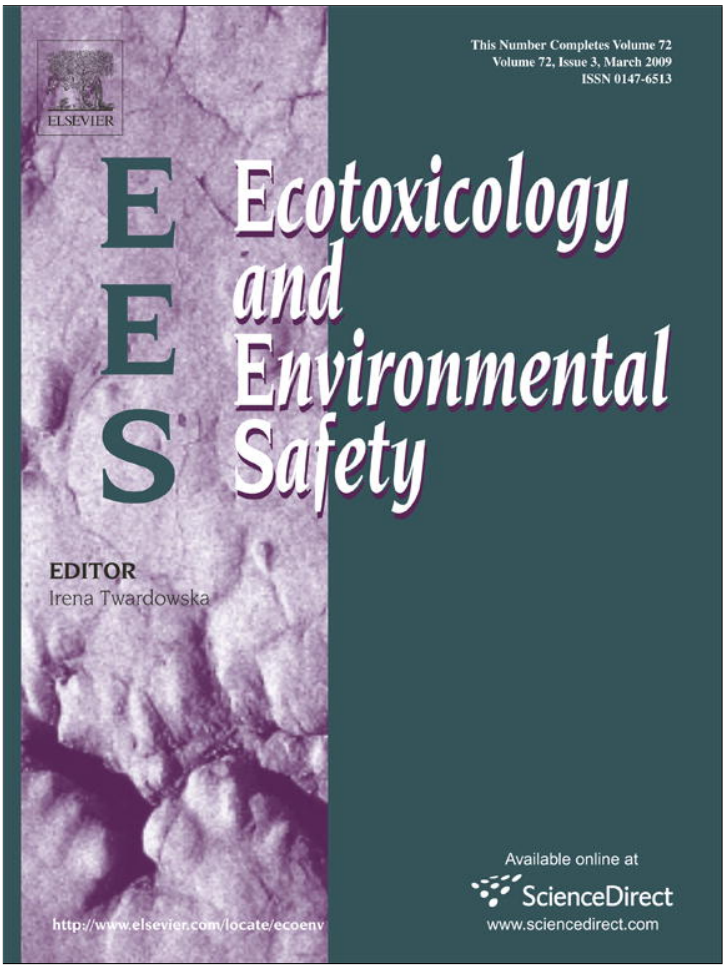

Ecotoxicology and Environmental Safety 72 (2009) 669–676

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Highlighted Article

A model to analyze effects of complex mixtures on survival ☆

Jan Baas *, Tjalling Jager, S.A.L.M. Kooijman

Department of Theoretical Biology, Vrije Universiteit of Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 16 April 2008
Received in revised form
30 July 2008
Accepted 7 September 2008
Available online 31 October 2008

Keywords:
Mixture
Survival
No-effect concentration
Hazard modeling

ABSTRACT

In ecotoxicology there is a growing interest in effects of mixtures. The aim of this research was to develop a biology-based model that describes effects of mixtures on survival in time.

The model works from the individual compounds in the mixture. Such an approach requires parameters for each individual compound in the mixture. For narcotic compounds we underpinned theoretical relations between the toxic parameters and the log K_{ow} with experimental data by analyzing almost 300 datasets from the open literature, allowing a vast reduction in effort in the assessment of effects of mixtures.

To illustrate the use of the model we simulated the effect of a mixture of 14 PAHs on the survival of *Pimephales promelas*. The simulation showed that due to the combined effect of the compounds in the mixture effects can be seen at very low concentrations.

1. Introduction

In ecotoxicology there is a growing concern that effects of mixtures are underestimated and that mixtures may produce unexpected effects (e.g. Broderius and Kahl, 1985; Nirmalakhandan et al., 1997; Silva et al., 2002). As there is no such thing as a single-compound exposure in the field (Yang et al., 1998), and as there is no way to test all possible mixtures, there is a need for models to predict effects of mixtures.

When the chemical composition of a mixture is known and constant, effects of mixtures can be described in a bottom-up approach, starting from the effects of the individual compounds that make up the mixture. Toxic effects are generally described by two standard models: concentration addition (CA) and independent action (IA). CA is used to predict effects of mixtures where the compounds in the mixture are assumed to have a common mechanism of action. In this case the effects of the mixture are not changed if one compound in the mixture is partially replaced, or diluted, by an equipotent amount of another. Mixture effects where the compounds in the mixture are supposed to act by a different working mechanism are described by the concept of IA. An elaborate description of these approaches can be found elsewhere (e.g. Altenburger et al., 2000; Backhaus et al., 2000; Jonker et al., 2005).

These models are used to predict mixture effects at a single point in time, assuming that there are no interactions, or they are used to find interactions (i.e., deviations from the modeled results) when the single compound and mixture effects are measured. The latter use is basically possible only for binary mixtures as the number of potential interactions in more complex mixtures and it rapidly becomes too high to handle. A major disadvantage of these models is that they are single time point models. It has been shown that the effects of mixtures may greatly vary at different points in time (Baas et al., 2007; Cedergreen and Streibig, 2005). In addition small random effects may also greatly influence the type of interaction found in a mixture (Baas et al., 2007). To overcome these drawbacks there is a need for a better understanding of the dynamics of the effects of mixtures with process-based models (Ashauer et al., 2006; Andersen and Dennison, 2004). A model of this type, describing the whole build up of effects in time by following the individual compounds that make up the mixture, was developed for the effect of binary mixtures and it proved to give an accurate description of measured survival data (Baas et al., 2007). It could also be used to identify synergistic or antagonistic interactions.

The aim of the present study was to expand the current survival models for binary mixtures into models that can cope with any number of compounds in a mixture, still based on biological assumptions about kinetics and effects. The starting point is a known chemical composition of the mixture and known effects of the individual compounds. A key point in an approach like this is that the amount of data needed to predict effects of mixtures is large, as for each individual compound in the mixture

☆ This study is based on data from the open literature and theoretical considerations. Effects of mixtures on survival are done by computer simulation.

* Corresponding author. Fax: +31 20 598 71 23.
E-mail addresses: Jan.Baas@falw.vu.nl (J. Baas), Tjalling@bio.vu.nl (T. Jager), Bas@bio.vu.nl (S.A.L.M. Kooijman).

0147-6513/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2008.09.003

parameters are needed to describe its effect. So a secondary aim of this study was to investigate the possibilities of data reduction, and thus a reduction in the experimental effort, by making use of theoretical relations between parameters and properties of compounds in a mixture.

