/A = synergism/antagonism, TR = toxicant ratio dependent deviation, EL = effect level dependent deviation. The conclusion describes the functional form of the dose response plain in comparison with the independent model.

Mixture	measurement	S/A $p(X^2)$	TR $p(X^2)$	EL $p(X^2)$	conclusion
Cu - Zn	Total	0.02	0.68	0.0002	Low EL => anta High EL => syn
	water	0.0002	0.38	0.20	antagonism
	CaCl2	3.58E-7	0.50	0.45	antagonism
Cu - Cd	Total	0.008	0.70	0.81	antagonism
	water	6.85E-5	0.84	0.99	antagonism
	CaCl2	0.0001	0.35	0.52	antagonism
Cd - Pb	Total	0.12	0.22	0.03	Low EL => anta High EL => syn
	water	0.012	0.88	0.70	antagonism
	CaCl2	7.19E-5	0.10	0.0002	Low EL => anta High EL => syn
Cu - Carb	Total	0.15	0.31	0.25	independent

Discussion

Sorption

For assessing exposure levels of toxic agents in soils analysing interactions of the chemicals with the soil matrix is important (Posthuma *et al.* 1997, Evangelou *et al.* 1999). These interactions depend on the soil characteristics (Janssen *et al.* 1997), and therefore data on sorption of metals to soil show large variability among and within different studies. For instance, partition coefficients of copper can range from 50 to 6400 L/kg after experimental application (Janssen *et al.* 1996), and from 25 to 4300 L/kg in the field, dependent on the soil type. Also in the presented experiments some variation was observed, despite the standardisation. The partition coefficients for copper, based on water-soluble concentrations ranged from 755 to 306. The lowest value coincided with the experiment where the lowest pH values were measured. The differences might be attributed to variability between experiments, maybe due to the different soil batches used. LUFA is highly standardised, but still a natural soil with differences between batches. For cadmium, reported partition coefficients range from 210 to 8900 (Sanchez-Martin and Sanchez-Camazano 1993), and from 5.5 to 755 L/kg

(Buchter *et al.* 1989) for various field soils. For lead, partition coefficients are reported to vary between 3.6 and 2.4E05 (Buchter *et al.* 1989) and 1135 and 5.5E05 L/kg (Hooda and Alloway 1998). Partition coefficients for zinc differ from 2.1 to 774 (Buchter *et al.* 1989) and from 29 to 2732 L/kg (Elrashidi and O'Connor 1982). The values found in this study did not deviate substantially from the partition coefficients found in literature, but the large variability blurs comparative conclusions.

In this study the sorption characteristics of the metals based on $CaCl_2$ extractable concentrations was more consistent than based on water-soluble concentrations, when different experiments are compared. The order of retention was approximately Pb > Cu > Zn > Cd. Harter (1983) did not study cadmium, but found the same order of retention for the other three metals. It should be noted that the order of retention can vary, it was for instance found to depend heavily on the dissolved organic compound (DOC) concentration present in the soil solutions (Gooddy *et al.* 1995).

When metals were combined it was found that the presence of lead and copper affected the retention of cadmium, whereas a reverse effect was not observed. Similarly, copper had a significant effect on zinc, whereas zinc less affected copper retention (only when the ions were extracted with $CaCl_2$). Thus the data indicated that the relative competitive strength for binding sites in the soil was quite well represented by the partition coefficient. The metal ions with the highest partition coefficients affected the retention of the metals with the lower partition coefficients in all cases, whereas the reverse was less likely. This might serve as a predictive tool to assess the potential risk of metal mixtures in field samples. It may therefore be worthwhile to investigate how the predictability of the relative competitive strength of metals, based on the partition coefficient, relates to soil characteristics.

Toxicity of individual compounds

The sorption characteristics described above changed the relative toxicity at the EC50 level of the compounds within each experiment. For instance, based on total concentrations, no substantial difference was found between the toxicity of copper and zinc (table 6). Based on water-soluble concentrations the EC50's were also very similar, but copper appeared to be more toxic when the $CaCl_2$ extractable concentrations were considered. A higher toxicity of copper than zinc was also reported by Dhawan *et al*. (Dhawan *et al.* 2000) and Tatara *et al*. (Tatara *et al.* 1997) for lethality and behavioural responses, who performed detailed toxicity tests in agar and liquid medium.

Compared to the range finding experiments cadmium was less toxic in the actual mixture experiments, which resulted in a relatively large uncertainty in the EC50 (large 95% confidence interval), when the data was analysed. Variability in toxic effect between experiments is a known phenomenon in ecotoxicity studies and can lead to experimental difficulties (Posthuma *et al.* 1997, Van der Geest *et al.* 2000). Fortunately, it was still possible to draw conclusions from these mixture studies. When *C. elegans* was exposed to cadmium

and copper, cadmium was more toxic when the effect was related to total concentrations, no difference was found when the effect was related to water-soluble concentrations, and copper was more toxic when the effect was related to $CaCl_2$ extractable concentrations (table 7). In literature copper is reported to be more toxic than cadmium with regard to survival (Tatara *et al.* 1997, Freeman *et al.* 1998), movement, feeding, growth and reproduction (Dhawan *et al.* 2000, Anderson *et al.* 2001).

Relating the toxicity of cadmium-lead mixture with total concentrations indicated that cadmium was more toxic than lead (table 8), whereas the opposite was observed when the toxicity was related to $CaCl_2$ extractable concentrations. Detailed toxicity tests in agar and liquid medium showed that lead was more toxic for *C. elegans* than cadmium with respect to movement, feeding, growth and reproduction (Dhawan *et al.* 2000, Anderson *et al.* 2001) and to survival (Tatara *et al.* 1997). Thus, the toxicity based on $CaCl_2$ extractable concentrations seems to reflect the relative toxicities of the individual chemicals best. These observations agree with the hypothesis that the 0.01 M $CaCl_2$ extractable concentrations generally correlate best with biological effects and therefore might represent the labile sorbed "bioavailable" metal fractions (Houba *et al.* 1996, Spurgeon and Hopkin 1996).

