Insects in polluted rivers: an experimental analysis

van der Geest, H.G.

Publication date
2001

Citation for published version (APA):
PART 4.

SURVIVAL OF RIVERINE INSECTS UNDER COMBINED STRESSORS

Published as:


and based on:

4.1 Mixture Toxicity of Copper and Diazinon to Larvae of the Mayfly *Ephoron Virgo*, Judging Additivity at Different Effect Levels

**Summary**

The toxic unit concept is commonly applied for determining the effects of a mixture of toxicants. The use of this concept is, however, limited to compounds with similarly shaped dose-response relationships. In the present study, a method is proposed to gain insight in the influence of differences in the shapes of dose-response relationships on the applicability of the concentration addition model, by judging additivity at different effect levels. To this purpose, two model toxicants with different modes of action and dose-response relationships were selected: copper and diazinon. Using mortality of the mayfly *Ephoron virgo* as the endpoint, it was demonstrated that the two compounds act less than concentration additive. Application of the proposed calculation method revealed that the less than concentration addition effect was independent from the effect level on which the mixture was judged.

**Introduction**

Aquatic ecosystems are often polluted with a large number of chemicals originating from many industrial, agricultural and domestic activities (RIWA, 1992-1996; Boedeker *et al.*, 1993). In mixture toxicity testing, a common method for determining effects is the toxic unit (TU) concept (Sprague, 1970), in which the concentrations of the various compounds present in the mixture are expressed as fractions of their EC50 values. The joint action of the mixture is then judged by comparing the EC50 of the mixture with the corresponding sum of toxic units. If the sum of toxic units which equals unity causes 50 % effect in the mixture (EC50mix = 1 TU), the joint action is defined as concentration additive. The applicability of the concentration addition model was demonstrated by Deneer *et al.* (1988) by accurately predicting the joint acute toxicity towards *Daphnia magna* of a mixture of 50 non-reactive organic chemicals, all present at a concentration of only 2 % of their LC50. The use of the toxic unit concept requires, however, that the dose-response relationships of the individual compounds have similar shapes (De March, 1987).
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Whereas this may hold for compounds exhibiting the same mode of action, compounds with different modes of action often do not have comparable dose-response relationships. Nevertheless, in most mixture toxicity studies, no attention is paid to this fact or no solution is given when the problem is recognized and the TU concept is applied although its limiting conditions are violated.

In the present study, a method is proposed to gain insight into the influence of differences in the shapes of dose-response relationships on the judgment of mixture toxicity using the concentration addition model, by judging additivity at different effect levels. To this purpose, two environmentally relevant model toxicants with different modes of action and dose-response relationships were selected: a metal (copper) and an organophosphorous insecticide (diazinon). Copper is involved in many metabolic pathways and is essential for a variety of physiological processes within organisms, but when the essential levels are exceeded it becomes toxic (Van der Geest et al., 1999). For diazinon, an insecticide used to control a wide variety of insects in agriculture, the primary mode of toxic action is the inhibition of the enzyme acetylcholine-esterase. Considering these different modes of action of copper and diazinon, a joint action deviating from concentration addition is expected (Kraak et al., 1999; Hermens et al., 1984).

The mayfly *Ephoron virgo* was chosen as a test organism because it may play a key role in assessing the ecological status of rivers in Western Europe which suffer from a diffuse pollution with a wide variety of toxicants, like for example the River Meuse. It has not been found in The Netherlands for more than fifty years and although it recently returned to the River Rhine (Bij de Vaate et al., 1992), in the River Meuse at the Dutch-Belgian border this species has not yet returned (Bij de Vaate, 1995). A short-term toxicity test using *Ephoron virgo* has recently been developed (Greve et al., 1999) and has proven to be a suitable tool for testing the adverse effects of toxicants (Van der Geest et al., 2000) and for determining the water quality of field collected water and pore water samples (Van der Geest and Greve, submitted).

The aim of the present study was to determine the effects of a mixture of copper and diazinon on larvae of the mayfly *Ephoron virgo* in order to extend the applicability of the concentration addition model.
MATERIALS AND METHODS

Toxicity tests

The joint toxic effects of copper and diazinon on larvae of mayfly *Ephoron virgo* were analyzed by determining the survival after 96 h of exposure to equitoxic mixtures of these compounds in a newly developed standardized laboratory test (Greve *et al.*, 1999). The experimental set-up consisted of a glass vessel (180 mL) filled with 100 mL Dutch Standard Water (DSW; (NNI, 1980)), a standardized synthetic analogue of common Dutch surface waters, containing 200 mg CaCl₂·2H₂O, 180 mg MgSO₄·7H₂O, 100 mg NaHCO₃ and 20 mg KHCO₃ per liter demineralized water (pH 8.2, hardness 210 mg/L CaCO₃, alkalinity ca. 1.2 meq/L). At the start of an experiment, twenty 0-2 day old *E. virgo* larvae, originating from field collected egg masses (Greve *et al.*, 1999), were placed in each vessel and were fed 0.1 mL of a suspension of 750 mg dried and ground *Urtica* in 25 mL DSW. No aeration was supplied and the vessels were closed with perforated plastic foil in order to avoid evaporation. Pilot experiments revealed, nevertheless, that the oxygen level is relatively constant during the 96 h incubation period and never deviates much from saturation. A 16:8 h light dark regime was provided and the temperature was maintained at 20 °C. After 96 h, surviving larvae were counted.