2. Methods

First, we provide a short description of the survival model, starting with a single compound, then extending it to two compounds and then to any number of compounds. A detailed mathematical description of the many-compound model for survival can be found in Appendix A.

2.1. Short description of the survival model

2.1.1. Description of the model for a single compound

The model is based on internal concentrations and explicitly considers toxicokinetics and toxicodynamics. As internal concentrations are rarely known a one-compartment model is used to link internal and external concentrations. A hazard model is used to link internal concentration and survival. Depending on the application of the model and the availability of data on bio-transformation, pulsed exposure and different exposure routes could be included. In this paper, we assume a constant exposure bio-available concentration, one major exposure route and no bio-transformation. A more elaborate description of the model can be found elsewhere (Bedaux and Kooijman, 1994).

The model assumes that the hazard rate is proportional to the internal concentration once a threshold is exceeded. This leads to three parameters to describe the effect: the external concentration that corresponds to the internal threshold, the no-effect concentration (NEC). When the NEC is exceeded the effect is described by the killing rate (b). A higher killing rate implies that the compound of interest is more toxic. The dimension of b is environmental concentration^{-1} time^{-1}. The third parameter is the elimination rate (k_e). The k_e describes how fast the equilibrium between internal and external concentration is reached (dimension time^{-1}). Note that (in practice) all three parameters are independent of time.

These parameters, with an extra parameter to describe the mortality in the control, give the hazard rate for any point in time, which is integrated to obtain the survival probability. As the model describes the development of the toxic effects over the entire time course, measurements at intermediate time points are necessary to estimate parameters from the experimental data.

It should be stressed that the NEC is a concept very different from the no observed effect concentration (NOEC) that is often reported in literature. The NEC is by definition the concentration below which no effects occur, even when an organism is exposed over its entire lifetime. So the NEC is a real toxicological threshold, which might be zero. In contrast to the NEC, the NOEC is one of the concentrations in the dose range and is time dependent. In addition actual effects at the NOEC level can be quite large (e.g., Crane and Newman, 2000). The NOEC strongly depends on the experimental design (e.g., Laskowski, 1995) and the statistical test that is used (e.g., van der Hoeven et al., 1997). Moreover, in contrast to the NEC the NOEC does not have a confidence interval.

2.1.2. Extension of the model to more compounds

If an organism is exposed to more than one compound, different concepts are applied to describe mixture effects. Basically each compound in the mixture can exert its own effect and act independently of other compounds (compare IA). It is also possible that the compounds in the mixture have a similar working mechanism and therefore can act as dilutions of each other (compare CA). In the latter case each compound will contribute to the NEC. We proposed two different mechanisms or models to describe this, the irreversible binding model and the reversible binding model. In the irreversible binding model it is assumed that once the NEC is exceeded the fractions that the different compounds take in occupying the NEC are fixed. The idea behind this is that a compound acts on a receptor inside the organism. Once the receptor is occupied it stays that way. In the reversible binding model it is possible to allow the internal concentration of the different compounds to be exchanged, depending on the composition of the mixture. In this case there is a competition for occupation of the receptor by different compounds.

The principal question that had to be answered first, however, was if the hazard rate of more than one compound in a mixture could be combined in the total survival probability to describe a mixture effect. This proved to be the case for binary mixtures (Baas et al., 2007), where the models gave a very accurate description of survival data.

For a mixture containing more than two compounds, the number of possible interactions and with this the number of parameters, increases rapidly. In our approach, for a mixture of n compounds the number of parameters is: $1+3n+n(n-1)/2$; one parameter to describe the control mortality, $3n$ for the toxicological parameters for each compound and $n(n-1)/2$ for the interaction parameters. As it is impossible to take all possible interactions into account and it has been shown that interactions are not expected to play a major role in mixtures containing large numbers of toxicants at low doses (Carpy et al., 2000) or as synergistic and antagonistic effects are expected to cancel out (Warne and Hawker, 1995), we start from the assumption that no interactions take place in the derivation of the different many compound survival approaches. We derived three different expressions for the hazard rate, i.e. one for the reversible binding model, one for the irreversible binding model and one for the assumption that the NEC does not depend on the concentration of other compounds (see Appendix A for details). The derivation is based on Baas et al. (2007), where a mathematical description of the models for binary mixtures is given.

2.2. Application of the model

In principle the model can be applied to a mixture of any complexity, e.g. mixtures of narcotic compounds like PAHs, mixtures of PAHs combined with metals, or mixtures of specifically working toxicants like pesticides. To run the model for each compound in the mixture the toxicological parameters have to be estimated. This can be done for each of the compounds in the mixture independently, by exposing organisms to different concentrations of the individual compounds making up the mixture and measuring the effect on survival at different time points. Of course this is very labor intensive.

A very attractive application of the model is for mixtures of non-specific acting compounds, as relations between the properties of the compounds in the mixture and the toxicological parameters of the compound are expected (Kooijman et al., 2007). The NEC, killing rate and the elimination rate are expected to depend on the potential of a compound to accumulate in an organism's tissue, and the accumulation potential is expected to be related to the n-octanol–water partition coefficient (K_{ow}), which is known for a large number of compounds. These relations will be worked out in the next section.

If there is a strong relation between toxicological parameters and K_{ow} values, then in principle, effects of only one compound in the mixture have to be measured to predict the mixture effect. The toxicological parameters for other compounds in the mixture can then be derived from their K_{ow} values. In this way the number of parameters and hence the experimental effort needed to assess the effect of a mixture can be greatly reduced.

We will simulate the effects of a mixture of PAHs to illustrate this and then show how to model effects of mixtures containing more than one narcotic compound.

3. Relations between toxicity parameters and K_{ow}

3.1. Theoretical considerations

In the model it is assumed that the internal concentration is directly responsible for the effect. The K_{ow} value is taken as a starting point to act as a measure of bio-concentration of a compound (e.g. Mackay, 1982; Baussant et al., 2001). For non-specific acting chemicals the amount of effect solely depends on the number of molecules in an organism, irrespective of which chemical is in the organism (van Wezel and Opperhuizen, 1995). The difference in toxicity (expressed as external concentration) between different compounds is caused by their different bio-concentration, and thus the efficiency with which they are taken up and reach the target. A more hydrophobic chemical will thus have a lower NEC (less available external concentration is needed to reach the threshold of the target) and a higher killing rate (which is also expressed on the basis of external concentrations). Furthermore, the NEC and the killing rate will be inversely proportional; if compound A has a NEC two times less than that of compound B, compound A will have a killing rate twice as high as that of compound B. Because NEC and killing rate are both expressed on the basis of external concentrations, differences between compounds result in the same factor of difference for both parameters. For simple narcotics, this factor of difference between compounds is equal to the factor difference in membrane–water partition coefficient (which is for non-polar compounds generally close to the K_{ow}, see e.g., Escher and Hermens, 2002).