For carbendazim an EC50 of 16.3 µg/g was observed (table 9). This is in the same order of magnitude, but somewhat higher than the 21 day LC50 for the earthworm *Eisenia andrei* in OECD soil (5.7 µg/g) and the 28 day LC50 reported for *Eisenia fetida* (9.3 µg/g) (Van Gestel 1992). The exposure concentration of carbendazim is difficult to assess. Based on a bioavailability study of Matser and Leistra (Matser and Leistra 2000) in LUFA 2.2 soil, the pore water concentration could be estimated as approximately 1 µg/ml. That makes *C. elegans* relatively tolerant compared to various aquatic invertebrates. Wijngaarden et al. (Van Wijngaarden *et al.* 1998) report many LC50's and EC50's of carbendazim, and the most sensitive species is a flatworm, giving an EC50 of 0.025 µg/ml.

Similar modes of action?

It is often stated that the additive model is only a suitable reference model in case of similar modes of action (and/or similar shape of the individual dose response curves). In addition, the independent model is a suitable reference in case of independent modes of action. Obviously in complex systems as described above both assumptions are violated. The substances differ with respect to complexation with soil organic matter and clay, hydrolisation characteristics, precipitation properties and other retention mechanisms in soil (Harter 1983). In addition physiological interaction might be promoted or reduced by defence and regulation mechanisms in the organism (Beeby 1991). Due to the violation of the model assumptions they are indeed unsuitable for effect prediction. Therefore, the only way to use these models is to describe the data in relation to a reference. As shown in this study, both can be applied, but the additive model compares relative toxicities, whereas the independent model compares the data with the assumption of independent probabilities of response.

Mixture effects

It can be stated that for assessing risk of combined toxicity probably the most relevant model is the additive model, since it compares relative toxicities. It is also more frequently used than the independent model. Enhanced risk is indicated by synergism, and synergism was only found at higher exposure levels (> EC50). Comparing these observations to other studies is difficult, because in most toxic mixture analyses the combined effect is only evaluated at a certain toxic level (mostly LC50 or EC50), leaving deviations from additivity at other toxic levels undetected. However, a few studies explicitly mention effect level dependent deviations from additivity. For instance cadmium and zinc combined affected fresh and dry weight (related to total, water-soluble and tissue concentrations) and reproduction (related to tissue concentrations) of *Folsomia candida* differently at EC10 level than at EC50 level (Van Gestel and Hensbergen 1997). The effects on weight changed from synergism at low effect levels to antagonism at high effect levels (Van der Geest *et al.* 2000). It has also been reported that the cytotoxic effect of four metals (As, Cd, Cr and Pb) on human epidermal keratinocytes was synergistic at low effect levels and antagonistic at high effect levels (Gennings *et al.* 2002).

The synergistic effect at higher effect levels observed for the copper-zinc mixture related to total concentrations was not found when the toxicity was related to water-soluble and $CaCl_2$ extractable concentrations. Associated with these concentrations, the toxicity was (mainly) antagonistic in comparison with the additive model. Thus, the synergism could be explained from the increased availability of the metals, and hence the retention characteristics of the soil. The toxicant ratio dependent deviation from additivity in relation with the water-soluble concentrations of copper and zinc consisted of a relative increase in toxicity associated with a relative increase of copper in the mixture. In other words, relatively less copper was required for obtaining the same effect. This was not observed when the toxicity was related to $CaCl_2$ extractable concentrations. Yet, the $CaCl_2$ extractable mixture contained relatively more copper, which was detected by a significant decrease in K_f for copper (table 2). Since relatively more copper was available in the $CaCl_2$ extracts than the water solutions, relatively more copper was associated with the same toxic effect, which reduced the copper related ratio effect in the $CaCl_2$ extracts.

The synergistic effects at higher effect levels observed for the cadmium-lead and copper-cadmium mixtures are more difficult to interpret. Various aspects may play a role. The metals might act more than additive due to physiological interaction, or they might affect different toxicological endpoints that deteriorate the population effect when combined. Another factor considers the biology of nematodes. Compared to their size the chemicals were possibly heterogeneously distributed over the soil particles. Nematodes are known to avoid toxic chemicals (Croll 1970), which might play a role in "detoxification", explaining antagonism at low effect levels. Possibilities of avoidance might decrease as the concentration of toxic agents increases, which might deteriorate toxic effects at higher dose levels. Another aspect to consider is that the metals were added as chloride salts, and that at high concentrations the

chloride might have increased the relative toxicity. However, the highest concentrations of chloride in the mixtures were 967 µg Cl^-/gr dry wt soil (copper-zinc mixture), 627 µg Cl^-/gr dry wt soil (copper-cadmium mixture) and 772 µg Cl^-/gr dry wt soil (cadmium-lead mixture), whereas the NOEC for *Enchytraeus crypticus* has been found to be 1380 µg Cl^-/gr dry wt soil for reproduction (Posthuma *et al.* 1997). Unless *C. elegans* was more sensitive or chloride and metals acted synergistically, the effect of chloride ions was expected to be minimal.

The effect of the mixture of copper and carbendazim was adequately described by the independent, as well as the additive reference model despite the different modes of action, illustrating the difficulties of drawing conclusions with respect to modes of action from the model fit (De March 1988). A synergistic effect as found in aquatic systems (Vasseur *et al.* 1988, Bonnemain and Dive 1990) was not observed in this soil study. Unfortunately, measuring pore water (or water-soluble) concentrations of the pesticides was not possible within this experiment. This complicates the interpretation of how combined effects are influenced by soil sorption. It has been reported that the transformation of carbendazim in the soil was delayed in the presence of copper and/or iprodione (Matser and Leistra 2000). Extrapolating these findings to the presented data would presumably yield an antagonistic effect of copper and carbendazim when the toxicity would have been related to water-soluble concentrations. Unfortunately, there is no general agreement in the literature on the possibility of predicting the sorption in soil of organic compounds (Almendros 1995). Yet, since synergistic effects have been reported for various combinations of organic compounds with metals (Forget *et al.* 1999), it is recommended to put more research effort in studying the effects of those mixtures in soil systems.