Treatments

The effects of copper singly, diazinon singly and of copper and diazinon jointly, were determined simultaneously. Equitoxic mixtures were prepared applying the toxic unit (TU) concept (Sprague, 1970), using previously determined 96 h LC50 values of 11.8 and 77 µg/L for *E. virgo* exposed to diazinon and copper (Van der Geest *et al.*, 2000). At the start of the experiments, toxicants were added. Copper was added as CuCl₂ (Titrisol, Merck, Darmstadt, Germany) and diazinon (derived from Luxan, Inc., 99.7 % purity) was added using a generator column derived stock solution in DSW (Bleeker *et al.*, 1998). The nominal test concentrations for copper were 0 (control), 20, 50, 100, 150 and 300 µg/L and for diazinon 0 (control), 1, 3, 10, 30 and 100 µg/L. The nominal test concentrations for the mixture (as diazinon/copper) were 0/0 (control), 3/19 (0.5 TU), 4.5/25 (0.75 TU), 6/39 (1 TU), 9/58 (1.5 TU), 12/77 (2 TU) and 18/116 (2.5 TU) µg/L (tested in triplicate).
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Chemical analysis

Water samples (3 x 1 mL for copper analysis and 1 x 10 mL for diazinon analysis) were taken after 1 h and at the end of each experiment in order to determine the actual toxicant concentrations in the water. Depending on the copper concentration in the water, samples were analyzed by air-acetylene Flame (Perkin-Elmer 1100B equipped with an impact bead) or Graphite Furnace Atomic Absorption Spectrometry (Perkin-Elmer 5100PC/HGA600/AS60 equipped with Zeeman background correction) after acidification with 20 µL 65 % nitric acid P.A (Merck, Darmstadt, Germany). Since no filtration or centrifugation was applied, actual concentrations reflect total copper concentrations in the water. Quality control of copper analysis was carried out by analyzing blanks and reference material (NIST SRM 1643d) and measured values were in good agreement with the certified values (<10 % deviation). Before water samples were analyzed for diazinon, 50 µL of a solution of 1.14 mg chlorpyrifos/L hexane was added as an internal standard to each 10 mL water sample and extraction was performed with 3 x 1 mL glass distilled hexane (Rathburn, Walkerburn, Scotland). The concentration of diazinon was quantified by gas chromatography using a calibration series of diazinon in hexane. Measurements were performed using a Carlo Erba GC 8000 series Gas Chromatograph equipped with a Carlo Erba NPD-80-FL NP-detector and a J&W Scientific (DB-1701, 30 m long, 0.25 µm film thickness and 0.25 mm inner diameter) column. From the actual toxicant concentrations in the water at t=1 h and the end of each survival test, actual average exposure concentrations were calculated for both compounds separately assuming a 1st order exponential decrease of the toxicant concentration over time.

Data analysis

In the single compound experiments, survival was expressed as percentage of the corresponding controls and plotted against the actual average toxicant concentration in the water. From the obtained dose-response plots, LC50 values and their corresponding 95 % confidence limits were calculated by a non-linear curve-fitting procedure with the computer program Kaleidagraph (Synergy Software) using the logistic response model after Haanstra et al. (1985):
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\[
Y = \frac{c}{1 + e^{b(X-a)}} \quad \text{(equation 1)}
\]

in which \(Y\) = survival (\%), \(c\) = survival in control (set to 100\%), \(a\) = log \(\text{LC50}\) (\(\mu\text{g/L}\)), \(b\) = slope and \(X\) = log concentration (\(\mu\text{g/L}\)).

In most mixture toxicity studies, TU are based on previously determined LC50 values and potential variation in the sensitivity of the test organisms is ignored. To correct for this potential variation, LC50 values for copper and diazinon were determined simultaneously with the mixture and the TU in the mixture were re-calculated according to newly determined LC50 values, based on the previously (Van der Geest et al., 2000) and simultaneously (this study) performed experiments. Survival in the mixture experiment was plotted as percentage of the corresponding controls against the re-calculated TU. From this dose-response plot an LC50\(_{\text{MIX}}\) was calculated following the method described above. The joint toxic effect of copper and diazinon was defined as concentration additive (LC50\(_{\text{MIX}}\) = 1 TU), or as more or less than additive (LC50\(_{\text{MIX}}\) respectively<1 TU or>1 TU).

The differences in slopes of the dose-response relationships between treatments (copper, diazinon and mixture) were tested for significance by fitting the toxicity data of the treatments simultaneously to logistic models that differed in their EC50 values but had the same slope parameter, using Systat 5.2.1 software. A likelihood ratio test was used to test the hypothesis of similarity of shape by comparing these results to those obtained when each model had its own slope parameter (Van Gestel and Hensbergen, 1997). The same method, but then with fixed LC50 values was used to test for differences in LC50 values between different experiments.

RESULTS

Exposure concentrations

During the 96 h toxicity tests, the recoveries, defined as the quotient of the concentration at \(t=96\) h and the concentration at \(t=0\) h, were 80 % (± 11 % SD) and 95 % (± 11 % SD) for copper and diazinon respectively, indicating that exposure concentrations were relatively constant during the 96 h exposure period. Mixture toxicity testing, however, also requires
to evaluate the exposure concentrations relative to the 'toxic strength' of the compounds in a mixture (Hermens et al., 1985). Therefore, the ratios of measured and nominal concentrations were also calculated. In the present study, the average measured diazinon concentration in the mixture experiment was 54 % (± 13 % SD) of the nominal concentration, while for copper this was 36 % (± 13 % SD).

**Toxicity**

Average control survival of newly hatched *Ephoron virgo* larvae was 95 % (± 7 % SD). Figure 4.1 shows the survival of *Ephoron virgo* larvae at different measured diazinon concentrations in the water as percentages of the corresponding controls. A comparison is made between the results as reported by van der Geest et al. (2000) and the results obtained in the present study, performed simultaneously with the mixture experiment. In figure 4.2, the same comparison is made for the survival of *E. virgo* larvae after exposure to different copper concentrations. For copper, the newly determined dose-response plot is in good agreement with the previously determined ones and no significant differences between LC50 values and slopes were found (p<0.05; table 4.1). For diazinon, however, a relatively scattered dose-response relationships was obtained in the simultaneously performed experiment. This resulted in a high 95 % confidence interval of the LC50 value, overlapping with the 95 % confidence interval of the previously determined LC50 value. Applying the likelihood ratio test, however, a small but significant difference was observed between the previously and simultaneously determined LC50 values (p<0.05). Considering the limited number of observations, which fall within the previously observed survival data (figure 4.1), we have chosen to re-calculate the TU for the mixture experiment based on both experiments, resulting in a more accurate estimation of the effect concentrations. The re-calculated LC50 values based on the previously reported and presently determined survival data are presented in table 4.1.