These compounds differ only in their toxicokinetics. The efficiency, with which they interact with the target, once they reach it, is the same for all compounds.

Also for other mechanisms of action, we expect to see a similar inverse proportionality between NEC and killing rate for compounds with the same mechanism of action. For different mechanisms of action, it is likely that the proportionality relations between NEC and killing rate are different.

If the assumption of a one-compartment model is valid, k_e is expected to be inversely proportional to the square root of K_{ow} (Kooijman et al., 2004, 2007). This follows from the skew-symmetry argument, namely that uptake and elimination are opposite processes at the same surface and from the assumption that at equilibrium the ratio of internal and external concentrations of the toxicant is proportional to K_{ow}.

In summary, it is expected that for narcotics NEC is inversely proportional to K_{ow}, b is proportional to K_{ow} and k_e is expected to be inversely proportional to the square root of K_{ow}. These theoretical relations will be tested against experimental data in the next section.

3.2. Testing the theoretical relations against data

3.2.1. Data

To test the theoretical dependences we need survival data, measured at different time points for different concentrations that allow an estimate of the toxicological parameters. In addition we need these data for a large number of compounds, with known K_{ow} values.

We used acute toxicity data for *Pimephales promelas*, which were taken from the original publications of the Center for Lake Superior Environmental Studies (Brooke et al., 1984; Geiger et al., 1985, 1986, 1988, 1990). These data result from 4-day tests with juvenile minnows (approx. 2 cm in length) at constant exposure (flow-through, generally five doses and a blank, exposure concentrations measured at several time points) and at a temperature around 25 °C. The specific experimental setup varies somewhat between tests, with a variable number of observations in time (generally 3–8), variable number of organisms per dose group (generally 10–100). What makes these publications special is that the raw survival data for each time point are reported (and not only the LC_{50} after 96 h). As such, they are directly useful for our model analysis. Exposure concentrations are measured and reported in these publications (we use the average measured values, corrected for recovery and translated to mmol L^{-1}).

Log K_{ow} values were taken from Epi Suite 3.12 (USEPA, 2004). Estimated values were used to provide consistency.

The one-compound model (in the form of DEBtox, Bedaux and Kooijman, 1994) was fitted to the raw survival data for the almost 300 available compounds. This yielded estimates of four parameters: NEC, killing rate, elimination rate and background hazard rate for each compound. Confidence intervals were generated using profile likelihoods, which are much more robust than the more familiar symmetric intervals resulting from asymptotic methods. Not in all cases, the DEBtox procedure was able to accurately identify all parameter values from the data. This was judged from the width of the confidence intervals. When the intervals spanned less than one order of magnitude, the estimate was considered to be of sufficient confidence for our purpose.

3.2.2. Results

In Fig. 1 the $^{10}\log$NEC (NEC in mmol L^{-1}) is plotted against $^{10}\log K_{ow}$. The NEC shows a very good correlation with $\log K_{ow}$, with a slope that is very close to the expected theoretical value of −1.

The killing rate (in mmol L^{-1} d^{-1}) shows a general increase with K_{ow} with a slope of 0.72 (see Fig. 2). The slope is close to the expected theoretical value of 1, but with much more scatter than with the NEC. This is partly caused by the fact that many of the data sets do not allow for an accurate identification of the killing rate. This would ask for smaller time increments than were used in the actual measurements. In Fig. 2 only estimates with a confidence interval (95% confidence) of less than a factor of 10 are included. For comparison, a typical confidence interval for NEC is less than a factor of 2.

The elimination rate is the most difficult parameter to accurately identify from the data. In addition to this, for high values of K_{ow} we should consider bias in the data set because of the limitations of the 4-d acute test. Suppose that we have narcotic compounds in the data set with a $\log K_{ow}$ between 5 and 6 that do adhere to the general relations. These compounds would have an elimination rate of 0.01 h^{-1} or less, which means that 95% of steady state is only achieved after 12 d. Further, these compounds have a NEC of 0.001 mmol L^{-1} or less. To see effects, we need to test chemicals at a level such that NEC is exceeded well within the 4 d of the test. However, between $\log K_{ow}$ of 5 and 6 the water solubility decreases from approximately 0.09 to 0.0006 mmol L^{-1} (using the regression equation based on K_{ow}, reported by Briggs (1981)). Exposure to a concentration well above NEC within 4 d may thus be impossible.

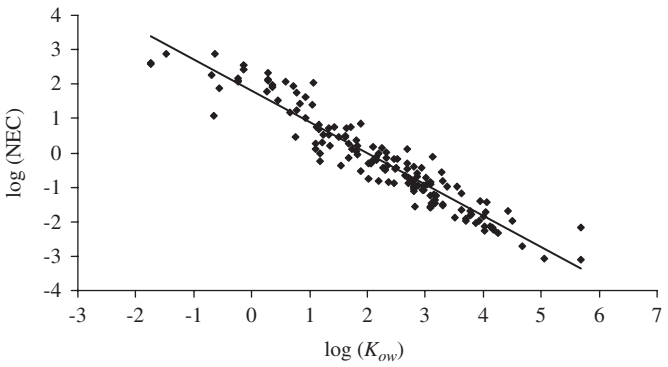

Fig. 1. Plot of log no-effect concentration (NEC) versus $\log K_{ow}$ (NEC in mmol L^{-1}). Best fitting line (microsoft excel) $\log(\text{NEC}) = -0.907 \log K_{ow} + 1.79$ ($R^2 = 0.904$) (see Section A.2.1 for further details about the NEC).

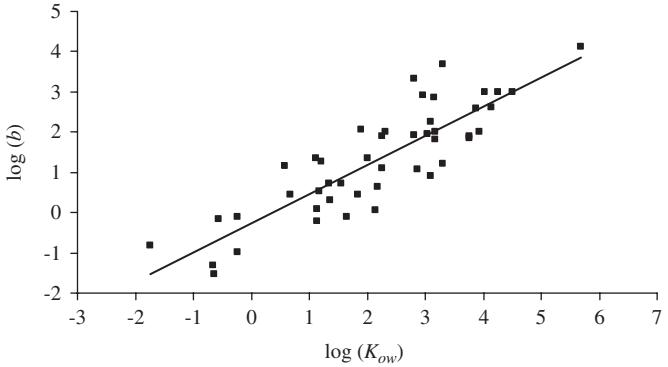

Fig. 2. Plot of log killing rate (b) versus $\log K_{ow}$ (b in mmol L^{-1} d^{-1}). Best fitting line (Microsoft Excel) $\log(b) = 0.72 \log K_{ow} - 0.26$ ($R^2 = 0.739$) (see Section A.2.1 for further details about the killing rate).