It would be of great value if the effect of soil retention characteristics on joint toxicity patterns related to available (assumed to be the water solutions or $CaCl_2$ extracts) concentrations would be grossly predictable (Posthuma *et al.* 1997). Especially if the relative competitive strength of metals can be adequately deduced from the soil properties (see above). In this study the influence of soil retention characteristics on joint toxicity patterns could be evaluated by comparing changes in partition coefficients with changes in toxicity patterns. Unfortunately, this comparison did not give general results. For instance, comparing table 2 and 6 (Cu-Zn) indicated that changes in partition coefficients were accompanied by changes the joint toxicity pattern, in contrast to comparing table 4 and 8 (Cd-Pb). Thus, there did not seem to be a general pattern revealing how changes in K_f or n changed joint toxicity patterns.

One aspect that complicates the search for general patterns is that the ratio of compounds in the mixture depends on the hypothesised bioavailability. This can only be taken into account with the quantification procedure that was applied in this study, where the complete dose response surface was judged. It may be a robust tool for developing and analysing future experiments and protocols for toxic mixture risk assessment.

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Chapter 6

Concluding remarks: predictability and risk assessment

For effective and successful risk assessment of toxicant mixtures it is required to have the disposal of tools that enable the prediction of combined effects, which covers both simple and complex mixtures. Yet, the type and magnitude of interaction can vary, because of its dependence on dose, duration, type of components, exposure sequence and route, test species and measured type of response, as illustrated in the preceding chapters. Therefore analyses of the joint toxicity of toxic chemicals yield no prediction of the effects for conditions other than the ones defined in the study. In addition, the interaction observed yields little information about the pharmacological processes involved, but has only a statistical meaning. In general, mechanistic pathways of the chemicals are poorly known, therefore the choice of the non-interaction model is arbitrary from a pharmacological point of view. It should nevertheless be well defined, or else statistical operations are meaningless, as stated in chapter 2. In this chapter current developments of mixture toxicity risk assessment will be discussed and potential directions for future research will be proposed.

Risk assessment

Current risk assessment protocols with regard to chemical mixtures are mainly based on analyses whether responses deviate from additivity. For instance, within human toxicology hazard indices are calculated for quantifying the toxicity of complex mixtures. A hazard index is equivalent to toxic strength, which is introduced in chapter 1. Several calculation procedures have been proposed to include binary interactions between the individual mixture components in the hazard index. For instance, (Woo *et al.* (1994) published on a large computer database of binary interactions of carcinogenic chemicals and proposed a calculation procedure to include them in risk assessment. Similarly, Mumtaz and Durkin (1992) developed a calculation model called WOE (Weight of Evidence), adopted by the US EPA, to include binary interactions in the risk assessment model. In line of this work, Borgert *et al.* (2001) determined 5 criteria for an interaction study to be useful in toxic mixture risk assessment. For instance, the study conditions should be relevant for the assessment scenario

(e.g. the experimental dose range should overlap the environmental dose range), the non-interaction model should be clearly defined, and statistical methods should be used. In addition, Feron and Groten (2002) developed a scheme for the safety evaluation of toxic mixtures, where for different types of mixtures different approaches were proposed, dependent on the exposure scenario and the amount, type and quality of the available data (Groten *et al.* 2001). It includes the evaluation of complex mixtures consisting of tens or hundreds of chemicals, which are qualitatively and quantitatively not fully known. In general, these risk assessment protocols are designed in such a way that three types of interaction can be taken into account: synergism, additivity and antagonism. The observation that statistical interactions can be dose level, toxicant ratio and exposure time specific raises questions about how these detailed aspects should be taken into account.

For environmental toxicants it has been argued that the effect of complex mixtures approaches additivity as the number of mixture components increase (figure 1). The model, called funnel hypothesis (Warne and Hawker 1995), was designed for equitoxic mixtures of narcotics, and supported by aquatic toxicity data from literature. It is difficult to assess to what extent the hypothesis can be generalised. For instance, the chemical composition of soils and sediments can influence the joint effect of toxic chemicals (chapter 5), which raises questions such as: should the funnel hypothesis be applied to available or to total toxicant concentrations in the soil? Secondly, it can also be questioned whether the theory holds when exposure conditions differ from the optimal laboratory conditions. Thirdly, narcotics have non-specific modes of action, but what happens if a non-specific and specific acting compounds are combined? Despite these uncertainties, current environmental mixture risk assessment is based on additive structures, such as the calculation of toxic equivalency factors (Logan and Wilson 1995, Neumann 1996, Mumtaz *et al.* 1997). Alternatively, the independent and additive model can be combined, applying a mixed model approach (Junghans *et al.* 2002, Posthuma *et al.* 2002). Nevertheless, in both calculation procedures interactions are ignored.

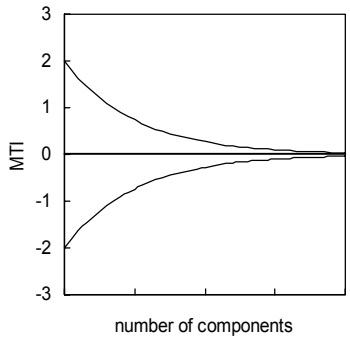

Figure 1. A graphical representation of the Funnel Hypothesis (Warne and Hawker 1995). The x-axis represents the number of compounds in the mixture, and the y-axis a mixture toxicity index (MTI) where additivity is indicated by 0. The larger the amount of mixture components, the more the response to the mixture approaches additivity.

More mechanism?

Obviously, the limited understanding and, more importantly, the condition specific consequences of mixture toxicity, results in a low predictability of combined effects and to pragmatic risk assessment approaches that are not entirely biologically sound. This is rather unsatisfactory and bothers mixture toxicity researchers in both toxicology and ecotoxicology. For toxicological research, Hertzberg and MacDonell (2002) argue that researches should cease investigations of synergism and antagonism and should instead look for mechanisms of interaction, and fundamental principles of joint toxicity from which predictive models can be assembled. In their opinion, physiologically based, pharmacokinetic (PBPK) models have great potential to serve as future framework to model combined toxicity. Two obstacles limit the application for risk assessment purposes. First is the complexity. PBPK models of simple three chemical mixtures can have over 20 model parameters. Since not all of the parameters can be independently determined, some parameters have to be estimated by fitting, entailing uncertainties. Second is the expense. PBPK models have not been parameterised for most of the relevant toxic chemicals, and information on interactions in organs is scarce.

