

Prediction of Daphnid Survival after in Situ Exposure to Complex Mixtures

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Received April 9, 2009. Revised manuscript received May 25, 2009. Accepted June 23, 2009.

We applied a mechanistically based model to predict the effects of complex mixtures as occurring in the field on the survival of *Daphnia magna*. We validated the model by comparing predicted survival with observed survival of in situ exposed laboratory cultured daphnids to polluted surface waters, in which over 90 chemical contaminants were measured. Using the chemical composition of the surface water at each sampling site, we calculated whether or not any of the individual or shared no effect concentrations were exceeded. If they were, we calculated the effect on survival. In 34 out of 37 cases (92%) we correctly predicted daphnid survival in surface waters. In the case of mortality we could also appoint the compound or group of compounds causing the effect. It is concluded that the proposed mechanistically based model accurately predicts effects on daphnids in the field, given the chemical composition of the water. Hence it is a powerful tool to link the chemical and ecological status of surface waters.

1. Introduction

In contaminated ecosystems, organisms are often exposed to mixtures of numerous chemicals originating from industrial, agricultural, and domestic activities. Hence, there is an increasing concern about the possibility that exposure to low concentrations of multicomponent mixtures may induce large unexpected effects (1–4). Yet, toxicity studies generally focus on single compounds and especially the effect of complex mixtures as occurring in the field is scarcely studied.

Different models have been developed for predicting mixture toxicity. The most common models are concentration addition (CA) (5, 6) and independent action (IA) (7). CA is used to predict the effect of substances with a common target site and similar modes of action. The effects of substances with dissimilar mode of action and different target sites are described by the concept of IA. An elaborate description of

these approaches can be found elsewhere (e.g., refs 1, 8–10). Both models are descriptive in nature and lack a strong mechanistic basis, and consequently are not suitable to extrapolate to other points in time, to other organisms, to other compounds or to fluctuating concentrations. Hence there is a need for more mechanistically based methods to predict the toxic effect of mixtures. Such a mechanistically based model was introduced by Baas et al. (11) and showed a very strong predictive power for effects of binary mixtures using only a few parameters. This model was expanded to cope with multicomponent mixtures (2), showing the potential to predict the effect of a mixture of any number of narcotic compounds on survival. The aim of the present study was to expand this approach further to complex mixtures consisting of a large number of compounds with different modes of action as occurring in the field. To validate the expanded model we predicted survival of *Daphnia magna* after one week of in situ exposure to complexly polluted surface waters and compared the model predictions with the observed survival. If successful this approach allows us to link the chemical and the ecological status of water bodies.

2. Materials and Methods

2.1. In Situ Exposure of Daphnids. In situ exposure of daphnids was carried out by the “Delfland Water Board”. This is one of the 27 regional water authorities in The Netherlands with the key tasks of maintenance of dikes and dunes and water level and water quality control. The Delfland region is situated between The Hague and Rotterdam and includes an area called “het Westland”, an agricultural area of about 80 km², of which 70% is covered by greenhouses. Due to the absence of sewage treatment, this area is contaminated with heavy metals, pesticides, nutrients, minerals, etc. But there are also more rural areas in the same region where the surface water is much less contaminated.

The Delfland waterboard regularly takes water samples at 20 different sites in this region. The sampling sites were selected to give a representative view of the water quality, the contamination caused by the agricultural activities and how the contaminants spread through the area. In addition three reference sites were chosen that were not influenced by agricultural activities. Monthly chemical analysis of water samples is performed and between May and October laboratory cultured 10 day old *Daphnia magna* were in situ exposed at 17 different sampling sites. An elaborate description of the monitoring network can be found in ref 12 and an elaborate description of the in situ exposure of the daphnids can be found in ref 13.