Therefore, we used elimination rates that were reported by De Voogt et al. (1991). These elimination rates are based on whole body residue data for guppy *Poecilia reticulata*. For narcotic compounds, whole body residue data are expected to reflect the mechanism of action.

The result is a relationship between $\log K_{ow}$ and elimination rate with a slope (−0.60) that is very close to the expected value of −0.5 (for $\log K_{ow}$ values between 1 and 6) (Fig. 3). This relation for the elimination rate, however, is based on different organisms under different circumstances. To estimate elimination rates for *P. promelas* we used the slope of this fit and derived the value of the intercept by using the elimination rate for naphthalene, which is part of the dataset on *P. promelas* (the k_e for naphthalene was estimated to be 5.5 d^{-1}). This leads to the following expression: $k_e = -0.6 \log K_{ow} + 1.76$.

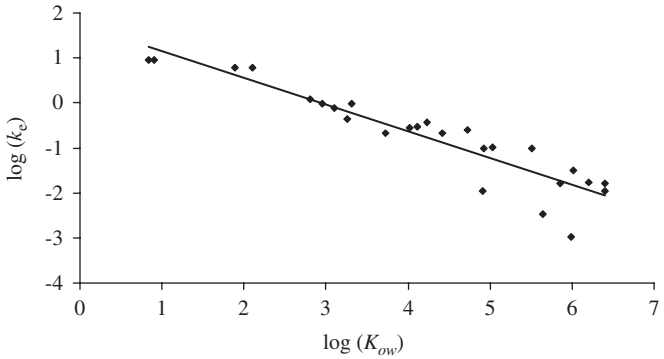

Fig. 3. Plot of log elimination rate (k_e) versus $\log K_{ow}$ (k_e in d^{-1}). Best fitting line (Microsoft Excel) $\log(k_e) = -0.60 \log K_{ow} + 1.76$ ($R^2 = 0.853$) (see Section A.2.1 for further details about the elimination rate).

Table 1
Molar mass, $\log K_{ow}$ and relative concentrations in Dutch sediments for the selected PAHs

PAH	Molar mass ($g\,mol^{-1}$)	Log K_{ow}[a]	Relative occurrence of PAHs
Phenanthrene	178.2	4.57	11.1
Anthracene	178.2	4.54	2.6
Fluoranthene	202.3	5.22	20.5
Benz[*a*]anthracene	228.3	5.91	9.1
Chrysene	228.3	5.86	9.1
Benz[*k*]fluoranthene	252.3	6.0	1.0
Benz[*a*]pyrene	252.3	6.04	4.3
Benzo[ghi]perylene	276.3	6.5	3.1
Naphthalene	128.2	3.37	3.2
Acenaphthene	154.2	3.98	1.1
Fluorene	166.2	4.18	1.4
Pyrene	202.3	5.18	14.8
Benzo[*b*]fluoranthene	252.3	5.8	10.9
DiBenzo[ah]anthracene	278.4	6.75	1.2

[a] Mackay et al. (1992).

4. Application of the model

4.1. Parameter estimates

As an example of the use of the model we derive a NEC for a mixture of 14 PAHs for *P. promelas*. For the relative occurrence of the PAHs we used the fingerprint that was found for 14 individual PAH in Dutch sediments (Jager et al., 2003) (see Table 1). There is no limitation to the number of compounds or the relative abundance of compounds in the mixture. We made this choice to show what we can do using the relations given in the previous section and the models.

4.1.1. Deriving toxicity parameters for the selected PAH

The relationships derived in Section 3 were used to derive values for NEC, killing rate and elimination rates for the compounds in the mixture, given the K_{ow} values of the individual PAH, assuming all PAHs have the same mode of action. The result is shown in Table 2.

Table 2 shows that for some of the PAHs water solubility is less than NEC for *P. promelas*. In these cases there will be no effect of an individual PAH on survival as the NEC cannot be exceeded.

Naphthalene and acenaphthene were taken up in the measurements from the Centre for Lake Superior Environmental Studies. So a comparison could be made between the predicted value and the parameter values directly derived from the experiment. The result of this is shown in Table 3.

It shows that we have quite a good resemblance between the results derived from theory and the results obtained by directly deriving data from the survival experiments.

4.2. The effect of a 14 PAH mixture on survival in time

With all toxicity parameters modeled for each individual compound in the mixture, simulations can be run. In the simulations PAHs were diluted by a constant factor, keeping the fingerprint constant. Simulations were run with the reversible

Table 2
Estimates of the no-effect concentration (NEC), killing rate (b) and elimination rate (k_e) based on theoretical relations between K_{ow} and the toxicological parameters for the individual PAHs for *Pimephales promelas*

Compound	$\log K_{ow}$	Water solubility (mM)	NEC (mM)	b ($mM^{-1}\,d^{-1}$)	k_e (d^{-1})
Phenanthrene	4.57	6.7E−3	4.4E−03	1.1E+03	1.0
Anthracene	4.54	4.3E−4	4.7E−03	1.0E+03	1.1
Fluoranthene	5.22	1.1E−3	1.1E−03	3.2E+03	0.42
Benz[*a*]anthracene	5.91	4.4E−5	2.6E−04	9.9E+03	0.16
Chrysene	5.86	1.2E−5	2.9E−04	9.1E+03	0.18
Benz[*k*]fluoranthene	6.0	3.0E−6	2.2E−04	1.1E+04	0.14
Benz[*a*]pyrene	6.04	9.1E−6	2.0E−04	1.2E+04	0.14
Benzo[ghi]perylene	6.5	9.4E−7	7.7E−05	2.6E+04	0.07
Naphthalene	3.37	2.3E−1	5.4E−02	1.5E+02	5.5
Acenaphthene	4.15	1.3E−2	1.1E−02	5.3E+02	1.9
Fluorene	4.18	1.1E−2	9.9E−03	5.6E+02	1.8
Pyrene	5.18	3.8E−4	1.2E−03	2.9E+03	0.45
Benzo[*b*]fluoranthene	5.8	4.8E−6	3.3E−04	8.2E+03	0.19
DiBenzo[ah]anthracene	6.75	1.8E−6	4.5E−05	4.0E+04	0.05