Survival of *E. virgo* larvae was clearly affected by increasing concentrations of copper and diazinon in the mixture (figure 4.3). The estimated LC50 for the mixture was significantly higher than 1 TU (1.3 TU with 95 % confidence limits ranging from 1.2-1.4 TU), suggesting that copper and diazinon were less than concentration additive with respect to survival of *Ephoron virgo* larvae (p<0.05).
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![Graph showing survival of 0-2 day old Ephoron virgo larvae after 96 h exposure to diazinon during previously reported experiments (Van der Geest et al., 2000) and presently performed experiments, plotted as percentages of the corresponding controls against measured diazinon concentrations. Lines indicate the logistic response model after Haanstra et al. (2000).]

**Figure 4.1.** Survival of 0-2 day old *Ephoron virgo* larvae after 96 h exposure to diazinon during previously reported experiments (Van der Geest et al., 2000) and presently performed experiments, plotted as percentages of the corresponding controls against measured diazinon concentrations. Lines indicate the logistic response model after Haanstra et al. (2000).

![Graph showing survival of 0-2 day old Ephoron virgo larvae after 96 h exposure to copper during previously reported experiments (Van der Geest et al., 2000) and presently performed experiments, plotted as percentages of the corresponding controls against measured copper concentrations. Lines indicate the logistic response model after Haanstra et al. (2000).]

**Figure 4.2.** Survival of 0-2 day old *Ephoron virgo* larvae after 96 h exposure to copper during previously reported experiments (Van der Geest et al., 2000) and presently performed experiments, plotted as percentages of the corresponding controls against measured copper concentrations. Lines indicate the logistic response model after Haanstra et al. (2000).

**Table 4.1.** 96 h LC50 values for 0-2 day old *Ephoron virgo* larvae exposed to copper and diazinon as reported previously by van der Geest (2000), as determined in this study and based on both experiments together. 95% Confidence limits are given between parentheses.

<table>
<thead>
<tr>
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<th>96 h LC50 values (µg/L)</th>
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<td></td>
<td>diazinon</td>
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<tr>
<td>previously</td>
<td>11.7 (9.7-14.1)</td>
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<tr>
<td>this study</td>
<td>6.9 (4.7-10.1)</td>
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<td>together</td>
<td>10.7 (8.8-13.1)</td>
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**DISCUSSION**

*Experimental and statistical considerations*

Although exposure concentrations were relatively constant during the exposure time, a difference was observed between the ratios of the nominal and measured concentrations of copper and diazinon. This difference is most likely a consequence of the uncertainty of the concentrations of the stock solutions, since no actual measurements of these solutions were performed before the experiments were started. This influences the relative contribution of the two compounds to the re-calculated TU in the mixture (figure 4.4). It is demonstrated that the ratio of copper and diazinon in the mixture, based on measured concentrations and re-calculated TU, is ca. 2 to 3, therewith being different from equitoxicity (1 to 1 TU). Although equitoxic mixtures are preferred since the discriminating power is highest when two compounds are present in concentrations with equal toxic strengths, the deviation from equitoxicity observed in the present study does not influence the judgment of additivity of the mixture because the analysis is based on measured concentrations, expressed as toxic units. Deviations from equitoxicity could also be caused by variation in the sensitivity of the test organism in time (Van Gestel and Hensbergen, 1997), since toxic units are based on previously determined effect concentrations, when added to the treatments. By testing the sensitivity to the individual compounds simultaneously with the mixture and re-calculating the TU in the
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mixture accordingly, however, this factor was eliminated.

An important variable influencing additivity of a mixture using the concentration addition concept is the difference in the slopes of the dose-response curves of the individual compounds. The concentration-addition model requires that dose-response curves of compounds in a mixture have similar shapes (De March, 1987). In general, this holds for compounds with a similar mode of action (Van Straalen and Verkleij, 1991). Although attempts have been made to extend the concentration addition concept for compounds with different shapes in dose-response relationships (De March, 1987), in most mixture toxicity studies no attention has been paid to this limiting condition. However, Van Gestel and Hensbergen (1997) clearly demonstrated the effect of differences in shapes of dose-response relationships on the additivity of a mixture. Determining the effects of a mixture of Cd and Zn on the reproduction of the collembolan Folsomia candida, they demonstrated that at the EC10 level Cd and Zn act more than concentration additive, whereas at the EC50 level a less than concentration additive effect was observed. Also in the present study significant differences in the shape of the dose-response relationships of copper and diazinon were observed (p<0.05) (figure 4.5), therewith violating the conditions of the toxic unit concept. Therefore, following van Gestel and Hensbergen (1997), mixture toxicity of copper and diazinon to larvae of Ephoron virgo were evaluated, applying the TU concept, using effect concentrations other than the LC50. To this purpose, the logistic response model of Haanstra et al. (1985) (equation 1) was used to calculate effect concentrations of the single compounds ranging from the LC10 to the LC90:

$$\ln \left( \frac{100}{100-i} \right)^{-1} + a$$

in which a and b are the log LC50 (µg/L) and the slope respectively (estimated using equation 1), i is the percentage effect and LCi is the concentration which causes i % mortality.