In short, the daphnids were exposed in cohorts of 10 individuals, in stainless steel jars of about 1 L with a double gauze cover. The jars float just below the water surface and the design of the cover allows fresh surface water to enter the jars, keeps the daphnids inside the jars and keeps debris and predators outside. At most sites one jar with daphnids was exposed, at some sites this was done in duplo. Survival of the in situ exposed *D. magna* was monitored after one week of exposure. The flow of the surface waters is carefully controlled by the Delfland Waterboard, during sampling most waters (about 75%) were stagnant.

Survival data for *D. magna* are available from 1990 to 2007. In general the surface waters became less polluted over this period and consequently *D. magna* mortality decreased. On the other hand the number of contaminants, taken up in the chemical analysis increased over the years. We chose the year 2000 as a case study, as there were still several sites showing (total) mortality, while the increased number of

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measured compounds allowed for an evaluation of the predictive power of the model.

2.2. Chemical Analysis. Oxygen concentration, pH, temperature and visibility were determined in the field. Water samples for chemical analysis were taken and conservation of the samples occurred in the field following ISO 5667-3 (14). Samples were cooled to 7 °C and stored in the dark. The concentration of over 90 different contaminants was measured: biphenyls, PAHs, heavy metals, pesticides, and minerals (see Supporting Information (SI)). The choice of the compounds that were taken up in the measurements was largely based on land use. But also some priority compounds from the Dutch Government, like the PAHs, were taken up in the measurements.

Metals were extracted with aqua regia and analyzed with inductively coupled plasma atomic emission spectroscopy. PAH were liquid/liquid extracted and analyzed with high performance liquid chromatography and fluorescence detection. The organophosphorous pesticides were analyzed using gas chromatography with mass spectrometry. The carbamates and the phenyl urea herbicides were analyzed using liquid chromatography and mass spectrometry. Ammonium (15), chloride (16), nitrate and nitrite (17), ortho phosphate (18), Kjeldahl nitrogen (19) and total phosphate (20) were analyzed photochemically. Not all compounds were always measured at all locations. A complete list can be found in the SI, and an elaborate description of the measurements can be found in ref 12.

2.3. Modeling Daphnid Survival. **2.3.1. Model Description.** The model consists of two parts, a one compartment model to link external concentrations to internal concentrations and a hazard model to link internal concentrations to survival. For each compound three parameters are needed to describe its toxic effects: the no effect concentration (NEC, unit μM), the killing rate (b_k , unit $\mu\text{M}^{-1}\text{hr}^{-1}$) and the elimination rate (k_e , unit hr^{-1}). The killing rate is a measure for the toxicity of the compound of interest, the higher the more toxic the compound (once the NEC is exceeded). The elimination rate determines how fast the equilibrium between internal and external concentrations is achieved. With these three parameters and a blank hazard rate to correct for control mortality a complete description of the effect of a single compound on survival at any moment in time can be calculated (21).

A key feature of the model is the NEC; by definition the NEC is the concentration below which no effects occur even after prolonged exposure. Once the NEC is exceeded there will be an effect on survival. We showed that compounds with the same mode of action in a mixture contribute to a single NEC (similar to the classical idea of CA where different compounds can be seen as dilutions from one another). So for similar acting compounds we assume that all compounds contribute to one single NEC and dissimilar acting compounds all have their own independent NEC. An elaborate description of the mixture model can be found in refs 2, 11.

2.3.2. Estimating Toxicity Parameters. The toxicity parameters of a specific compound can be obtained from different sources. The most reliable estimates are obtained if raw data on survival at different points in time at different concentration levels are available, then the toxicity parameters can be derived directly from measurements as was described by ref 21.

If these survival data are not available the toxicity parameters can be derived from data about the course of the LC_{50} values over time. The LC_{50} values at known points in time are then used to obtain the toxicity parameters by setting the survival probability (S) to 0.5 as a function of time and concentration (21). The starting value of the NEC in the estimates was obtained by fitting a curve described by

function 1 through the LC_{50} values measured at different points in time (22).

$$\text{LC}_{50,t} = \text{LC}_{50,\infty} / (1 - e^{-kt}) \quad (1)$$

The $\text{LC}_{50,\infty}$ is the infinitive LC_{50} value, which has the same numerical value as the NEC (23).

