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PART 1. GENERAL INTRODUCTION

"If we give rivers room to breathe, protect and use them wisely, and restore nature which has been lost, the benefits for people and wildlife are considerable. Living rivers and their floodplains offer better flood control, productive natural resources, flood regulation, natural purification and a richer diversity of wildlife: ecologically-based river management has been proven to work and it makes economic sense."

World Wildlife Fund (Europe's Living River Campaign, 2000).
LARGE RIVERS

Rivers are the most important freshwater resource for man (Chapman, 1992). Large rivers and their flood plains have several important functions (figure 1.1). Besides draining the land and carrying away the water, numerous economically important functions depend on the geological, hydrological and chemical properties of our large rivers: for example, rivers provide shipping routes, connecting coastal ports with our main cities, and hydro-electric power stations provide electricity. The water is used for industrial applications, irrigation of agricultural land and as a main source for drinking water. Sand, gravel and clay are used for construction works and the flood plains as recreational and agricultural areas (Middelkoop and van Haselen, 1999).

But also in terms of biodiversity, large rivers are of main importance, despite the intensive anthropogenic impact.

![Figure 1.1. Services of river ecosystems](image)

**Riverine ecosystems**

Large rivers and their flood plains are complex ecosystems consisting of numerous habitats and biotic communities (Chapman, 1992). They form living areas and migration routes for many terrestrial and aquatic organisms, including birds, amphibians, mammals and insects. The high level of spatio-temporal heterogeneity makes riverine floodplains among the most species-rich environments known (for example Ward *et al.*, 1999; Hubalek, 1999).

A pristine and resilient riverine ecosystem has been, amongst others, characterized by a high biodiversity of especially the macro-faunal community (Ward, 1992). Macro-invertebrates play a key role in the dynamic riverine food web (figure 1.2) as an important link in the
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The turnover of organic material (Jonsson and Malmqvist, 2000). They also provide a food source for many fish and bird species.

![Diagram of carbon fluxes in riverine ecosystem]

**Figure 1.2.** Example of a simplified model of principal carbon fluxes in riverine ecosystem, in which the plankton and the benthos interact dynamically.

**Threats**

"More than one-half of the world's major rivers are being seriously depleted and polluted, degrading and poisoning the surrounding ecosystems", says the World Commission on Water for the 21st Century (www.worldwatercommission.org). Riverine biota are clearly suffering from these threats. A century ago large river ecosystems were still characterized by a high biodiversity, especially of benthic communities. Due to regulation of streambeds and the construction of weirs and dams, however, the surface area of suitable habitats for invertebrate species decreased dramatically (for example Stanford et al., 1996; Kingsford, 2000; Kondolf, 1997). Secondly, discharges of many industrial, agricultural and domestic activities resulted in serious pollution of the water (Admiraal et al., 1993). These threats resulted in impoverished ecosystems with a low biodiversity in which opportunistic species are dominant (for example Nijboer and Verdonschot, 1997).
Since this thesis focuses on water quality issues as a potential cause of an impoverished riverine community, no attention will be paid to habitat deterioration. One should in keep in mind, however, that in order to establish and maintain an ecosystem with natural characteristics, all requirements must be met, including a suitable habitat.

**Impact of poor water quality on riverine communities**

Not only the availability of suitable habitats, but also the chemical characteristics of the river water (including the presence of toxicants) determine to a great extend the presence or absence of species as well as the physiological performance of individual organisms in that river (for example Leland and Fend, 1998; Chapman, 1992; Alba-Tercedor et al., 1995).

In some large European rivers, the climax of pollution was reached in the late 1960s, early 1970s. The river Rhine, for example, was excessively polluted at that time (Beurskens et al., 1994; Tittizer et al., 1994). This was indeed clearly reflected in the changes in species richness during the previous century: an analysis of the development of the benthic macro-fauna in the 20th century reveals a drastic decline in the number of species from the mid-1950s to the early 1970s (Tittizer et al., 1994). In figure 1.3, the changes in biodiversity in the river Rhine during the previous century are presented, clearly coinciding with the changes in concentrations of two selected model contaminants (a metal and a PAH).