An example of this procedure is given for the LC10 values: all concentrations of copper and diazinon in the mixture were divided by their corresponding LC10 value, resulting in LC10 based toxic units (TU10). The mixture survival data were plotted against these TU10 (figure 4.6),
the logistic response model (equation 1) was fitted through the dose-response plot and the LC10 of the mixture was then calculated using equation 2. 95 % Confidence limits for this effect concentration were calculated after Miller and Miller (1984). The same procedure was then applied for other effect concentrations, in steps of 10 %, up to the LC90. In figure 4.7A, LCI values of the mixture (TU i) are plotted against the effect level i on which the toxic units are based. Over the entire effect level range (from the LC10 to the LC90), the corresponding effect levels in the mixture were significantly higher than 1 TU (p<0.05), indicating a less than concentration additive lethal effect of copper and diazinon on larvae of the mayfly *Ephoron virgo* throughout the entire effect range. A similar pattern was observed for the terrestrial worm *Enchytraeus crypticus* exposed to a mixture of two compounds with similar shapes of the dose-response relationships (copper and zinc): less than additive effects of this mixture were demonstrated at both the EC10 and the EC50 level (Posthuma et al., 1997). However, in the experiments of Van Gestel and Hensbergen (1997) mentioned above, differences were demonstrated, when the EC10 and the EC50 level were used as the basis of the TU concept. In order to determine the extend of this influence, the original data of Van Gestel and Hensbergen was re-evaluated to determine the additivity of the mixture over the entire effect level range. In figure 4.7B, the ECi values of the Cd+Zn mixture (6 wk reproduction of *F. candida*; TU i) are plotted against the effect level i (EC10, EC20... EC90) on which the toxic units are based. At effect levels lower than 45 %, mixture effects did not differ significantly from concentration additivity (ECi MIX=1 TU; p<0.05), whereas at higher effect levels a strongly diminished concentration additive effect (ECi MIX>1 TU; p<0.05) was demonstrated. It is therefore concluded that the additivity of a mixture may depend on the effect level on which the mixture is judged. The presently proposed effect concentration dependent judgment method led, however, to different results when applied to the experiments in the present study and when applied to the experiments of Van Gestel and Hensbergen (1997). This can, however, not only be explained by a difference in shape of the dose-response relationships of the separate compounds or by a difference in the primary mode of toxic action, because such differences were observed in both studies. In this case, the difference in slopes of the mixture dose-response relationships and the expected dose-response relationships based on the survival data of both individual compounds together are most likely the responsible factor influencing the concentration dependency of additivity: The slope of the
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copper and diazinon mixture dose-response relationship was not significantly different from the expected slope based on the survival data of both individual compounds together (p<0.05), while for the mixture of Cd and Zn such a difference was significantly apparent (Van Gestel and Hensbergen, 1997). Although no alternative is given for applying the toxic unit concept to mixtures that violate its limiting conditions, by applying the mathematical method presented here, insight is given in the concentration dependency of the additivity of a mixture of chemicals.

![Graph](image1)

**Figure 4.4.** Relative contribution of copper and diazinon to the toxic units in the mixture, based on re-calculated LC50 values based on the survival data from the previously and presently performed toxicity experiments, illustrating the deviation from aimed equitoxicity in the present mixture experiment.

![Graph](image2)

**Figure 4.5.** Difference in shape between dose response relationships for copper and diazinon after standardization against LC50 values.
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**Figure 4.6.** Survival of 0-2 day old *Ephoron virgo* larvae after 96 h exposure to a mixture of copper and diazinon, plotted as percentages of the corresponding controls against toxic units (TU10) based on re-calculated LC10 values of the individual compounds.

**Figure 4.7.** 96 h lethal effect concentrations (LC; TU) for 0-2 day old *Ephoron virgo* larvae exposed to a mixture of copper and diazinon, and sublethal effect (6 wk reproduction) concentrations (EC; TU) for *Folsomia candida* exposed to a mixture of cadmium and zinc, plotted against the effect level (%) on which the toxic units in the mixture were calculated. Dotted lines indicate the corresponding 95% confidence limits.

*Copper and diazinon mixture toxicity*

Concentration addition or less than concentration addition are the two most frequently observed effects when combinations of chemicals are tested and hence, more than concentration additive effects are not
commonly observed (Deneer et al., 1998; Könemann, 1981; Broderius et al., 1995; Faust et al., 1993; De Wolf et al., 1985; Bailey et al., 1997). The mixture effects of copper and diazinon observed in the present study corroborate these findings. As hypothesized in the introduction, the observed deviation from concentration addition was expected, since the primary modes of toxic action are different for both compounds (Kraak et al., 1999; Hermens et al., 1984). Strongly synergistic mixture effects of metals and organophosphorous insecticides are, however, also observed: Forget et al. (1999) determined the joint effects of nine binary mixtures of different metals (copper, arsenic or cadmium) and pesticides (carbofuran, dichlorvos or malathion) towards the marine micro-crustacean Tigriopus brevicornis. In almost all cases, using both mortality and AChE inhibition as endpoints, synergistic effects were observed, with copper and malathion being the most toxic combination. Since the compounds used were almost similar to the ones used in the present study, but with mixture effects being in contrast to the present study, it is concluded that the outcome of a mixture experiment is not only influenced by intrinsic properties of the compounds and possible interactions between the compounds, but also by differences in the response of different test organisms.

Using mortality as the endpoint, it is most likely that the mixture effects are a result of the malfunctioning of a wide variety of processes within the organism caused by both specific and narcotic effects of the individual compounds (Hermens et al., 1985). Although copper and diazinon have a different mode of action, secondary effects cannot be excluded. For example, it is demonstrated that not only organophosphorous insecticides but also metals can inhibit AChE activity in organisms (Olson and Christensen, 1980). The complexity of possible interactive effects of metals and organophosphorous insecticides are described by Flammarion et al. (1996), who demonstrated differences in the joint effects of copper and methidation using different physiological endpoints. Following Plackett and Hewlett (1967) and Broderius et al. (1995) it can therefore be concluded that “similar and dissimilar actions should be thought of as being at opposite ends of a continuum of joint actions for binary mixtures of chemicals”. The presently observed partial concentration addition of a binary mixture of two compounds with dissimilar modes of primary action corroborate this statement.
Ecological relevance