Table 3
Comparison of the NEC and the killing rate based on the theoretically derived relationships and those directly derived from the measurements

Compound	NEC (mM)		b ($mM^{-1}\,d^{-1}$)	
	Theoretically derived	Derived from experiments	Theoretically derived	Derived from measurements
Naphthalene	5.4E−2	4.4E−2	1.5E+2	1.0E+2
Acenaphthene	1.1E−2	6.9E−3	5.3E+2	4.2E+2

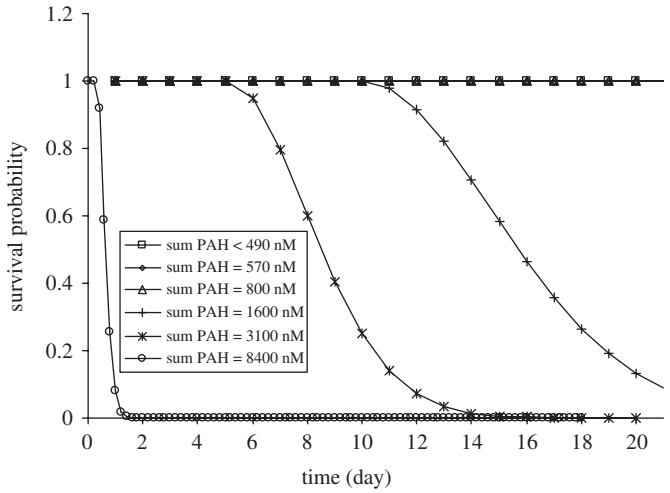

Fig. 4. Simulated effect if the compounds in the mixture compete for the no-effect concentration for a mixture of 14 PAHs with a fingerprint as shown in Table 1 on the survival of *Pimephales promelas*.

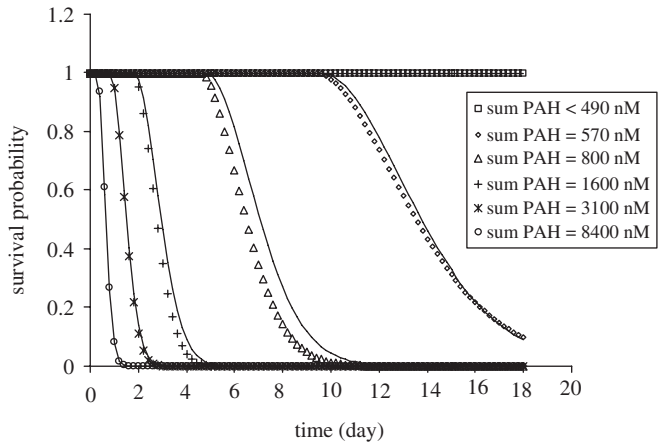

Fig. 5. Comparison of the effect of the 14 compound PAH mixture built up from its individual compounds with a competition for the no-effect concentration (lines) and the mixture effect approximated as if exposure was to one aggregated compound for *Pimephales promelas* (symbols).

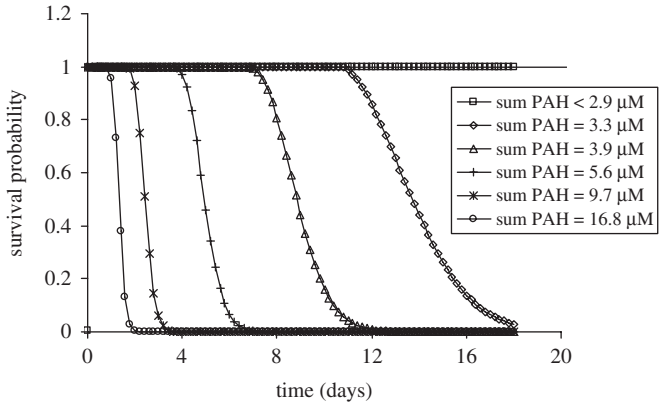

Fig. 6. Simulated effect if each compound in the mixture has its own no-effect concentration, independent of all other compounds for *Pimephales promelas* exposed to 14 individual PAH with the fingerprint as shown in Table 1.

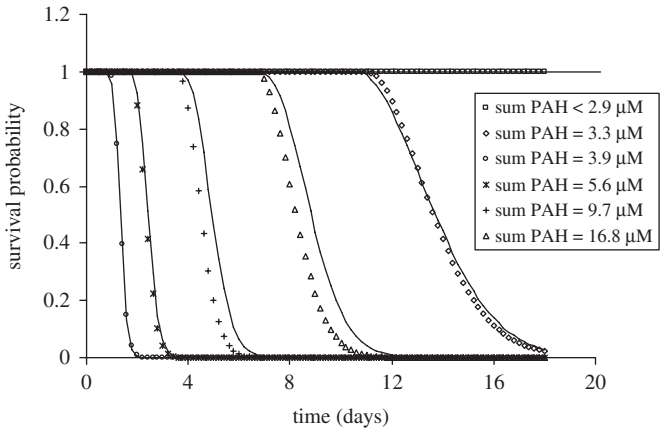

Fig. 7. Comparison of the effect of the 14 compound PAH mixture built up from its individual compounds with each compound having its own individual no-effect concentration (lines) and the mixture effect approximated as if exposure was to one aggregated compound for *Pimephales promelas* (symbols).

binding model, where the toxicants act additive and with the IA model, where each compound has its own effect.

4.2.1. Additive model

An example of a simulated survival experiment using the models where toxicants act as dilutions from one another for the 14 PAH is shown in Fig. 4.

A water concentration where NEC for *P. promelas* is not exceeded is 490 nM of total PAH with the fingerprint as given in Table 1. The exact composition of the PAH mixture, however, is not expected to have a large effect on NEC if the number of compounds in the mixture is high and K_{ow} values are relatively close. However, the more components in the mixture, the lower the NEC of the mixture. The mixture effect can also be approximated as the effect exerted by one aggregated compound in a top-down approach (see Fig. 5). This aggregated compound has a NEC, b and k_e of respectively, 0.49 μM, 6.2E+3 $mM^{-1}\,d^{-1}$ and 0.21 d^{-1}.

4.2.2. Independent model

If the compounds in the mixture act independently, the simulated survival as a result of the 14 PAH mixture is shown in Fig. 6.