In ecotoxicology, PBPK models are only developed for a few standardised test species (Thomann *et al.* 1997), and it is therefore not an obvious generally usable tool for predicting mixture effects. Alternatively, by combining one-compartment toxicokinetics with survival analysis, biological mechanism can be included in bioassays data quantification, an approach successfully applied to single toxicity data (Kooijman and Bedaux 1996). In this modelling context it is assumed that an individual has a certain internal tolerance concentration, and as long as the internal concentration is below this tolerance, no toxic response will be elicited. If the internal concentration exceeds the tolerance concentration, it is hypothesised that the hazard rate is proportional to the difference between the internal and tolerance concentration (figure 2). Note that this model is designed to analyse mortality (quantal) data. Applying the approach to mixture toxicity analysis may yield some mechanistic insight, but requires addressing two aspects: i) a decision on using separate tolerance concentrations for all mixture constituents or one single joint tolerance concentration, and ii) an interpretation of how one chemical alters the uptake of the other.

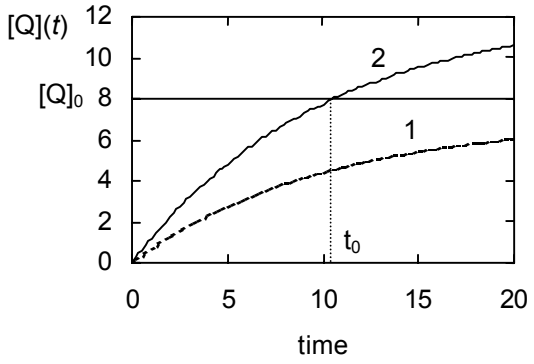

Figure 2. Two accumulation curves for two environmental toxicants. The internal concentration is indicated by [Q]. Curve 1 does not exceed the tolerance concentration $[Q]_0$, and does not induce a response. Curve 2 exceeds the tolerance concentration at $t = t_0$, and the hazard rate is proportional to $[Q](t) - [Q]_0$.

Experimental data on how one chemical alters the uptake of the other for invertebrates is difficult to generalise at this stage, and may be organism and mixture specific. For instance, Ireland and Fischer (1978) found that lead reduced iron concentrations in the earthworm *Lumbricus terrestris*. Ahsanullah *et al.* (1981) reported that the uptake of copper by the shrimp *Callianassa australiensis* was inhibited by the presence if cadmium, but cadmium uptake was unaffected by copper. Van Capelleveen (1987) found that cadmium and zinc increased each others concentration in the isopod *Porcellio scaber*. Migula *et al.* (1989) found that zinc decreased lead and cadmium concentrations in the cricket *Acheta domesticus*. Posthuma *et al.* (1997) reported that copper concentrations in the worm *Enchytraeus crypticus* were not influenced by the presence of zinc, whereas zinc concentrations increased in the presence of copper. Absence of interactions was reported by Berger *et al.* (1994), for zinc and cadmium concentrations in the snail *Helix pomatia*, and by Van Gestel and Hensbergen (1997) for cadmium and zinc in the springtail *Folsomia candida*. See Beeby (1991) for an extensive review on the subject. We analysed the effect of copper-cadmium uptake interactions and the effect of carbendazim on copper uptake in *C. elegans*. The results are shown in figure 3. Measuring internal metal concentrations in nematodes is difficult and the concentrations were somewhat higher than expected. The graphs should therefore be interpreted with care, however it can be concluded that we were not able to detect interactions.