Metals and pesticides are two common groups of contaminants in the aquatic environment (RIWA, 1992-1996; RIZA, 1996). Although international treaties (like for example the Rhine action plan; (Admiraal et al., 1993)) have led to a sharp decrease in the concentrations of a relatively small number of dominant toxicants, a diffuse pollution by a wide range of compounds, including metals and pesticides, remained. The present study demonstrated that combinations of copper and diazinon can produce adverse effects, and although a less than concentration additive mixture effect was observed over the entire effect range, it is clear that the toxicity of the mixture is higher than that of the individual compounds. At the lowest calculated effect level in the mixture (LC10mix), the concentrations of copper and diazinon were relevant to the concentrations of both compounds as measured in for example the River Meuse. It is therefore argued that attention needs to be paid to mixture effects in defining standards for water quality and risk assessment procedures.
4.2 COMBINED EFFECTS OF TOXICANTS AND LOWERED OXYGEN CONCENTRATIONS ON LARVAE OF THE MAYFLY Ephoron virgo

SUMMARY

In many large European rivers, the number of typical riverine insect species, such as mayflies, stoneflies and caddisflies, is greatly reduced compared to historic records. This can no longer be explained by high concentrations of a relatively small number of dominant toxicants since many rivers have changed from heavily polluted systems with a few selected key toxicants to systems with a complex pollution. This pollution consists of many substances in low concentrations coinciding with other unfavorable conditions, such as low oxygen concentrations. It was hypothesized that the joint adverse effects of such multiple stressors may be a steering factor in the distribution of riverine insect species. The aim of this study was therefore to determine the combined effects of toxicants and oxygen depletion. To this purpose, larvae of the riverine mayfly Ephoron virgo were exposed to two different model toxicants, copper and diazinon, under normoxia and hypoxia (50 % air saturation) conditions. It was demonstrated that the adverse effects of copper were more severe at a lowered oxygen concentration. For diazinon, however, such an effect was not observed, illustrating that such interactions between multiple stressors are compound specific. Since the effects of multiple stressors may have a stronger impact than can be expected based on the adverse effects of the individual factors, we argue that standard toxicity tests are no longer sufficient to determine the impact of human activities on the ecological state of riverine communities. Instead, attention needs to be paid to more environmental realistic non-optimal conditions in toxicity testing, to adequately fulfill the needs of ecological recovery programs.

INTRODUCTION

In the last few decades, river rehabilitation programs have resulted in an improvement of physical, chemical and biological conditions of several large river systems in Western Europe (Admiraal et al., 1993). The subsequent ecological recovery, however, took place only to a certain extent: in particular the number of typical riverine insect species, such as may-
flies, stoneflies and caddisflies, are still greatly reduced when compared to historic records (Nijboer, 1999; Bij de Vaate, 1995; Ketelaars and Frantzen, 1995). Besides the effects of habitat deterioration (Nijboer and Veronschot, 1997), the question remains if the water quality is still an important factor hampering further ecological recovery. Due to water quality improvements, potential adverse effects can no longer be explained by high concentrations of a relatively small number of dominant toxicants, and therefore it is hypothesized here that the answers to this question may be associated with mixture toxicity and in joint effects of different stressors (multiple stress (Heugens et al., in press)).

Mixture toxicity as a cause of retarded ecological recovery has recently been documented (Van der Geest et al., 2000), but besides toxicants, there are several other key factors limiting the distribution of riverine insect species. One of the variables in the aquatic environment of considerable importance to benthic communities is the oxygen concentrations of the water and the upper sediment layers (Ward, 1993). Fluctuating oxygen levels are often observed in inland waters, as a result of complex diurnal and annual variations, depending on both (a)biotic variables such as light intensity, current velocity or disintegration processes (Jacob and Walther, 1981), as well as human activities like hydrological and geo-morphological modifications or additional input of organic matter (Uncles et al., 2000). In most large European rivers, mean annual oxygen concentrations meet the environmental standards, but nevertheless temporary low oxygen conditions still occur frequently on a regional scale (for example the rivers Meuse, Scheldt, Mersey, Elbe, Tiber (anonymous, 1992)). In the River Meuse, for example, variations in oxygen concentration between almost 0 % and super-saturation are observed (RIWA, 1993-1997). Moreover, the periods of the lowest oxygen concentrations often coincide with the presence of the highest contaminant concentrations. It can be expected that, besides its direct adverse effects (for example Nebeker, 1972; Becker, 1987), low oxygen concentrations influence the response of aquatic insects to environmental contaminants since a variety of physiological processes in the organism may be affected by oxygen deficiency (for example Penttinen and Holopainen, 1995). The influence of lowered oxygen on the toxicity of compounds is at this moment, however, hardly investigated: possible adverse effects of chemicals are mostly determined in the laboratory under favorable conditions and optimal oxygen concentrations are required in most standard operating procedures (for example the OECD
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guidelines), which makes the extrapolation of laboratory toxicity data to field conditions difficult.

The aim of this study was therefore to determine the combined effects of low oxygen and toxicants, using larvae of the indigenous riverine mayfly *Ephoron virgo* as a test organism. Copper and diazinon were used as model toxicants, following studies on the effects of both model compounds separately (Van der Geest *et al.*, 2000) and jointly (Van der Geest *et al.*, 2000b).

*Ephoron virgo*, a typical mayfly species for large European rivers (Kureck, 1996), was chosen as a test organism because it may play a key role in assessing the ecological status of rivers in Western Europe. In The Netherlands, for example, it has not been found for more than fifty years and although it recently returned to the River Rhine (Bij de Vaate *et al.*, 1992), this species is still not recorded in the more polluted River Meuse at the Dutch-Belgian border (Bij de Vaate, 1995). A short-term toxicity test using *Ephoron virgo* has recently been developed (Greve *et al.*, 1999) and has proven to be a suitable tool for testing the adverse effects of toxicants (Van der Geest *et al.*, 2000) and for determining the water quality of field collected water and pore water samples (chapter 2). Two different model toxicants, copper and diazinon, were selected based on their observed presence in large European rivers (RIWA, 1993-1997) and differences in mode of toxicity. Copper represents a micro-nutrient which is essential for a variety of physiological processes within organisms, but toxic when essential levels are exceeded. Adverse effects have been demonstrated for a wide variety of aquatic organisms (anonymous, AQUIRE 2000). Diazinon is an organophosphorous insecticide used to control a wide variety of insects in agriculture (Giddings *et al.*, 1996) and direct adverse effects of these insecticides have been described for several aquatic organisms (anonymous, AQUIRE 2000). Organophosphorous insecticides are the most extensively used insecticides in the world and enter the aquatic environment via spray, drift, leaching, run-off or atmospheric deposition (Legierse, 1998).