The NEC of the mixture is the first NEC of any of the individual compounds that is exceeded, but concentrations of compounds

where NEC is not yet exceeded do add to total concentration. This leads to a NEC of the mixture of 2.9 μM. Also here the mixture effect can be approximated as exerted by one aggregated compound, with NEC, b and k_e of respectively, 2.9 μM, 3300 $mM^{-1}\,d^{-1}$ and 0.20 d^{-1}, as shown in Fig. 7.

5. General discussion

5.1. Comparing the different models

In general the independent model predicts a lower effect for the same concentrations than the additive model, though the plot itself looks similar (compare classical CA and IA). The water concentration where NEC is not exceeded is 2.9 μM for the independent model and 490 nM for the additive model.

If the toxicants act on the same target, as can be expected for a mixture of PAH, it was shown that all compounds contribute to the effect (e.g. Deneer et al., 1988; Grimme et al., 2000; Landrum et al., 2003). Therefore, the reversible binding model is the model that is most likely to be the appropriate one to use in cases like this.

For nine individual PAHs the maximum allowable concentration for surface water in the Netherlands (based on Kalf et al., 1997) could be compared with their NECs. The maximum allowable concentrations for the individual PAH are much lower than the individual NECs and therefore the maximum allowable concentrations give adequate protection against effects on survival for *P. promelas*. But a comparison of the maximum allowable concentrations for the PAH in the mixture at the NEC level gives a different view (see Table 4). Due to the combination effect of the 14 PAHs in the mixture the maximum allowable concentration still gives protection, but the margin of safety is much smaller for *P. promelas*. With a mixture containing more than 14 PAHs (as will occur in real life) effects at the level of maximum allowable concentration are possible.

5.2. Other mixtures

The model is not limited to either mixtures of narcotics or mixtures of independently acting compounds. There is no fundamental restriction in the type of mixture effects that can be calculated, though a choice in working mechanism has to be made, compare CA and IA.

The results also suggest that a mixture of (e.g.) some metals and a mixture of PAH can be approximated as a mixture containing one aggregated PAH and some metals, thereby reducing the theoretical and practical complexity of the mixture.

Table 4
Maximum allowable concentrations of individual PAHs in surface water and the no effect concentration (NEC) of individual PAH and the NEC of individual PAH when the no effect concentration of the mixture is reached *Pimephales promelas*

Compound	Maximum allowable concentration (nM)	NEC of the individual PAH (nM)	Concentration of individual PAH at the NEC for the mixture (nM)
Phenanthrene	1.68	4400	68
Anthracene	0.39	4700	16
Fluoranthene	1.48	1100	110
Benz[*a*]anthracene	0.04	260	43
Chrysene	1.31	290	43
Benz[*k*]fluoranthene	0.16	220	4
Benz[*a*]pyrene	0.20	200	19
Benzo[ghi]perylene	0.10	77	13
Naphthalene	9.36	5400	27

An elegant application of the model is in the so-called whole effluent applications. If the chemical constitution of the mixture is unknown or partly known, and effects are tested with the mixture as it occurs in the field, it is possible to predict effects from the compounds for which the concentration is known. If this is (close to) the measured effect there is no effort needed to further investigate the chemical constitution of the mixture. If only a (small) part of the measured effect is predicted, further attention to the chemical composition of the mixture is needed to find the compounds that are responsible for the measured effect. By simulations it is possible to determine the number of relevant compounds (or compound classes) that determine the effect (as seen in practice) of an unknown mixture. With this approach more information about the source(s) of pollution can be obtained.

6. Conclusions

A model was developed that predicts effects of mixtures on survival at any point in time with any number of compounds, assuming there are no interactions. The model works in a bottom-up approach, where the effect of the mixture is built up from the effect of the individual compounds.

An approach like this requires that the parameters that describe the toxic effect of the individual compounds are known and therefore an approach like this is labor intensive. For narcotics we showed that the theoretical relations between toxic parameters and affinity for a compound to be bio-concentrated (approximated with the $\log K_{ow}$ value of the compound) are in very close agreement with the relations that could be derived from data available in the open literature. This allows for a vast reduction in labor intensity of the assessment of effects of mixtures in an approach like this.

We performed simulations with a known mixture of 14 individual PAHs and predicted the NEC for *P. promelas* of 490 nM. A comparison with the maximum allowable concentration for individual PAHs in surface water shows that NEC of individual PAHs for *P. promelas* does not exceed the margin of safety. However, this decreases dramatically due to the combined effect of the compounds in the mixture. For a mixture containing more PAHs (as can be expected for PAHs) we do expect to find effects at concentrations as low as the maximum allowable concentration in surface water.

The model allows interesting possibilities in assessing effects of whole effluent applications in a combined bottom-up and top-down approach in evaluating mixture effects.

Acknowledgments

The study was supported by the EU Integrated project NoMiracle (Novel Methods for Integrated Risk assessment of Cumulative Stressors in Europe; http://nomiracle.jrc.it) contract no. 003956 under the EU-theme "Global Change and Ecosystems", topic "Development of risk assessment methodologies", coordinated by Dr. Hans Løkke at NERI, DK-8600 Silkeborg, Denmark.

Appendix A. Many-compound mixture

The basic idea behind the many models is that the organism is supposed to be able to cancel effects of (a mixture of) compounds with some limited capacity. For exposure to a single compound this is the NEC. In the case of a complex mixture it is somewhat unnatural to speak of a NEC. We prefer to speak of the effect-canceling capacity of an organism.

We propose three different ways to cancel effects: reversible binding, irreversible binding, and independent NEC (see also Baas et al., 2007). We start with the conceptually most straightforward case, the reversible binding, and then consider numerically more friendly alternatives. All models are based on internal concentrations, which are converted to external concentrations by a one-compartment model, assuming external concentrations are constant and bio-available, that bio-transformation does not play a role in the time where toxic effects build up and that there has been no previous exposure. Note that bio-transformation can also be modeled (at the cost of additional parameters) and that reliable data to enable this are usually lacking.