For some compounds there were no LC_{50} values available at different points in time, only a 48 h LC_{50} value. In these cases the NEC was estimated from a direct comparison of LC_{50} values with a related compound, as in eq 2.

$$\text{NEC}_a = (\text{LC}_{50,a} / \text{LC}_{50,b}) \text{NEC}_b \quad (2)$$

where NEC_a is the unknown NEC of compound a, NEC_b is the known NEC of compound b, $\text{LC}_{50,a}$ is the known LC_{50} value of compound a, and $\text{LC}_{50,b}$ is the known LC_{50} value of compound b.

The elimination rate was based on a comparison of log K_{ow} values for related compounds (as was described in ref 2). If no K_{ow} values were available the elimination rate was estimated by comparing the LC_{50} values in the same way as for the NEC. All LC_{50} values were taken from the PAN database (24) unless noted otherwise.

The environmental parameters pH and oxygen had a potential effect on survival of the daphnids. Especially in cases of high pH or low oxygen synergistic effects are expected (25, 26). A pH of 9.5 is considered to be a reasonable threshold for daphnid survival (27). The oxygen content in the surface waters can be very low. Daphnids are known to be able to survive under low oxygen conditions, but a minimum measured oxygen concentration of 2 mg/L is considered reasonable. In literature a minimum value of ca. 1 mg/L is given (28), but it is known that daphnids kept under low food, relatively high temperatures and toxic stress require more oxygen (29). In addition the oxygen concentration is expected to be lower at night than during the day when the measurements took place.

2.3.3. Predicting Daphnid Survival. For the exceedence of a NEC of a mixture we distinguished different groups of compounds, based on their mode of action. For pesticides we only considered the specific mode of action and assumed the contribution to the narcotic effects negligible.

For binary metal mixtures the best way to describe the effect on survival was to share the NEC (11). We distinguished the following groups of compounds sharing a NEC: PAHs and biphenyls with a narcotic mode of action (30), organophosphorous insecticides, inhibiting acetyl-choline esterases, and metals. All other compounds were assumed to have their own individual NEC or threshold (pH and oxygen content). Then using the chemical composition of the surface water at each sampling site we calculated whether or not any of the individual or shared NECs were exceeded and subsequently calculated the effect on survival after the exposure period of 1 week.

3. Results

3.1. In Situ Exposure of the Daphnids. In total 104 data sets on daphnid survival, were available, measured at 17 different locations. Of these data sets 31 showed complete mortality, 38 showed partial effects, and 35 showed no effects. In data sets with complete survival or with no survival the replicates showed very little variability (less than 10%), but data sets with partial effects showed such a large variation that the actual effect could not be determined. Consequently any model prediction would fall within the range of the observed mortality and therefore these data sets were discarded from further analysis.

From the 66 data sets with either complete survival or complete mortality, we could use 37 data sets. In the other

29 cases the metals and the PAHs and some pesticides were not measured and therefore these data sets were excluded from further analysis. Of the 37 data sets left, 20 showed complete survival, and 17 showed complete mortality.

3.2. Modeling Daphnid Survival. *3.2.1. Estimating Toxicity Parameters.* If the detection limit of the chemical analysis for a specific compound was never exceeded the compound was discarded from the effect analysis and no toxicity parameters were calculated. If the measured concentration exceeded the detection limit at least once, the detection limit was taken as the actual measured concentration in the cases where it was not exceeded. In the SI a complete overview of compounds that were taken up in the measurements and in the survival analysis is given.

Only for a small number of metals survival data at different points in time for different concentrations were available. For all other compounds the toxicity parameters were derived either from the course of the LC_{50} values in time or by comparison with related compounds within the same group. Below the parameter estimates for the different groups are presented.