The improvement of the water quality of the river Rhine at the end of the previous century was a direct result of an increasing interest from governments, industries and NGOs in environmental issues. For the Rhine basin, this resulted in the initiation of the 'Rhine Action Program' in 1987, triggered by a large accidental spill at the chemical company Sandoz in Basel, Switzerland. Nowadays the basic water quality of the river Rhine has improved to such an extent that no direct adverse effects can be revealed by standard bioassay experiments (Hendriks et al., 1994). For other large European rivers, like for example the rivers Meuse, Elbe and Oder, rehabilitation is, however, until now not as successful as for the river Rhine (Van Dijk et al., 1994), in spite of water quality improvements.
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![Graph showing changes in number of macro-fauna species and concentrations of two model contaminants, BaP and Pb, in the river Rhine during the previous century.](image)

Figure 1.3. Changes in number of macro-fauna species (from: Tittizer et al., 1994) and in concentrations of two model contaminants, a metal (Pb) and a PAH (BaP), in the river Rhine during the previous century. The toxicant concentrations are measured in sediment core samples (in mg/kg) and plotted against the estimated year of deposition (from: Beurskens et al., 1994).

Having solved the problems associated with high concentrations of a relatively small number of key toxicants (as in the 1960s-1970s), the question remains, however, if the present diffuse pollution with a wide range of toxicants in low concentrations (Hendriks et al., 1994; Heemken et al., 2000) hamper further ecological recovery of riverine communities. Many different micro-pollutants still enter the large rivers from point sources and effluent discharges from domestic and industrial sources, from diffuse sources and through atmospheric deposition (Chapman, 1992). But also other compounds, like for example metals, can in some rivers still be detected in elevated concentrations due to diffuse emissions (for example Rautengarten, 1993). Adverse effects of this 'cocktail of chemicals' (mixture toxicity) on riverine biota are difficult to determine and are therefore at this moment hardly investigated. Even when a relatively high number of compounds is measured in the river, it appears to be impossible to attribute the observed toxicity to specific compounds based on laboratory experiments (Hendriks et al. 1994). This is even more complicated by the fact that besides toxicants, several other variables influence the distribution of aquatic organisms: most large European rivers are nowadays, for example, affected by eutrophication (Van Dijk et al., 1994). Also lowered oxygen concentrations (anonymous, 1992), often as a result of this eutrophication, and changes in salinity and temperature are observed. These different environmental stressors can interact in a variety of ways, which are until now hardly explored. It
is, however, most likely that such interactions influence the response of aquatic organisms to polluted river water ('multiple stress' Heugens et al., in press).

To determine the potential impact of environmental contaminants in nature, single substance laboratory tests under favorable conditions (optimal temperature, oxygen regime etc.) are therefore no longer sufficient: the presence of mixtures of chemicals and multiple stressors in the field hampers the translation of results from standard laboratory tests to field situations, and insight in their joint effects is therefore required.

Besides the problems associated with diffuse pollution and multiple stressors, there is another serious threat to riverine biota: even when the basic water quality and the habitats are no longer limiting the distribution of riverine species, accidental discharges may still prevent the re-colonization of sensitive invertebrate species (Lindgaard-Jørgensen and Bender, 1994; Malle, 1994; Prat et al., 1999). One of the most clear and well studied calamities in de last decades in the west European rivers is the 'Sandoz accident' in 1986 when water contaminated with pesticides flowed into the river Rhine after a fire at Sandoz in Basel (Switzerland) causing massive kills of aquatic organisms (Capel et al., 1988). Monitoring of the benthic communities downstream of the spill showed that such a chemical disturbance may have catastrophic effects (Van Urk and Kerkum, 1987), despite the fact that in general riverine communities are adapted to cope with natural disturbances (like for example floodings; Hendrick et al., 1995). When the chemicals had disappeared from the river, however, a relatively rapid re-colonization by opportunistic species occurred, most likely due to the natural dynamics of the riverine populations (Van Urk and Kerkum, 1987). On a longer time-scale, therefore, not only the concentration and kind of chemical spilled are determining the effects on biota, but also the frequency of the accidents. In this light, also smaller accidents are potentially limiting the distribution of smaller or larger numbers of aquatic species. Incidental peak concentrations of toxicants, exceeding the environmental standards, are in some rivers still frequently observed. In the river Meuse (at the Dutch/Belgian border), for example, more than 100 incidents were reported by the Institute for Inland Water Management and Waste Water Treatment (RIZA) in the period 1994-1996 (Stuijfzand, 1999). The exact impact of such events on the riverine communities is, however, hardly investigated. To illustrate the potential adverse effects of such incidents, as an
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example a literature review was made investigating the potential impact of an accidental spill of the insecticide diazinon in the River Meuse:

Impact of a diazinon calamity in 1996 on the aquatic macro-invertebrates in the river Meuse, The Netherlands.