A.1. Notation

B_i	internal killing rate for compound i, C-mol mmol^{-1} d^{-1}
b_i	external killing rate for compound i, mM^{-1} d^{-1}
c_i	external concentrations for compound i, mM
C_i	external concentration used to describe time effects, mM
C_i^0	external no-effect concentration (NEC) for compound i, mM
h_0	hazard rate in the blank, d^{-1}
h_c	hazard rate caused by toxicant, d^{-1}
k_i	elimination rate for compound i, d^{-1}
n	the number of compounds in the mixture
P_{id}	bio-concentration factor for compound i, l C-mol^{-1}
Q_i^0	internal NEC for compound i, mmol C-mol^{-1}
Q_i	internal concentration for compound i, mmol C-mol^{-1}
$S(t)$	survival probability
t	time, d
t_0	point in time where the mixture-NEC is exceeded, d
w_i	fraction of compound i in the NEC

A.2. Reversible binding model

A.2.1. Calculation of the hazard rate

It is assumed that all compounds in the mixture compete for the capacity to cancel effects and that Q_i^0 is the internal NEC for compound i. No effects occur if (compare CA):

$$1 > \sum_{i=1}^{n} Q_i/Q_i^0$$

If this condition is not fulfilled, compound i takes fraction (w_i) of the effect canceling capacity:

$$w_i = Q_i/Q_i^0 \left(\sum_{i=1}^{n} Q_i/Q_i^0 \right)^{-1}$$

The internal concentrations that cause effect are

$$Q_i^e = \max(0, Q_i - w_i Q_i^0)$$

Notice that the internal concentrations Q_i change in time. So the concentrations that do not cause effects change in time, because the different compounds compete for the effect-canceling capacity. The internal concentrations and the hazard rate are given by

$$Q_i(t) = c_i P_{id}(1 - \exp(-tk_i))$$

$$h_c(t) = \sum_{i=1}^{n} B_i Q_i^e(t)$$

A.2.2. From internal to external concentrations

The internal concentrations typically are not known. So we define a concentration that is proportional to the internal concentration, but has the dimension of an external concentration, using the bio-concentration factor P_{id}:

$$\text{Substitute}: \quad C_i = Q_i/P_{id}, \quad b_i = B_i P_{id}$$

$$C_i(t) = c_i(1 - \exp(-tk_i))$$

$$w_i = C_i/C_i^0 \left(\sum_{i=1}^{n} C_i/C_i^0 \right)^{-1}$$

$$C_i^e = \max(0, C_i - w_i C_i^0)$$

$$h_c(t) = \sum_{i=1}^{n} b_i C_i^e(t)$$

We now have the hazard rate expressed in external concentrations. The complete hazard rate is given by $h(t) = h_0 + h_c(t)$, where h_0 is the blank hazard rate.

Effects occur from $t > t_0$, when the effect-canceling capacity is exceeded:

$$1 < \sum_{i=1}^{n} c_i/C_i^0$$

The value of t_0 must be obtained numerically from

$$1 = \sum_{i=1}^{n} C_i(t_0)/C_i^0 = \sum_{i=1}^{n} (1 - \exp(-t_0 k_i))(c_i/C_i^0)$$

A.2.3. From hazard rate to survival probability

The survival probability is given by

$$S(t) = \exp\left(- \int_0^t h(s)\,ds \right)$$

For $t < t_0$ the survival probability equals that of the blank

$$t < t_0: \quad S(t) = \exp(-h_0 t)$$

For $t > t_0$ integration is done numerically.

This formulation allows changes in the use of the canceling capacity after the moment effects show up. Alternatively the use of the effect canceling capacity can be frozen right after the moment effects show up. This is described in the following section.

A.3. The irreversible binding model

Alternatively the capacity to cancel effects can be frozen at the moment effects show up. Biologically this could be seen as an irreversible binding of the toxicant to a receptor. Numerically this is much simpler than the reversible binding model. Once the no-effect time is exceeded, the no-effect thresholds for the internal concentrations remain fixed, irrespective of the varying internal concentrations. When the effect-canceling capacity is frozen once effects show up, we have constant values for

$$C_i^0 = (1 - \exp(-t_0 k_i))c_i$$

$$w_i = (1 - \exp(t_0 k_i))c_i/C_i^0$$

This means that the survival probability can now be obtained analytically (given the numerically obtained value for t_0):

$$S(t) = \exp\left(-h_0 t - \sum_{i=1}^{n} b_i g_i(t) \right)$$

$$g_i(t) = -c_i t_i + (c_i - C_i^0)(t - t_0)$$

$$t_i = k_i^{-1}(\exp(-k_i t_0) - \exp(-k_i t))$$

A.4. The independent NEC model

A third alternative is to use the individual NEC values for each of the components in the mixture. These values do not depend on concentration of other compounds. The no-effect time is given by

$$t_0^i = -\log(1 - C_i^0/c_i)/k_i$$

$$t_0 = \max(t_0^i)$$

Expressions for $S(t)$, $g_n(t)$ and t_n are the same as for the irreversible binding model.

A.5. Programming

All programming has been done in Octave free available software (see http://www.octave.org/).

References

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., Grimme, L.H., 2000. Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: mixtures composed of similarly acting chemicals. Environ. Toxicol. Chem. 19, 2341–2347.

Andersen, M.E., Dennison, J.E., 2004. Mechanistic approaches for mixture risk assessment—present capabilities with simple mixtures and future directions. Environ. Toxicol. Pharmacol. 16, 1–11.

Ashauer, R., Boxall, A., Brown, C., 2006. Predicting effects on aquatic organisms from fluctuating or pulsed exposure to pesticides. Environ. Toxicol. Chem. 25, 1899–1912.

Baas, J., van Houte, B.P.P., van Gestel, C.A.M., Kooijman, S.A.L.M., 2007. Modeling the effects of binary mixtures on survival in time. Environ. Toxicol. Chem. 26, 1320–1327.

Backhaus, T., Altenburger, R., Boedeker, W., Faust, M., Scholze, M., Grimme, L.H., 2000. Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*. Environ. Toxicol. Chem. 19, 2348–2356.

Baussant, T., Sanni, S., Skadsheim, A., Jonsson, G., Borseth, J.F., Gaudebert, B., 2001. Bioaccumulation of polycyclic aromatic compounds: 2. Modeling, bioaccumulation in marine organisms chronically exposed to dispersed oil. Environ. Toxicol. Chem. 20, 1185–1195.

Bedaux, J.J.M., Kooijman, S.A.L.M., 1994. Statistical analysis of bioassays, based on hazard modeling. Environ. Ecol. Stat. 1, 303–314.

Briggs, G.G., 1981. Theoretical and experimental relationships between soil adsorption, octanol–water partition coefficients, water solubilities, bioconcentration factors, and the parachor. J. Agric. Food Chem. 29, 1050–1059.

Broderius, S.J., Kahl, M.D., 1985. Acute toxicity of organic chemical mixtures to the fathead minnow. Aquat. Toxicol. 6, 307–322.