Direct data for the effects of PAHs on daphnids were not available. For phenanthrene LC_{50} values at different time points were used to derive the toxicity parameters. The parameters of all other compounds were derived on the basis of their K_{ow} values, see eq 2. A table of the toxicity parameters for the PAHs is given in the SI. The concentration of biphenyls was never above the detection limit of the method of analysis ($0.005 \mu\text{g/L}$ or $\pm 0.02 \text{ nM}$). Therefore the biphenyls were not incorporated in the effect analysis and no toxicity parameters were derived.

For copper, cadmium, zinc, and nickel the toxicity parameters were derived directly from exposure data on daphnid survival carried out in our own laboratories. For lead data on LC_{50} values at different points in time were used to estimate the toxicity parameters. For chromium and mercury no LC_{50} values at different points in time were available. For arsenic only two LC_{50} values at different time points were available. Following the procedure described in section 2.3 we obtained the metal toxicity parameters, which are listed in the SI.

The concentration of a number of pesticides never exceeded the detection limit (see SI). These pesticides were not taken up in the effect analysis and no toxicity parameters were derived. For diazinon, dichlorophos and parathion, toxicity parameters could be derived from the course of the LC_{50} values in time. For dichlobenil, dieldrin, disulfoton, pirimiphos-methyl, and tolclofos methyl only a 24 or 48 h LC_{50} value was available. This was used to estimate the NEC, based on a comparison with diazinon (see section 2.2) because most data were available for diazinon. For mevinphos, a 24 and a 48 h LC_{50} value was available, also here the NEC was based on a direct comparison of LC_{50} values with diazinon. The elimination rates were based on a comparison with diazinon based on the log K_{ow} values as described in section 2 and the killing rate was based on a simulation with the derived values for the NEC and k_e . The toxicity parameters are given in the SI.

Several ions were measured in the surface waters: ammonium, calcium, chloride, phosphate, nitrate, nitrite, and ortho-phosphate. The toxicity of these compounds was considered individually. A comparison of the measured concentrations with the LC_{50} values left only chloride and calcium as having a potential effect on survival of the daphnids. For these two compounds toxicity parameters were derived based on the course of the LC_{50} values with time. The toxicity parameters are given in the SI.

3.2.2. Environmental Parameters. In general all waters were slightly basic with a typical pH of 8. The minimum pH was 6.4 and the maximum was 10.55. So the threshold of a

TABLE 1. Summary of Possible Effects on Survival Per Group of Compounds

compound group	expected effect	rationale behind expected effect
PAH	no	concentrations too low
metals	yes	occasional high concentrations, mixture effects
pesticides	yes	occasional high concentrations, mixture effects
salts	yes	occasional high concentrations of chloride
other measurements	yes	high pH, low oxygen

pH of 9.5 was exceeded in several occasions. The oxygen content also varied quite considerably from 0 mg/L to ca. 10 mg/L, so in some cases the daphnids died due to lack of oxygen.

3.2.3. Predicting Daphnid Survival. Using the chemical composition of the surface water at each sampling site, we calculated whether or not any of the individual or shared NECs were exceeded. If so we calculated the effect on survival after one week of exposure.

The calculations showed that PAHs were not expected to have an effect on the survival of the daphnids. The highest actual measured concentration equaled $4.9 \times 10^{-3} \mu\text{M}$ total PAH, while the no effect concentration for that mixture of PAHs equaled ca. $0.2 \mu\text{M}$ total PAH. Therefore effects from PAHs were not expected. Note that the solubility of most individual PAHs (and biphenyls) is not high enough to induce an effect per compound.

The exposure to copper, cadmium and zinc was typically between 10 and 20% of their respective NEC. At one location the NEC for Zn was exceeded. For the other metals the highest measured concentrations were much lower than their NECs. For arsenic, chromium, and mercury, the highest measured concentrations are $0.12 \mu\text{M}$ (0.3%), $0.15 \mu\text{M}$ (0.05%) and $2 \times 10^{-4} \mu\text{M}$ ($8 \times 10^{-4}\%$), respectively.