(...) In April 1996, high concentrations of the pesticide diazinon were measured in the water of the river Meuse at the Belgian-Dutch border (Eijsden). Diazinon is a non-systemic organophosphorous pesticide used to control a broad variety of leaf eating and sucking insects in agriculture (Giddings et al., 1996). The maximum concentrations measured in the spring of 1996 (ca. 1 µg/L on April 14, 0.8 µg/L on April 21 and ca. 1 µg/L on April 28; RIZA; unpublished data) exceeded the Dutch standard for drinking water quality (RIWA-A limit; 0.02 µg/L). The actual maximum concentrations of diazinon and the fluctuations in time are unknown, since water samples were not taken continuously, but only at certain time intervals. In biological warning systems at the RIZA monitoring station at Eijsden, a change in activity of the fish Leuciscus idus and the waterflea Daphnia magna was observed and consequently an alarm was sent out by RIZA. Despite the high concentrations and the biological alert, little attention was paid to this diazinon accident.

Although there are many uncertainties to take into account when determining the ecological impact of the diazinon discharge and no monitoring of natural invertebrate communities was carried out after the accident, it is most likely that the macro-fauna community in the river Meuse was severely damaged. LC50 values for several aquatic species were exceeded, a high mortality in bioassays with C. riparius and H. angustipennis occurred and changes in activity in the biological warning systems were observed. It is likely that the ecological impact was even more severe than can be expected from the results of laboratory toxicity tests, since the diazinon peak coincided with low oxygen concentrations. Moreover, the diazinon calamity occurred in the spring, when many species are present as sensitive first instars (Hynes, 1970). An inventory of the structure of the aquatic invertebrate community in the river Meuse before the accident took place (Bij de Vaate, 1995; Ketelaars and Frantzen, 1995) showed a poor community in which opportunistic species dominate, despite the recent recovery of the average water quality. Recovery of such a community after an accident is expected to take place within one generation, as happened after the Sandoz accident in 1986 in the river Rhine (van Urk et al., 1993). Frequent accidental discharges, a poor average water quality and the absence of suitable habitats hamper the rehabilitation of other species that require stable conditions. But even when the basic water quality and the habitats improve as results of action programs initiated under the Meuse Treaty, accidents like the diazinon discharge in spring 1996 may still prevent the re-colonization of sensitive invertebrate species in the river Meuse (...)

RIVERINE INSECTS AND WATER QUALITY

To answer the questions related to the present environmental problems in large river ecosystems, as described above, the integration of ecotoxicological and ecological techniques is needed (Admiraal et al., 2000). Especially since toxicant concentrations in the field are decreasing and because rehabilitation programs are location specific, there is a need for test organisms and bioassays which can be used for ecological water quality assessments. The selection of test organisms should be based on both the sensitivity to stressors and the representativeness for the ecosystem of concern.

In the process of rehabilitation of the large European rivers measures were taken to allow the number of aquatic species to increase. The number of riverine insects, however, is still strongly reduced when compared to the situation one century ago: in figure 1.4 the development of the number of species in the river Rhine during the previous century is presented. After a period of very low biodiversity in the 1970s, the Rhine Action Program has resulted in an improved water quality and consequently in an increasing number of macro-fauna species. The contribution of aquatic insects to the benthic community in the river Rhine at this moment, however, is much lower than it was at the beginning of the previous century.

![Graph showing the number of species in the Rhine river from 1900 to 1988.](image)

Figure 1.4. Number of macro-fauna species in river Rhine during the previous century (Landesamt für Wasser und Abfall Nordrhein-Westfalen, 1988).
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Although the introduction of exotic species (especially of crustaceans) may have contributed to these observations, we argue that riverine insects, which are representative for pristine riverine ecosystems, could play a key role in assessing the ecological status of aquatic communities and in indicating ecological recovery. This is also supported by the Dutch approach to indicate ecosystem quality: in the so-called AMOEBE, several representative plant and animals species have been chosen, the presence and abundance of which (relative to a defined target situation) should indicate a intact ecosystem. For the large rivers two midge larvae (Diptera), one caddisfly larvae (Trichoptera) and one mayfly larvae (Ephemeroptera) are incorporated (Van Dijk and Marteijn, 1993), indicating the importance of aquatic insects for water management. In addition, also many biotic classification systems use the distribution of aquatic insects to define water and/or habitat quality (Resh, 1992). Examples are the widely used EPT-index, the benthic index of biological integrity (B-IBI; Fore et al., 1996) and RIVPACS (River invertebrate Prediction and Classification System; Wright et al., 1998).