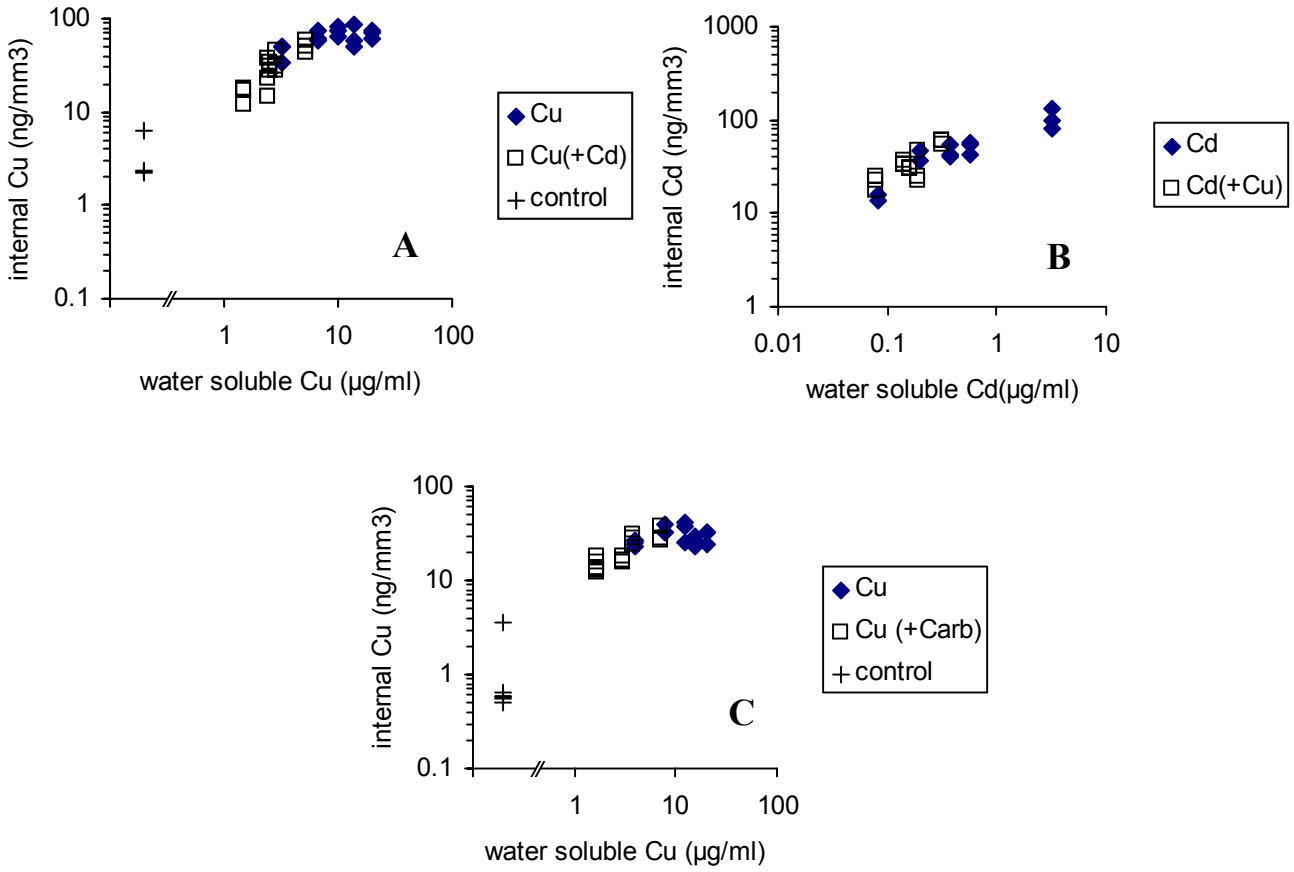

Figure 3. Relationships between metal concentrations in the agar and the tissue (internal) concentrations of *C. elegans* for copper applied singly and with cadmium (1A), for cadmium applied singly and with copper (1B) and for copper applied with carbendazim (1C), after 6 days of exposure. Cadmium concentrations in the controls were below detection limit.

Future research

The recommendation of Hertzberg and MacDonell (2002), to look for mechanisms of interaction and to identify fundamental principles of joint toxicity for developing predictive models, also applies to ecotoxicologists. However, ecotoxicologists are interested in other subjects than toxicology alone. Hence, there are numerous topics that can potentially be considered in the search for general mechanisms and fundamental principles, such as:

- The Funnel Hypothesis is underpinned by quantitative, mechanistic considerations, and may therefore provide a basis for delineating general concepts. Questions such as the ones posed above should be addressed to extend the model, which would make the theory more robust.

- Different metals alter each others availability in sediments and soils. At present, data indicates that at least qualitative conclusions can be drawn from the knowledge of the binding characteristics of the metals to soils and sediments. To some extent, prediction of the bioavailability of metal mixture components may thus be possible.
- The wealth of possible combinations of different toxic substances may become more digestible for environmental scientists if research is focussed on effects of different ecologically relevant combination groups. The classification of combinations can be based on chemical of physiological characteristics. An example of a rough chemical classification is: i) metal combinations, ii) organic compound combinations, iii) metal-organic compound combinations. An example of a physiological classification is to distinguish combinations based on their specificity of action, or target organs. Classes of combinations may result in similar effects, which may facilitate mixture risk assessment based on chemistry data from the field.
- It may be important to address the effects of stochastic environmental stressors on mixture toxicity, such as climatic conditions, soils conditions, pH changes, moist and drought. Can these additional stressors affect the mixture effect, and change for instance antagonism into synergism.
- Effects of combined exposure at higher levels of biological organisation such as populations and communities are virtually unknown. However, joint toxicity can potentially be analysed in microcosms, mesocosms or terrestrial model ecosystem studies (TME), to help filling this gap. Chapter 4 illustrates that it is possible to combine joint toxicity analysis with principles from population biology, and it may be considered to extent it to predator-pray interactions, species interactions and metapopulation models.

In contrast to the considerations of Hertzberg and MacDonell (2002), I think that within ecotoxicology the additive model will keep on playing an important role in the future, simply because there are hardly any alternative approaches available. At present, the bioassay is still one of the most important tools for ecotoxicologists, and the additive (as well as the independent) model fits perfectly well in this approach: it is basically an extension of the single dose response curves. Complex mixtures, which are qualitatively and quantitatively not fully known, still pose difficulties. At this moment I see no other option than to test them as a whole. Yet, this way of analysing yields no comparison between mixture effects and effects of the individual mixture components. It can be concluded that future research on joint toxicity should focus on the effects of combined exposure on ecological mechanisms. For developing risk assessment protocols it would be fruitful to enhance the co-operation with (human) toxicologists, because they face grossly similar conceptual problems as ecotoxicologists. To cope with the research topics mentioned above (and there are probably more), strong collaborations between experimental biologists, experimental toxicologists, biomathematicians, statisticians and pharmacologists are indispensable.

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Summary

In natural environments, contaminated sites are frequently polluted with mixtures of compounds rather than one toxicant only. In general organisms in polluted sites are thus exposed to combinations of toxicants. Nearly all regulatory toxicity testing is carried out using single individual chemicals, therefore it can be questioned whether ecological conclusions based on single toxicity studies suffices for predicting the combined toxic effects that occur in contaminated areas. Since the number of mixture toxicity studies with regard to soil systems is limited, in this thesis the research is focused on investigating ecotoxicological consequences of combined exposure for soil invertebrates. Therefore, four research topics were addressed: i) the development of a robust data quantification methodology, ii) the analysis of consequences of chronic exposure, iii) the effect translation of combined effects to levels of higher biological organisation and iiii) investigating the relationship between soil sorption and combined effect.