The NEC for individual pesticides or for the group of OP pesticides was exceeded on several occasions. In Table 1 a summary of the expected effects is given per group of compounds.

3.3. Comparison of Predicted and Observed Effects on Survival.

3.3.1. Observations with Complete Survival. In 19 out of 20 cases we made a correct prediction of the observed survival. Only in one case mortality was predicted, because the NEC for Zn was exceeded by 20%, while survival was observed.

3.3.2. Observations with Complete Mortality. In 15 out of 17 cases we made a correct prediction of the observed mortality. In two cases, survival was predicted while mortality was observed.

An example of the course of the calculated effect on survival with the concentrations as measured at location OW 306 in October 2000, is given in Figure 1. In this case the effect was (mainly) caused by diazinon, because the measured concentration of diazinon was $4.1 \times 10^{-3} \mu\text{M}$ while the total OP pesticide concentration was $4.4 \times 10^{-3} \mu\text{M}$. The no effect concentration of diazinon was $1.1 \times 10^{-3} \mu\text{M}$. Figure 1 shows that it takes about 20 h before the internal no effect concentration of diazinon was reached. Once it was reached the exceedence of the NEC was such (about 4-fold) that it took only a few additional hours to cause complete mortality.

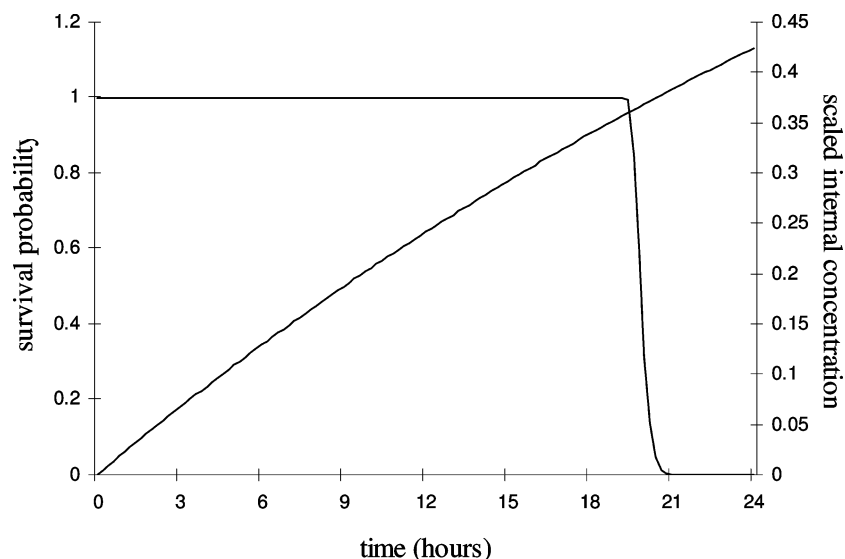


FIGURE 1. Calculate effect on survival of the measured mixture in October 2000 at location OW306. The effect is caused by diazinon, with a measured concentration of $4.1 \times 10^{-3} \mu\text{M}$ and a no effect concentration of $1.1 \times 10^{-3} \mu\text{M}$, the scaled internal no effect concentration equals 0.36.

TABLE 2. Cause of Predicted Mortality (See for the Location Code Delfland, 2005)