The construction of biotic indices that use riverine insects to assess pollution and other anthropogenic disturbances often requires insight in the sensitivity of taxa. In contrast to the above, aquatic insects (and especially Ephemeroptera, Plecoptera and Trichoptera) have rarely been included in standardized ecotoxicological test schemes and most sensitivity data is based on field observations only and often highly subjective (Metcalf, 1989). Consequently, the responsible environmental or anthropogenic variables limiting the distribution of aquatic insect species are at this moment hardly known. The following example demonstrates that the knowledge of the autecology and the sensitivity to toxicants of a relatively well known aquatic insect species is still missing: in 1993 and in 1994 two papers regarding the reappearance of the caddisfly *Hydropsyche contubernalis* in the River Rhine were published, both giving different explanations for the observed distribution pattern of this species: Van Urk et al. (1993) suggested that a decrease in concentrations of insecticides in the river Rhine was responsible for the reappearance of this caddisfly, whereas Neumann (1994) suggested that this re-colonization was due to the increasing oxygen levels.

The lack of toxicity data on riverine insects is also illustrated in figure 1.5, in which the number of laboratory derived LC50 values reported in
the U.S.EPA aquatic toxicology database AQUIRE is presented for riverine insects (mayflies, caddisflies and stoneflies) and the standard test organism *Daphnia*. Is it clearly shown that the number of toxicity tests using mayflies, stoneflies or caddisflies is much lower than the number of tests with daphnids, especially when one considers the fact that in figure 1.5 *Daphnia* represents only a few species whereas *mayfly*, *caddisfly* and *stonefly* represent complete orders. Moreover, the majority of the tests performed with the riverine insects were done using field collected larvae, while *Daphnia* tests use controlled laboratory cultures.

![Graph showing number of laboratory derived LC50 values](attachment:graph.png)

**Figure 1.5.** The number of laboratory derived LC50 values, reported in the U.S.EPA aquatic toxicology database AQUIRE (AQUIRE, 2000).

One obvious reason for the lack of toxicity data is the difficulty of handling riverine insects, like caddisflies, mayflies or stoneflies, in the laboratory. The most frequently used test organisms in standardized test procedures were traditionally selected because of their ease in culturing, handling and testing (for example McCahon and Pascoe, 1988; Watts and Pascoe, 1996). In general, species with little demands with respect to their habitat, are more easily kept under laboratory conditions. On the other hand, such opportunistic species are also likely to be more tolerant to changing environmental variables such as increasing levels of toxicants. Another complicating factor in the handling of riverine insects like mayflies or caddisflies is the length of their life-cycle: it takes only a few weeks to complete the life-cycle of chironomids (another group of frequently used test organisms) under laboratory conditions, but for univoltine species like many caddisflies and mayflies this may take much longer, up to several months.
The length of the life-cycle of an organism itself is an important variable determining the effects on populations of organisms in the field: as demonstrated in many field studies, accidental discharges have more effects on univoltine species than on multivoltine species with short life-cycles, because it will take at least a year before the number of individuals will increase again (for example Van Urk et al., 1993). Since riverine insects like mayflies and caddisflies have in general much longer life-cycles than the standard test organisms like chironomids or daphnids, sensitivity data for such species would give more insight in adverse effects of environmental contamination in the field situation. When the available toxicity data is related to the recovery times of the corresponding test organisms, however, again it is clear that at this moment only little attention is paid to species with long life-cycles (and consequently long recovery times) such as mayflies, caddisflies or stone-flies (figure 1.6).

![Median recovery times for several aquatic species after chemical or physical disturbances](image)

**Figure 1.6.** Median recovery times for several aquatic species after chemical or physical disturbances (Niemi et al. 1990) in relation to the total number of records (both laboratory and field studies, all endpoints) in the U.S.EPA aquatic toxicology database AQUIRE (AQUIRE, 2000).