Brooke, L.T., Call, D.J., Geiger, D.L., Northcott, C.E., 1984. Acute Toxicities of Organic Chemicals to Fathead Minnow (*Pimephales promelas*), vol. I. Center for Lake Superior Environmental Studies, University of Wisconsin-Superior, Superior, WI, USA.

Carpy, S.A., Kobel, W., Doe, J., 2000. Health risk of low-dose pesticides mixtures: a review of the 1985–1998 literature on combination toxicology and health risk assessment. J. Toxicol. Environ. Health Pt. B 1, 1–25.

Cedergreen, N., Streibig, C., 2005. Can the choice of endpoint lead to contradictory results of mixture-toxicity experiments? Environ. Toxicol. Chem. 24, 1676–1683.

Crane, M., Newman, M.C., 2000. What level of effect is a no observed effect? Environ. Toxicol. Chem. 19, 516–519.

Deneer, J.W., Sinnige, T.L., Seinen, W., Hermens, J.L.M., 1988. The joint acute toxicity to *Daphnia magna* of industrial organic chemicals at low concentrations. Aquat. Toxicol. 12, 33–38.

de Voogt, P., van Hattum, B., Leonards, P., Klamer, J.C., Govers, H., 1991. Bioconcentration of polycyclic hydrocarbons in the Guppy (*Poecilia reticulata*). Aquat. Toxicol. 20, 169–194.

Escher, B.I., Hermens, J.L.M., 2002. Modes of action in ecotoxicology: their role in body burdens, species sensitivity, QSARs, and mixture effects. Environ. Sci. Technol. 36, 4201–4217.

Geiger, D.L., Northcott, C.E., Call, D.J., Brooke, L.T., 1985. Acute Toxicities of Organic Chemicals to Fathead Minnows (*Pimephales promelas*), vol. II. University of Wisconsin-Superior, Superior, WI, USA.

Geiger, D.L., Poirier, S.H., Brooke, L.T., Call, D.J., 1986. Acute Toxicities of Organic Chemicals to Fathead Minnow (*Pimephales promelas*), vol. III. Center for Lake Superior Environmental Studies, University of Wisconsin-Superior, Superior, WI, USA.

Geiger, D.L., Call, D.J., Brooke, L.T., 1988. Acute Toxicities of Organic Chemicals to Fathead Minnow (*Pimephales promelas*), vol. IV. Center for Lake Superior Environmental Studies, University of Wisconsin-Superior, Superior, WI, USA.

Geiger, D.L., Brooke, L.T., Call, D.J., 1990. Acute Toxicities of Organic Chemicals to Fathead Minnow (*Pimephales promelas*), vol. V. Center for Lake Superior Environmental Studies, University of Wisconsin-Superior, Superior, WI, USA.

Grimme, L.H., Altenburger, R., Backhaus, T., Faust, M., Bödecker, W., Scholze, M., 2000. Kombinationswirkungen von Umweltchemikalien in der Ökotoxikologie. Z. Umweltchem. Ökotox. 12, 226–234.

Jager, D.T., Baerselman, R., Dijkman, E., de Groot, A., de Hogendoorn, E., de Jong, A., de Kruitbosch, J., Pijnenburg, W., 2003. Availability of polycyclic aromatic hydrocarbons to earthworms (*Eisenia adrei*, Oligochaeta) in field polluted soils and soil–sediment mixtures. Environ. Toxicol. Chem. 22, 767–775.

Jonker, M.J., Svendsen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J.E., 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose–response analysis. Environ. Toxicol. Chem. 24, 2701–2713.

Kalf, D.F., Crommentuijn, T., van de Plassche, E.J., 1997. Environmental quality objectives for 10 polycyclic aromatic hydrocarbons (PAHs). Ecotoxicol. Environ. Saf. 36, 89–97.

Kooijman, S.A.L.M., Jager, T.D., Kooi, B.W., 2004. The relationship between elimination rates and partition coefficients. Chemosphere 57, 745–753.

Kooijman, S.A.L.M., Baas, J., Bontje, D., Broerse, M., Jager, T., van Gestel, C.A.M., van Hattum, B., 2007. Scaling relationships based on partition coefficients and body sizes have similarities and interactions. SAR QSAR Environ. Res. 18, 315–330.

Landrum, P.F., Lotufo, G.R., Gossiaux, D.C., Gedeon, M.L., Lee, J.H., 2003. Bioaccumulation and critical body residue of PAHs in the amphipod, *Diporeia* spp.: additional evidence to support toxicity additivity for PAH mixtures. Chemosphere 51, 481–489.

Laskowski, R., 1995. Some good reasons to ban the use of NOEC, LOEC and related concepts in ecotoxicology. OIKOS 73, 140–144.

Mackay, D., 1982. Correlation of bioconcentration factors. Environ. Sci. Technol. 16, 274–278.

Mackay, D., Shiu, W.Y., Ma, K.C., 1992. Illustrated Handbook of Physical–Chemical Properties and Environmental Fate for Organic Chemicals, vol. II. Lewis, Boca Raton.

Nirmalakhandan, N., Xu, S., Trevizo, C., Brennan, R., Peace, J., 1997. Additivity in microbial toxicity of nonuniform mixtures of organic chemicals. Ecotoxicol. Environ. Saf. 37, 97–102.

Silva, E., Rajapakse, N., Kortenkamp, A., 2002. Something from "nothing"—eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ. Sci. Technol. 36, 1751–1756.

USEPA, 2004. EPI Suite, version 3.12.

van der Hoeven, N., Noppert, F., Leopold, A., 1997. How to measure no effect. Part I: towards a new measure of chronic toxicity in ecotoxicology. Introduction and workshop results. Environmetrics 8, 241–248.

van Wezel, A.P., Opperhuizen, A., 1995. Narcosis due to environmental-pollutants in aquatic organisms—residue-based toxicity, mechanisms, and membrane burdens. Crit. Rev. Toxicol. 25, 255–279.

Warne, M.S.J., Hawker, D.W., 1995. The number of components in a mixture determines whether synergistic and antagonistic or additive toxicity predominate—the funnel hypothesis. Ecotoxicol. Environ. Saf. 31, 23–28.

Yang, R.S.H., Thomas, R.S., Gustafson, D.L., Campain, J., Benjamin, S.A., Verhaar, H.J.M., Mumtaz, M.M., 1998. Approaches to developing alternative and predictive toxicology based on PBPK/PD and QSAR modeling. Environ. Health Perspect. 106, 1385–1393.