The research focused on comparing the effect of the mixture to the effect of the individual mixture constituents. It is the general approach upon which the mixture models are based. First, a modest overview was presented to illustrate the wealth of quantification methods that exist. The additive and independent action models were originally developed with a clear definition of the joint modes of action of chemicals, based on concentrations of the chemicals that reach the sites of action. In addition, they can be unified by addition of a similarity parameter. In ecotoxicology researchers frequently have to deal with limited information on the joint mode of action of the chemicals. There may be limited information on the concentrations that actually reach the site of action, and toxicants can have non-specific modes of action. Just as single dose response curves, the mixture models are applied to different complex systems and to quantify the effect of many different compounds. Hence, for ecotoxicological research the descriptive properties of the models are most useful. Both models define a mathematical reference relative to which the data can be analysed. In their mathematical definitions they do not constitute real alternatives. The independent model is a statement about relationships between probabilities of response, whereas the additive model is a statement about the relative toxicities. From that point of view, a rigid separation between the additive joint action model and independent joint action model is justified, and the models can be considered as mutually exclusive. The deviations from the model description had to be quantified accurately, and modelling procedures were proposed that enabled the quantification of four biologically relevant deviation patterns from either the additive or the independent reference:

- No deviation. The actual effect of the mixture is well described by one of the chosen reference situations.
- Absolute synergism or antagonism. When all combinations of a mixture are either more toxic or less toxic than the reference situation, synergism or antagonism is observed.
- Toxicant ratio dependent deviation. When the deviation pattern is subject to the relative amount of (one of) the toxicants in the mixture, the effect is toxicant ratio dependent. Considering two substances, synergism can be observed if toxicant A is relatively

dominant in the mixture whereas antagonism can be observed if toxicant B is relatively dominant in the mixture.

- Effect level dependent deviation. When, for example, antagonism is observed at low effect levels and synergism at high effect levels, an effect level dependent deviation from the reference is identified.

In the examples that were used to demonstrate the applicability of the quantification procedures, it was shown that the mixture effect, compared to the effect of its individual components, can vary over time, and that different response variables may yield different results. It illustrates the difficulties of drawing conclusions with respect to modes of joint action from the model outcome. It also raises the question how these aspects should be interpreted, and what it means from an ecotoxicological point of view.

To consider this last question it was decided to study sublethal effects of chronic stress of binary mixtures on life cycle events of *Caenorhabditis elegans*. *C. elegans* is a free living, soil dwelling, bacterivorous nematode with a relatively simple lifecycle, which facilitates the analysis of combined exposure on different response variables. The nematode is a frequently used test organism in ecotoxicology, it has been used for single toxicity experiments in liquid phase, on agar and in soils, and various endpoints have been measured. It may be considered as a model organism for studying fast reproducing opportunistic soil invertebrates. Additionally, it may represent the metazoa which are important for decomposition and nutrient cycling in soil systems.

We investigated the exposure to mixtures of copper and cadmium, and copper and carbendazim. The combined effects were compared to the effects of the individual mixture constituents. The life-cycle straits studied were age specific cumulative reproduction, length of the juvenile period, length of the reproductive period and growth. The cadmium-copper effect on reproduction varied over time: it changed from synergistic, to a toxicant ratio dependent deviation from additivity. The mixture was relatively less toxic if relatively more cadmium was available. The effect of copper-carbendazim was synergistic at low effect levels and antagonistic at high effect levels. In general, the juvenile period was relatively non-sensitive, prolongation was observed at concentrations of 2-3 times the EC50 for reproduction. The length of the reproductive period was relatively sensitive: prolongation was observed at concentrations similar as EC50 for reproduction. The combined effects on the juvenile period and the reproductive period were similar as the effects of the individual compounds. Thus, for *C. elegans* it was found that mixture toxicity may be transient and that interactions may differ among life history traits.

The assessment of the ecological relevance of the observed patterns proceeded by demographic analyses. To explore possible effects on population growth rate (λ), combined effects on a hypothetical life history were discussed, and the consequences of different response patterns of life history parameters for λ were evaluated. The response scenarios illustrated that the combined effect on λ, compared to the effect of the individual mixture constituents depends on three factors: i) the sensitivity of each life history trait to each of the toxicants, ii) the combination effect of the toxicants on each life history trait, iii) the sensitivity of λ to changes in each life history trait. A detailed analysis of mixture effects on

the life history of nematode *C. elegans* showed that synergistic effects on reproduction were transferred to the population level. Toxicant exposure changed the elasticity to the stage-classified model parameters, where combined exposure induced the changes at lower dose levels. It was shown that the application of population models in mixture toxicity research puts interactive effects at the individual level in an ecological perspective. It incorporates time dependent mixture effects. In addition, the importance of synergistic and antagonistic effects on different response parameters can be weighted according to their contribution to population growth rate.

Population consequences of chemical combinations were also studied in soil. Yet, sorption to soil may render a fraction of the chemicals inaccessible for uptake by the nematodes. Soil characteristics and the presence of other chemicals influence sorption. Therefore a standardised well-characterised soil (LUFA 2.2) was used to minimise soil quality variation. Chemical interactions in the soil were analysed by comparison of Freundlich isotherms. To compare interactive effects on soil sorption to joint toxicity, single and combined toxicity of copper-zinc, copper-cadmium, cadmium-lead, copper-carbendazim and copper-carbendazim-iprodione to *C. elegans* were studied. The one-week population increase was estimated as toxicity endpoint. Joint toxicity patterns were quantified by comparing mixture effects to the effect of its individual constituents, and were related to total concentrations in the soil, water-soluble concentrations and 0.01 M $CaCl_2$ extractable concentrations. Differences in partition coefficients were found between the metals when applied individually. The metal with the highest partition coefficient affected the sorption of metal with the lowest partition coefficient when both were combined. Consequently, both the ratio and the relative toxicity of individual mixture constituents depend on assumptions about exposure conditions, which was taken into account in the data quantification procedure applied. It was found that both the additive and the independent model were generally inadequate to describe metal mixture effects. Compared to the additive model, synergism was observed at dose levels higher than the EC50's of the individual compounds. Compared with the independent model antagonism was mostly detected. Analysing the interactive sorption effects on joint toxicity yielded no general pattern.

For risk assessment of toxicant mixtures to be successful it is required to have the disposal of tools that enable the prediction of combined effects, of both simple and complex mixtures. Literature and the presented studies indicate that the type and magnitude of statistical interaction can vary dependent on dose, duration, type of components, exposure sequence and route, test species and measured response parameter. However, current risk assessment protocols are designed such that only three types of interaction can be taken into account: synergism, additivity and antagonism. A more detailed risk assessment requires more research and the collaboration of specialists from different life science disciplines.