**MATERIALS AND METHODS**

**Test organism**

Eggs of the mayfly *Ephoron virgo* were collected from a population of the River Waal, a branch of the River Rhine at the German-Dutch border, and kept in the laboratory in an artificial diapause at 4 °C according to the method described by Greve *et al.* (1999). Five days prior to an experiment, eggs were transferred to 20 °C after which the larvae hatched. The larvae used for each experiment, were 0-2 days old at the start of the experiment.

**Experimental setup**

Two different sets of experiments were performed to test the effects on the survival of 0-2 day old *Ephoron virgo* larvae of 1) lowered oxygen 2) lowered oxygen in combination with a toxicant, either copper or diazinon. In all experiments, the following setup was used to keep the oxygen concentration in the water at a constant user defined level (figure 4.8): a glass aquarium (45 L) was filled with 18 L Dutch standard water (DSW), a standardized synthetic analogue of common Dutch surface waters, containing 200 mg CaCl₂·2H₂O, 180 mg MgSO₄·7H₂O, 100 mg NaHCO₃ and 20 mg KHCO₃ per L demineralized water (pH 8.2, hardness 210 mg/L CaCO₃, alkalinity ca. 1.2 meq/L). The oxygen concentration in the water in the aquarium was kept at a constant level by an oxystat that compared the actual oxygen concentration, measured by an oxygen electrode (YSI), with the preset concentration. When the measured oxygen concentration was too high, an electrical gas valve was automatically opened to pass nitrogen into the water. When the measured oxygen concentration was too low, air was passed into the water and when the measured oxygen concentration was the same as the preset concentration, both valves were closed. The aquarium was covered with a glass plate and nitrogen and air were brought into the water by means of air stones. The temperature was maintained at 20 °C and a light dark regime of 18:6 h was applied in all experiments.

In the first set of experiments, the larvae were exposed to lowered oxygen concentrations in 12 glass vessels which were placed in the aquarium (figure 4.8). At the start of an experiment, twenty 0-2 day old *E. virgo* larvae were placed in each glass vessel (180 mL) using a
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Pasteur pipette and a stereomicroscope and were fed 0.1 mL of a suspension of 750 mg dried and ground *Urtica* in 25 ml DSW. The glass vessels were closed with plankton gauze (70 μm) to prevent larvae from escaping. The oxygen concentration inside the glass vessels was continuously monitored by a data-logger connected to a second oxygen electrode (YSI), which received water from the glass vessels using a peristaltic pump (figure 4.8).

At t=0 h, the 12 glass vessels (each containing 20 larvae) were placed in the aquarium. As controls, 5 glass vessels were placed outside the aquarium (100 % air saturation), under the same ambient conditions (temperature, light-dark regime). After 24, 48, 72 and 96 h of exposure, three glass vessels were taken out of the aquarium to count the number of surviving larvae. Survival of the larvae kept under control conditions outside the aquarium was also determined each day (1 control per day and 2 controls at t=96 h). This experimental setup allowed to test one lowered oxygen concentration and a control (100 % air saturation) per experimental run. The tested oxygen concentrations were 50, 20 and 10 % air saturation.

![Diagram](image)

**Figure 4.8.** Schematic view of the experimental setup used to determine the survival of *Ephoron virgo* larvae at different oxygen concentrations.

In the second set of experiments, the larvae were exposed to 50 % air saturation in combination with either copper or diazinon. In these multiple stress experiments, the same experimental setup as described above was used, with the only difference that toxicants were added to the water in the aquarium at the start of the experiments. Copper was
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added as CuCl₂ (Titrisol, Merck) and diazinon (derived from Luxan Inc., 99.7 % purity) was added using a generator column derived stock solution in DSW. This setup allowed to test one toxicant concentration at 50 % air saturation in one experimental run. Each run was accompanied by a control (100 % air saturation, no toxicant) and a toxicant control, consisting of the same toxicant concentration at 100 % air saturation. An oxygen control (50 % air saturation without a toxicant) was derived from the first set of experiments (see above). In total, five different concentrations of copper and diazinon were tested in combination with 50 % air saturation.

Chemical analysis

Water samples (3 x 1 mL for copper analysis and 1 x 10 mL for diazinon analysis) were taken after 1, 24, 48 and 72 h and at the end of each experiment (96 h) in order to determine the actual toxicant concentrations in the water. Depending on the copper concentration in the water, samples were analyzed by air-acetylene Flame or Graphite Furnace Atomic Absorption Spectrometry (respectively Perkin-Elmer 1100B and Perkin-Elmer 5100PC/HGA600/AS60) after acidification with 20 µL 65 % nitric acid (Merck P.A.). Since no filtration or centrifugation was applied, actual concentrations reflect total copper concentrations in the water. Quality control of copper analysis was carried out by analyzing blanks and reference material (NIST SRM 1643d) and measured values were in good agreement with the certified values (<10 % deviation). Before water samples were analyzed for diazinon, 50 µL of a solution of 1.14 mg chlorpyrifos/L hexane was added as an internal standard to each 10 mL water sample and extraction was performed with 3 x 1 mL glass distilled hexane (Rathburn). The concentration of diazinon was quantified by gas chromatography using a calibration series of diazinon in hexane. Measurements were performed using a Carlo Erba GC 8000 series Gas Chromatograph equipped with a Carlo Erba NPD-80-FL NPD detector and a J&W Scientific (DB-1701, 30 m long, 0.25 µm film thickness and 0.25 mm inner diameter) column. From the actual toxicant concentrations in the water at the start and the end of each exposure period (24, 48, 72 or 96 h), actual average exposure concentrations were calculated assuming a 1st order exponential decrease of the toxicant concentration over time.
Data analysis