In earlier attempts to use riverine insects for water quality assessments, field-collected late instars have been used (Frick and Herrmann, 1990; Diamond et al. 1992). The major disadvantage of this approach is the relative unknown and probably low sensitivity of late instars compared to young instars (Hutchinson et al. 1998; Williams et al. 1986). Also a continuous supply of larvae with a known history and age is needed to advance the use of key species of invertebrates.
**SCIENTIFIC CHALLENGE AND OBJECTIVES OF THIS THESIS**

To understand the key factors limiting the distribution of insect species in rivers, a basic understanding of their dependence on water quality variables is needed. These variables are natural (for example oxygen concentrations) as well as man made (for example toxicants). It is argued that the questions on the distribution of species are similar in natural gradients and pollution gradients. Ward (1992) points to the extreme richness of insect species in flowing waters that is partly due to the stabilization of oxygen supply and temperature. This species richness is hypothesized to be lost during the downstream transition to lower rivers and lakes; environmental variations in oxygen, nutrients etc. are likely to explain this. The loss of insect species over this gradient can be regarded as analogous to the loss of species over gradients of man-made perturbations (eutrophication and subsequent oxygen depletion, toxicants).

This thesis aims to generate insight in how specialized river insects cope with large variations in water quality. Based on this understanding, the key environmental standards could be defined that allow rehabilitation of the original biodiversity in degraded rivers. Therefore in this thesis, basic eco(toxico)logical knowledge on representative riverine species will be provided. Riverine insects that play a key role in indicating ecological recovery, as argued above, are selected as model species. These selected model species are to be developed as sensitive tools for the ecological management of rivers.

Until now only a very limited number of aquatic insects, representative for undisturbed riverine ecosystems, can be cultured in the laboratory. Therefore, investments in rearing riverine insect species in the laboratory is needed and insight into their basic requirements and life-cycles is to be gained. Furthermore standardized methods for toxicity testing with these species are essential to evaluate the effects of environmental contaminants. Such toxicity tests can then be used to determine the sensitivity of the selected test organisms to different model toxicants or to complex conditions present in situ. This information is to be analyzed in conjunction with ecotoxicological data from literature, in order to determine the sensitivity of these specialized river insect species relative to other ubiquitous or opportunistic organisms. In this way, the newly developed ecotoxicity tests may be developed as tools for environmental risk assessments. As mentioned before, most large rivers are nowadays suffering from a diffuse pollution by a wide range of compounds in low
concentrations, often coinciding with sub-optimal environmental conditions (such as low oxygen concentrations). Therefore, this thesis also aims to gain insight in the joint effects of chemicals (mixture toxicity) and multiple stressors on riverine insects. In this way, adverse effects of environmental relevant concentrations of contaminants can be quantified and compared to field observations on the occurrence of river insects.

**TEST ORGANISMS**

*Selection*

The selection of the insect species that are used as test organisms in the present study was based on two criteria: the past and present distribution of the species in the large European rivers and the ability to keep the organisms in the laboratory under controlled conditions. Attempts have been made to develop laboratory cultures for several caddisfly, mayfly and midge species. Based on the results of these attempts, two caddisflies (*Hydropsyche angustipennis*; Hydropsychidae, Trichoptera and *Cyrnus trimaculatus*; Polycentropodidae, Trichoptera) and one mayfly (*Ephoron virgo*; Polymitarcidae, Ephemeroptera) were selected (figure 1.7). More information on the ecology and distribution of these species is given below.

![Figure 1.7.](image)

*Figure 1.7.* (From left to right) larvae of the caddisflies *Cyrnus trimaculatus* and *Hydropsyche angustipennis* (from: Eddington and Hildrew, 1981) and of the mayfly *Ephoron virgo* (from: Kureck, 1996).

*Ecology*

In natural ecosystems, the case-less and net-spinning caddisflies *Hydropsyche angustipennis* and *Cyrnus trimaculatus*, play an important ecological role as a decomposer of organic material and as a predator respectively. On their turn, they are a food source for fish and birds.
Caddisflies are insects with a complete metamorphosis. The flying adult female usually deposits an egg mass, containing a few hundred eggs, on a submerged boulder. After hatching, the larva develops through a series of five instars. The fully developed larva constructs a stony pupal case which is firmly attached to hard substrate. When the pupa is fully developed, it swims to the water surface where it emerges from the pupal skin. The adult flies from the surface, ready to mate.