Samenvatting

Ecotoxicologie is het vakgebied dat zich bezig houdt met het bestuderen van mogelijk schadelijk effecten van toxische stoffen op het milieu en (natuurlijke) ecosystemen. Milieuvervuiling bestaat vaak uit coctails van verschillende toxische stoffen. Vandaar dat in vervuilde gebieden de daar levende organismen vaak worden blootgesteld aan meerdere toxische stoffen tegelijk, in plaats van één enkele chemische stof. Voor de risicobeoordeling van milieuvervuiling worden toxiciteitstesten gedaan, maar daarbij wordt nagenoeg geen rekening gehouden met combinatietoxiciteit. Vandaar dat de vraag gesteld kan worden of binnen de ecologische risicobeoordeling rekening moet worden gehouden met de effecten die het gevolg zijn van blootstelling aan toxische mengsels. Het aantal combinatie toxiciteits studies met betrekking tot de bodem en daarin levende organismen is beperkt. Vandaar dat deze studie zich richt op het bestuderen van (eco)toxicologische effecten, als bodem invertebraten worden blootgesteld aan combinaties van toxische stoffen.

Bij het onderzoeken van combinatietoxiciteit is het in de eerste plaats van belang om het effect van het mengsel te vergelijken met de effecten van de individuele mengsel componenten. Op basis hiervan zijn gedurende de afgelopen decenia vele combinatie mechanismen geclassificeerd. Twee basismodellen staan daarbij centraal: het additief model (gelijke werking) en het model voor onafhankelijke werkingsmechanismen. Afwijkingen van de modelvoorspelling worden vaak respectievelijk complexe gelijke werking en afhankelijke werking genoemd. De onderliggende werkingsmechanismen bij deze modellen zijn goed gedefinieerd, en de twee basis modellen zijn zelfs mathematisch aan elkaar gerelateerd door middel van een interactie parameter. Bij de toepassing kunnen echter interpretatieproblemen opduiken. Het grootste probleem is dat een bepaald dosis-respons patroon niet specifiek toegewezen kan worden aan een bepaald werkingsmechanisme. Bijvoorbeeld, het antagonistisch effect kan net zo goed beschreven worden door het model voor onafhankelijke werking. Daarnaast betekent een additief effect niet dat er geen fysiologische interactie is opgetreden.

We kunnen dus het model voor onafhankelijke werking en het additief model als twee verschillende referentiemodellen beschouwen. Het vergelijken van de data met één van de modellen maakt het vervolgens mogelijk de data te karakteriseren. Daarbij kan alleen de mathematische betekenis van de referentiemodellen gebruikt worden. Het onafhankelijke model maakt het mogelijk om te analyseren of de kans op respons door blootstelling aan het ene toxicant onafhankelijk zou kunnen zijn van de kans op respons door blootstelling aan een andere. Het additief model maakt het mogelijk om te analyseren of het mengsel relatief giftiger is dan de relatieve toxiciteit van de individuele mengsel componenten. Vervolgens zijn er modellen ontwikkeld voor het kwantificeren van vier typen afwijkingspatronen van deze referentie modellen:

- Geen afwijking, wat betekent dat de data goed beschreven kan worden door één van de referentiemodellen.
- Synergisme/antagonisme, wat betekent dat alle combinaties/verhoudingen van een bepaald mengsel meer dan wel minder giftig zijn dan de referentie aangeeft.

- Toxicant-ratio afhankelijke afwijking. Dit betekent dat het afwijkingspatroon van een referentie afhankelijk is van de ratio van de mengsel componenten. Het zou bijvoorbeeld giftiger kunnen zijn als er meer van stof A in het mengsel zit, en minder giftig als er meer van stof B in het mengsel zit.
- Effect niveau afhankelijke afwijking. Dit betekent dat het afwijkingspatroon van een referentie afhankelijk is van het effect niveau dat geanalyseerd wordt. Er zou bijvoorbeeld antagonisme op kunnen treden bij lage effect niveaus, en synergisme bij hoge effect niveaus.

De data die gebruikt was om het gebruik van de modellen te illustreren, gaf aan dat het effect van het mengsel, vergeleken met de effecten van de individuele stoffen, kan variëren in de tijd, en dat het afhankelijk is van de response parameter die gemeten wordt.

Om vervolgens (eco)toxicologische effecten van mengseltoxiciteit op bodem invertebraten te kunnen bestuderen is er gekozen voor *Caenorhabditis elegans* als testorganisme. *C. elegans* is een vrij levende, bacterie etende nematode die in de bodem voorkomt. Het beestje is makkelijk te kweken, en heeft een relatief simpele levenscyclus, waardoor het zeer geschikt is als test organisme in ecologische experimenten. Het wordt dan ook erg veel gebruikt voor toxiciteits-testen in water, op agar en in de bodem.

In het laboratorium is *C. elegans* chronisch blootgesteld aan koper en cadmium, en aan koper en carbendazim, om de sub-letale effecten op de levenscyclus te kunnen bestuderen. De levenscyclus eigenschappen die bestudeerd zijn, zijn cumulatieve reproductie, de lengte van de juveniele periode, de lengte van de reproductieve periode en groei. Het effect van cadmium en koper op cumulatieve reproductie varieerde in de tijd: daarbij veranderde het van synergisme naar een toxicant-ratio afhankelijke afwijking van het additief model. Het mengsel was relatief minder toxisch als er meer cadmium in het mengsel beschikbaar was. Het effect van koper en carbendazim op de cumulatieve reproductie was afhankelijk van het effect niveau: op lage effect niveaus werkten de stoffen synergistisch en op hogere effect niveaus werd het mengsel minder toxisch. De effecten op groei kwamen overeen met de effecten op reproductie. De lengte van de juveniele periode was een relatief ongevoelige response parameter. Het verlengen van de juveniele periode begon pas bij concentraties die 2 tot 3 keer hoger lagen dan de EC50 voor reproductie. De lengte van de reproductieve periode was daarentegen gevoeliger. Het verlengen van de reproductieve periode begon bij concentraties die lager/vergelijkbaar waren met die van de EC50 voor reproductie. De effecten van de mengsels op zowel de juveniele als de reproductieve fase waren echter vergelijkbaar met de effecten van de individuele stoffen.