To quantify the effect of lowered oxygen on the toxicity of copper and diazinon, 24-96 h LC50 values (for example the toxicant concentration at which 50 % mortality occurs) were calculated based on the survival data from the toxicity experiments at 100 % and 50 % air saturation. Although the replicates (separate vessels) were placed in the same aquarium, the different treatments were not considered as pseudo-replicates, similar to flow-through systems in which the different treatments receive test-medium from one batch. Survival was expressed as the number of surviving individuals as percentage of the total number of recovered individuals and plotted against the actual average toxicant concentration in the water. From the obtained dose response plots, LC50 values were calculated by a non-linear curve-fitting procedure with the computer program SPSS 9.0 for Windows (SPSS Inc.) using the logistic response model after Haanstra et al. (1985):

\[ Y = \frac{c}{1 + e^{b(X-a)}} \]

in which \( Y \) = survival (%), \( c \) = survival in control, \( a \) = log LC50 (µg/L), \( b \) = slope and \( X \) = log concentration (µg/L).

The differences in LC50 values, determined at 100 % and 50 % air saturation, were tested on significance by fitting the toxicity data of the treatments simultaneously to logistic models that differed in their slope parameters but had the same LC50 parameter, using the computer program SPSS 9.0 for Windows (SPSS Inc.). A likelihood ratio test was used to test the hypothesis of similarity of LC50 values by comparing these results to those obtained when each model had its own LC50 parameter (Van Gestel and Hensbergen, 1997).

RESULTS AND DISCUSSION

Experimental considerations

In all experiments, control mortality (100 % air saturation, no toxicant) was less than 10 %. Also recovery of the number of larvae (both dead and alive) at the end of an exposure period was high in most treatments: on
average, 96% of all larvae that were placed in the glass vessels at the start of the experiments were found again. In less than 4% of all treatments, less than 75% of the initial number of larvae was recovered and because it was unknown whether these were escaped or decayed, these cases were omitted from the data analysis. The recovery of the toxicants in the different treatments (defined as the concentrations at the end of an experiment as percentage of the concentration at the start) was on average 82% and 58% for copper and diazinon respectively.

*Lowered oxygen*

Figure 4.9 shows the survival of newly hatched *Ephoron virgo* larvae after 0-96 h of exposure to different oxygen concentrations. Exposure to 50% air saturation did not result in any increased mortality of *E. virgo* larvae during the 96 h exposure period. Exposure to 10% air saturation resulted in a slightly increased mortality after 24 h and almost 100% mortality after 96 h of exposure. The strongest effect was observed after exposure to 0% oxygen: after 48 h of exposure, none of the larvae survived. When compared with 96 h LC50 values for other mayfly and caddisfly species reported by Nebeker (1972), ranging from 5 to 40% air saturation, the sensitivity of *E. virgo* to lowered oxygen concentrations is in the middle range of sensitivities.

![Figure 4.9. Survival of newly hatched Ephoron virgo larvae after 0-96 h of exposure to different oxygen concentrations (0, 10 and 50% air saturation). Error bars indicate standard deviations.](image)
Multiple stress

Figure 4.10 shows the survival of newly hatched *Ephoron virgo* larvae after 0-96 h of exposure to different copper concentrations at 100 % and 50 % air saturation. In both treatments, mortality increases with increasing copper concentrations and with increasing exposure times. In the 50 % treatment, however, the observed mortality at each given exposure time and copper concentration was higher than the corresponding point in the 100 % treatment. This is reflected in the LC50 values presented in table 4.2: after 24 h and 48 h exposure to different copper concentrations at 100 % air saturation, no LC50 values could be calculated since almost all larvae survived. In contrast, at 50 % air saturation, an increased mortality at increasing copper concentrations was observed after 48 h of exposure (figure 4.10, table 4.2). After 72 and 96 h of exposure, clear dose response relationship were observed in both the 100 % and 50 % treatment, but the corresponding LC50 values determined at 100 % air saturation are significantly higher (ca 1.7 times) than at 50 % (p<0.05). At specific points in the time-dose-response surface (figure 4.10), the difference between the 100 % and 50 % treatment are even much higher: after 96 h of exposure to ca. 50 μg copper/L, all larvae survived in the 100 % treatment, but in the 50 % treatment survival was already reduced to 50 %. Since 50 % reduction in the oxygen content and 50 μg copper/L separately did not resulted in any mortality (figures 4.9 and 4.10), it is concluded that the combined effect of copper and low oxygen is much higher than can be expected based on the effects of both factors separately. The combined effects observed in the present study, corroborate the tentative results of Eriksson and Weeks (1994), who observed similar patterns after exposing field collected amphipods (*Corophium volutator*) to three different copper concentrations in combination with lowered oxygen. But also other compounds, like ammonia (for example Magaud *et al.*, 1997; Wajsbrot *et al.*, 1991), anthracene (McCloskey and Oris, 1991), phenol (Hlohowskyj and Chagnon, 1991), multiple effluent stressors (Lowell and Culp, 1999) and even fish kariomones (Hanazato and Dodson, 1995) exhibited different effects to organisms at lowered oxygen concentrations, then at normoxia. The higher sensitivity to toxicants could be explained by the increase in gill movements at lower oxygen concentrations, resulting in a higher volume of toxicant containing water passing across the absorbent membranes of the gill surface (Sijm and Van der Linde, 1995). For aquatic insects, this increase in undulatory movements at low oxygen...
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Concentrations has indeed been demonstrated (for example Philipson and Moorhouse, 1974). But also copper itself may be responsible for increased ventilation rates: Gerhardt and Palmer (1998) demonstrated that copper exposed mayflies (Adenophlebia auriculata) ventilated more than the mayflies in the unexposed controls. Either way could result in an increased uptake of copper and subsequent increased adverse effects. Another, complicating, factor may be whether or not the toxicant itself causes damage to the gill structure, therewith influencing the respiratory efficiency. Several authors have described hypoxia-like effects, physiological effects and/or physical damage of copper on the functioning of gills of for example mussels (Viarengo et al., 1993), fish (Pilgaard et al., 1994; Heath, 1991), crustaceans (Hebel et al., 1999; Nonnotte et al., 1993) and caddisflies (Leslie et al., 1999). If copper induced similar effects to the respiratory membranes of E. virgo (the young 1st instar E. virgo larvae do not have tracheal gills yet), this could also have contributed to the observed joint effects of copper and lowered oxygen.