location, month	observed effect	predicted effect	cause of death in predicted effect				
			metals	pesticides	pH	diss ox	chloride
OW0056, sept 2000	mortality	survival	zinc	parathion-ethyl pirimiphos-methyl	pH pH pH	oxygen oxygen	chloride chloride chloride
OW058, Oct 2000	mortality	mortality					
OW090, Oct 2000	mortality	survival					
OW115, June 2000	mortality	mortality	mixture OP pesticides parathion-ethyl pirimiphos-methyl pirimiphos-methyl pirimiphos-methyl	pirimiphos-methyl diazinone	oxygen oxygen	oxygen oxygen	chloride chloride chloride
OW115, July 2000	mortality	mortality					
OW115, Aug 2000	mortality	mortality					
OW119, Sept 2000	mortality	mortality					
OW221, July 2000	mortality	mortality					
OW306 a, Aug 2000	mortality	mortality					
OW306a, Oct 2000	mortality	mortality					
OW306b, Juny 2000	mortality	mortality					
OW306b, Sept 2000	mortality	mortality					
OW306b, Oct 2000	mortality	mortality					
OW310, May 2000	mortality	mortality					
OW310, Aug 2000	mortality	mortality					
OW310, Sept 2000	mortality	mortality					
OW310, Oct 2000	mortality	mortality					

In Table 2, a complete list of the cases with observed complete mortality is shown with the cause of death according to the model.

4. Discussion

4.1. Reliability of the Model Predictions. In 34 out of 37 cases we made a correct prediction of the observed survival or mortality. And in case of mortality we could identify which contaminant or group of contaminants caused the mortality. High pH, individual pesticides (pirimiphos-methyl and parathion-ethyl) and low oxygen were the most common causes of mortality of the daphnids.

In the one case where we predicted survival, but mortality were observed, none of the NECs was exceeded. Several explanations are possible: mortality may have been caused by compounds that were not measured, the compounds causing the effect reached high concentrations during the one week exposure period that were not captured by the actual water sampling and chemical analysis or uncertainty in the parameter estimates. Also the sensitivity of the daphnids used in the present study may have differed from the sensitivity of the daphnids from which toxicity parameters were calculated (31).

In one of the two cases where we predicted mortality but survival was observed, the NEC of zinc was slightly exceeded (NEC: $3.1 \mu\text{M}$, actual concentration: $3.7 \mu\text{M}$), which should have led to mortality within 1 week of exposure. In the second case the NEC and LC_{50} for pirimiphos-methyl were exceeded. This should have led to complete mortality within 16 h of exposure, but no mortality was observed. In both cases the most likely explanation is again the variation of the chemical composition of the water over time.

The key feature in our approach is the estimate of the NEC for the individual compounds. Especially for the pesticides mostly limited data were available and LC_{50} data were sometimes conflicting. For instance for dichlorvos 48 h LC_{50} values between 720 and 45 nM were found in literature (32, 33). As the highest actual measured concentration for dichlorvos equaled 0.36 nM, the sole effect of dichlorvos was negligible. This example shows however that there can be large differences in available data that could influence the result significantly. Also the assumptions made on the effect of high pH and low oxygen content on the survival of daphnids were poorly underpinned. Nevertheless the predictability of our approach is high, with 92% correct predictions.

4.2. Partial Effects and Build up of Effects in Time. In situ exposure generally leads to a higher variability in the results because the circumstances of the exposure are much less controlled than in a toxicity test in the laboratory. Also in the present study variation between replicates showing partial effects was high and for this reason partial effects were not considered. Since we used a mechanistic model, there is however no principal difficulty to assess partial effects (or effects of pulsed exposures).

To allow an evaluation of the prediction of partial effects, these first need to be quantified more precisely. This may be achieved by increasing the number of replicates in the in situ exposure or to follow the build up of effects over time. Both imply a substantially higher experimental effort, making this costly.

On the modeling side we would need better estimates of parameter values. In the current study we calculated that if a NEC was exceeded a complete effect would occur within 36 h of exposure. Therefore a somewhat rough estimate of the kinetics hardly influences the result (predicted survival after 1 week of exposure). Moreover, we expect that partial effects are, much more than complete effects, caused by mixtures of groups of compounds, making accurate estimates of parameters even more important. Parameter estimates could be improved by making raw data of toxicity tests available (as appendices in papers for instance or in special databases). This would also greatly facilitate the development of QSAR-like approaches for parameter estimates (e.g., refs 34, 35).