Wat betekenen deze verschijnselen nu voor een populatie van nematoden? Dit kan geanalyseerd worden door middel van populatie modellen. De responsparameter die indicatief is voor de populatie effecten is de populatie groeisnelheid, die vaak wordt aangegeven door het symbool λ. Door eerst een hypothetische levenscyclus te analyseren kon vastgesteld worden dat het effect van een toxisch mengsel, in vergelijking met de effecten van de individuele stoffen, op de populatie groeisnelheid voornamelijk afhangt van drie aspecten: i) de gevoeligheid van iedere respons parameter voor iedere toxicant, ii) het combinatie effect van de toxicanten op de response parameter, en iii) de gevoeligheid van λ voor veranderingen

in elk van de respons parameters. Uit de analyse van de *C. elegans* data bleek dat de synergistische effecten op reproductie ook effect hadden op populatie niveau. De demografische analyse gaf ook aan welke levenscyclus eigenschap het belangrijkste is voor de populatie groeisnelheid, en welke minder belangrijk zijn. De blootstelling aan toxicanten veroorzaakte veranderingen in deze patronen. Het synergistische effect op reproductie had als gevolg dat deze veranderingen bij relatief lagere concentraties plaats vonden. Het moet wel opgemerkt worden dat de synergistische effecten op reproductie op populatie niveau "afgevlakt" werden, doordat de populatie groeisnelheid van *C. elegans* niet zo erg gevoelig was voor veranderingen in deze respons parameter.

C. elegans is ook blootgesteld in de bodem. Na 1 week blootstelling aan verschillende toxische mengsels zijn de nematoden uit de bodem geëxtraheerd om een schatting te kunnen maken van het effect op de populatie groeisnelheid. Er moet echter rekening gehouden worden met het feit dat een deel van de toxicanten niet beschikbaar is, omdat dit hecht aan de bodem deeltjes. De beschikbaarheid van een toxicant wordt beïnvloed door de eigenschappen van de stoffen zelf, van de bodem, en door de aanwezigheid van andere (toxische) stoffen. Deze processen zijn bestudeerd in een gestandaardiseerde bodem, genaamd LUFA 2.2. De binding van metalen, alleen of in combinatie met een andere metaal, werd gekwantificeerd door middel van de Freundlich isotherm. De effecten van de volgende mengsels zijn geanalyseerd: koper en zink, koper en cadmium, cadmium en lood, koper en carbendazim en koper, carbendazim en iprodion. De toxische effecten van de mengsels zijn vergeleken met de effecten van de individuele stoffen, en zijn gerelateerd aan totale concentraties in de bodem, water oplosbare metaal fracties, en 0.01M CaCl2 extraheerbare metaal fracties. Er zijn verschillen gevonden in partitie coefficiënten tussen de metalen, die de verschillen in bindingscapaciteit kwantificeerden. Als de metalen als mengsel werden toegediend, dan werd de binding van het metaal met de laagste partitie coefficiënt beïnvloed door het metaal met de hoogste partitie coefficiënt, terwijl andersom maar één keer voorkwam. Het gevolg van deze processen is dat de verhouding van stoffen in het mengsel afhankelijk is van wat men aanneemt als zijnde "beschikbaar". Bij de toxiciteitsanalyse werden afwijkingen gevonden van zowel het additief model, als het model voor onafhankelijke werking. Synergistische effecten ten opzichte van het additief model werden alleen gevonden op effect niveaus die hoger lagen dan de EC50's. Het vergelijken van toxiciteitspatronen met bindings-eigenschappen van de metalen leverde helaas geen generieke resultaten op.

Voor de ecologische risicoanalyse van toxische mengsels is het eigenlijk nodig om de gecombineerde effecten te kunnen voorspellen. Dit proefschrift, en andere reeds gepubliceerde studies geven echter aan dat gecombineerde effecten van een mengsel ten opzichte van additiviteit, onder andere afhankelijk kunnen zijn van de dosis, de duur van de blootstelling, de mengselcomponenten zelf, de volgorde van blootstelling, test organisme en de gemeten response parameter. Deze aspecten kunnen nog niet meegenomen worden in de huidige risicobeoordeling. Meer onderzoek is nodig voor het ontrafelen van mengseltoxiciteits-concepten die als basis zouden kunnen dienen voor het voorspellen van combinatietoxiciteit. Een goede samenwerking tussen verschillende toxicologische en eco(toxico)logische disciplines is daarvoor onontbeerlijk.

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Curriculum Vitae

Martijs Johannes Jonker werd geboren op 27 december 1971, en studeerde van 1990 tot 1996 Biologie aan de Universiteit van Amsterdam. Daar begon de specialisatie in de richting ecotoxicologie met de stage "The effect of joint exposure to toxicants and humic acids on river inhabiting invertebrates: the mussel *Dreissena polymorpha* and the caddisflies *Hydropsyche angustipennis* en *H. siltalai*", bij vakgroep Aquatic Ecology and Ecotoxicology. Na de studie bleef hij een jaar bij de betreffende vakgroep werken aan een project genaamd "Differences in metal tolerance between benthic diatom species, isolated from metal polluted and clean river sites". Vervolgens werd hij aangenomen als phD-student bij de vakgroep Nematologie van de Wageningen Universiteit, en aangesteld op het project "Conceptualising the effect assessment of toxicant mixtures to soil organisms". De relevante resultaten die uit dit onderzoek kwamen staan beschreven in dit proefschrift. Thans zijn de voorbereidingen in volle gang om in Engeland te gaan werken bij The Centre for Ecology and Hydrology (CEH), Monkswood, UK, aan een project met de titel "Identifying conserved gene expression changes functionally linked to lifecycle parameters and predictive for fitness of soil invertebrates". Het één en ander hangt af van de toekenning van een Marie-Curie Fellowship.