Figure 4.10. Survival of newly hatched Ephoron virgo larvae after 0-96 h of exposure to different concentrations of copper, at 100 % (left graph) and 50 % (right graph) air saturation. The black dots represent the average survival per treatment.

The mortality of E. virgo after 0-96 h of exposure to different concentrations of diazinon at 100 % and 50 % air saturation is presented in figure 4.11. Similar to the copper experiments, mortality increases with increasing concentrations and with increasing exposure times. In contrast to the copper experiments, no significant difference in response was observed between the 100 % and 50 % treatment (p>0.05; table 4.2). This
difference between the influence of lowered oxygen on the toxicity of copper and diazinon is most likely caused by the difference in the modes of toxic action of both compounds. Inhibition of the enzyme acetylcholineesterase (AChE) by an oxidative product of diazinon (diazoxon) is considered to be the most important mode of toxicity of diazinon (Legierse, 1998; Keizer et al., 1993). A possible inverse relationship between the oxygen concentration and the formation of this toxic oxygenated product (or of any other product causing oxidative stress (Choi et al., 2000)) at lower oxygen concentrations, may even reduce the toxicity of diazinon. For anthracene, a similar process was observed by McCloskey and Oris (1991): an interaction among the level of respiratory stress and the rate of oxygen-dependent production of toxic (photo-) products of anthracene resulted in an increased toxicity at intermediate oxygen concentrations. At lower oxygen concentrations, the toxicity of anthracene was reduced by a decreased formation of toxic products. Since this trade-off between respiratory stress and oxygen dependent transformation does not play a role in the toxic mode of action of copper, whereas it possibly does for diazinon, this could explain the observed differences in the influence of lowered oxygen on the toxicity of both compounds.

![Figure 4.11. Survival of newly hatched Ephoron virgo larvae after 0-96 h of exposure to different concentrations of diazinon, at 100 % (left graph) and 50 % (right graph) air saturation. The black dots represent the average survival per treatment.](image)
Table 4.2. LC50 values of 1-2 day old *Ephoron virgo* larvae after 24-96 h of exposure to copper and diazinon at different oxygen concentrations (100 and 50 % air saturation). 95 % confidence intervals are given in parentheses. ne = no effect at tested concentrations; p<0.05 indicates significant differences between the 100 and 50 % treatment; ns = no significant differences.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Exposure time (h)</th>
<th>LC50 at 100 % (µg/L)</th>
<th>LC50 at 50 % (µg/L)</th>
<th>significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>24</td>
<td>ne</td>
<td>ne</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>ne</td>
<td>70.9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>98.3</td>
<td>57.9</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>89.0</td>
<td>51.5</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Diazinon</td>
<td>24</td>
<td>ne</td>
<td>ne</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>ne</td>
<td>ne</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4.7</td>
<td>8.0</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>1.0</td>
<td>2.4</td>
<td>ns</td>
</tr>
</tbody>
</table>

*Environmental relevance.*

In freshwater ecosystems, minimal contents of oxygen are an important factor limiting the distribution of benthic organisms. For example, Neumann (1994) and Becker (1987) demonstrated that increasing oxygen levels in the River Rhine allowed the re-colonization of the caddisfly *Hydropsyche contubernalis.* In addition to such direct adverse effects of lowered oxygen concentrations, the present study clearly demonstrated that the combination of stressors (copper and lowered oxygen) may have an stronger impact than can be expected based on the adverse effects of the individual factors. To determine the environmental relevance of these findings, insight is required in the level and duration of the periods of low oxygen concentrations as well as in the simultaneous occurrence of oxygen depletion and toxic compounds in the field. Figure 4.12 gives the minimum oxygen concentrations, the number of days with oxygen concentration below 50 % air saturation and the duration of the longest period with oxygen concentration below 50 % measured in the River Meuse at the Dutch-Belgian border (Eijsden) in the period 1990-1999. Although the situation is improving, it is shown that the periods of lowered oxygen in the River Meuse comparable to those used in the present study still frequently occur. Also in other large European rivers like the Elbe (DUH, 1997), Scheldt (Baeyens et al., 1998) and Mersey (Jones, 2000) temporary periods of low oxygen concen-
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trations still occur. Figure 4.13 shows that the lowest oxygen concentrations in the field indeed often coincide with the highest toxicant concentrations, stressing the ecological relevance of the conditions tested in the present study. It is concluded that multiple stress circumstances actually occur in complexly polluted large rivers and that under such conditions adverse effects on riverine biota can be unexpectedly high.

![Graphs showing minimum oxygen concentrations, number of days with oxygen concentration < 50%, and duration of the longest period with oxygen concentration below 50%](image)

**Figure 4.12.** Minimum oxygen concentrations, number of days with oxygen concentration < 50% air saturation (n) and duration of the longest period with oxygen concentration below 50% (based on average day measurements), measured in the River Meuse (Eijsden) in the period 1990-1999. Data from the Dutch Institute for Inland Water Management and Waste Water Treatment (RiZA).
In many river systems, the question remains which factors are responsible for the observed impoverished community. Results of standard ecotoxicity tests, do often suggest that the present levels of contaminants have no impact on riverine biota. Based on the results presented here, however, we argue that the joint effects of multiple stressors are a potential key factor hampering the progress of ecological recovery. This observation is of major importance for future ecotoxicological assessment studies: since many rivers have changed from heavily polluted systems with a few selected key toxicants to systems with a complex pollution (with many substances in low concentrations), standard high dose single substance tests are no longer valid to determine the impact of human activities on the ecological state of riverine communities. To adequately fulfill the needs of ecological recovery programs, attention needs to be paid to more realistic conditions in toxicity testing, especially to mixture toxicity (Van der Geest et al., 2000) and multiple stress (this study).
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