4.3. Comparison with Other Methods. As discussed in the Introduction, traditional methods to address effects of mixtures (concentration addition and independent action) lack a mechanistic basis, which makes extrapolation to other compounds, other organisms, other points in time or fluctuating concentrations very difficult if not impossible. Applying CA or IA approaches to this in situ exposure data involving large numbers of compounds requires LC_{50} values for all compounds after a one week exposure period. For the majority of compounds, however, only LC_{50} values for 24, 48, and 96 h (sometimes also 72 h) are available. This means that traditional methods immediately fail because of lack of data. But a comparison based on the available 48 h LC_{50} values is possible. We did this for toxic units ranging from 0.25 to 3, demonstrating that there is no toxic unit which gives better predictability than the process based approach. Either the predictive power for mortality fails or the predictive power for survival fails (see section 4 of the SI). Apart from the better predictive power of our model, it also does not require the arbitrary choice of specific toxic units, and LC_{50} values can be transferred to time independent parameters enabling the calculation of the whole dose–response curve for the whole mixture at any point in time.

Apart from the approach we chose, different process based models are in use to assess effects on survival, the best known are those by Lee and Landrum 2006 (36) and Ashauer et al. 2008 (37). In a recent paper (38) the different modeling approaches were compared, and the underlying assumptions for traditional methods are also discussed. The methods by Ashauer and Lee require one additional toxicokinetic parameter compared to our method. This additional parameter has to be derived from elaborate experimental data and is needed to assess the overall effects. The type of experimental data needed is not available for (nearly all) the components in this mixture and therefore these methods can not be applied (as yet) to the type of complex mixtures as we discuss in this paper. In addition, our approach is embedded in an elaborate theoretical framework that can also be extended to include effects on growth, reproduction or food stress within a consistent framework (see eg Jager et al. (22)).

4.4. Linking Chemical and Ecological Status of Water Bodies. Although the model was expanded such that it accurately predicts the effect of complex mixtures, only in one case the observed complete mortality was actually caused by mixture toxicity. In all other cases where complete mortality occurred, it was caused by the exceedence of the NEC (or threshold) of a single compound. This general picture, that high concentrations of relatively few compounds can be linked to observed effects in field situations has been reported earlier (39–41). This is in apparent contrast to the idea of a “grey veil” of numerous compounds all in low concentrations jointly causing effects (42). However if partial or sub lethal effects would be evaluated using the present approach we expect mixtures effects to play an important role.

Approaches that try to link the chemical and ecological status of water bodies are scarce. Several attempts have been made for mixtures of pesticides (see the recent review by Belden (41)) and PAH (e.g., ref 43). Yet, these studies include relatively small numbers of compounds (4–10) and are not suitable for complex mixtures as occurring in the field. In our approach we successfully predicted the effects of a complex mixture containing over 80 components, but only for one species, though our approach could be expanded to other species. The method proposed by de Zwart and Posthuma (44) is also directed at predicting effects of complex mixtures. This approach directly addresses effects on multiple species, based on species sensitivity distributions. Their line of reasoning, where toxicants are divided in classes of compounds with the same mode of action is very similar to our approach. In contrast to our model, their approach is more suited to predict effects on species assemblages, but the causes of the observed effects on fish communities remained largely unknown (45). This approach might be more suitable to rank polluted sites, rather than predict the quantitative effect in the field given a defined mixture of chemicals.

The very strong point in our approach is that it is possible to predict the effect of a complex mixture given the chemical composition of the water and to identify which chemical or group of chemicals was responsible for the observed mortality. Hence it is concluded that the model is a powerful tool to link the chemical and ecological status of surface waters.

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 theme 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 We also like to thank the master students of the University of Amsterdam for deriving toxicity parameters for heavy metals for *Daphnia magna* in their ecotoxicology experiments.

Supporting Information Available

A complete list of all the compounds taken up in this study with their measured concentration ranges and (if applicable) their toxicity parameters, is given. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ES901083V