In order to filter particles out of the water *H. angustipennis* larvae spin a net in between hard substrates. The larvae are omnivorous; they eat algae, detritus and small invertebrates from their nets and occasionally it has been observed that they scrape periphyton off the substrate. Nets spun by *C. trimaculatus* larvae consist of a silken tube, in which they hide, and have catching surfaces at both ends. They are usually described as carnivorous, but it has been observed that they also feed on plant material.

In addition, one mayfly (Ephemeroptera) species, *Ephoron virgo* (Plecoptera) was selected. *E. virgo* is one of the large mayfly species typical for large rivers and also plays an important ecological role in natural riverine ecosystems. The univoltine life-cycle of *E. virgo* in the River Rhine is described in detail by Kureck (1996). The eggs of *E. virgo* hatch in spring followed by a larval stage of 3-4 months. When the larvae reach the sub-imago stage they swim to the water surface where they emerge. *E. virgo* adults occur in mass swarms over the rivers just after twilight at the end of August and the beginning of September. The males emerge earlier than the females and land on the river banks where they molt their sub-imago exuviae after which they return to the river to fly horizontally above the water surface searching for emerging females. The females remain sub-imagoes during their adult lives and are fertilized in flight. After mating, the female deposits two egg masses, containing in total 2000-3000 eggs, on the water surface. The adults die after the flight period, which last for approximately one hour. The eggs sink to the bottom of the river were they attach to the substrate with a sticky polar cap to prevent drifting (Kureck, 1996). During winter the eggs are in diapause which is deactivated in spring by the rising temperatures.

The larvae of *E. virgo* live on and in the river sediment. The first instars do not have tracheal gills and live freely in the substrate. Later instars
start burrowing U-shaped-tubes in the river sediment. By generating wave-like movements with their feathered tracheal gills a water current is generated through the U-tube providing oxygen and food, such as detritus and algae which are filtered from the water.

There is little known about the habitat preferences of *E. virgo* larvae. Literature on required stream velocities and oxygen demands is not available, while data on the substrate requirements of *E. virgo* are divergent and all based on field observations. Schleuter (1989) observed that a combination of fine sediment and stones was the most favorable substrate in the River Main. In contrast, Bij de Vaate *et al.* (1992) concluded from a field survey that the river sediment from which larvae were collected mainly consisted of sand. Tobias (1996) reported stable layers of clay and Gysels (1991) loamy river banks as the most suitable substrate. Before they became extinct in the River Rhine, Schoenemund (1930) reported that *E. virgo* larvae could be found in muddy or sandy depositions and clay banks. In the River Rhine *E. virgo* larvae were found by Kureck (1996) in fine sediment between groynes as well as in the main channel where fine sediment was obviously stabilized by stones. Based on all these different observations it can be concluded that the substrate preference of *E. virgo* larvae is not very strict.

**Distribution**

The utility of the three selected species to monitor ecological rehabilitation is based on their distribution in two large European rivers, the rivers Rhine and Meuse, during the previous century. Around 1900, both rivers had a species-rich caddisfly and mayfly fauna, but during the previous century the diversity of these insect species declined (figure 1.8) (Nijboer and Verdonschot, 1997; Van den Brink *et al.*, 1990).

*E. virgo* was in the beginning of this century present in mass numbers in the Dutch rivers (Schoenemund, 1930; Albarda, 1889) but was observed for the last time in 1936 (Mol, 1985). It was extinct in The Netherlands for more than fifty years until Bij de Vaate *et al.* recorded some larvae near the German/Dutch border in 1991 (Bij de Vaate *et al.*, 1992). A survey afterwards concluded that the Rhine branches and a small part of the Meuse were already colonized by *E. virgo* (Bij de Vaate *et al.*, 1992).
The colonization of the River Rhine took place in downstream direction, probably starting from the River Main (Bathon, 1983). E. virgo is nowadays present in the River Rhine (and some of its large tributaries, the Mosel, Main and Neckar) downstream from Mannheim where the River Neckar flows in the River Rhine (Schöll, 1996). The colonization of the lower part of the River Meuse does probably not originate from upstream locations of the River Meuse, but from a branch of the river Rhine, the River Waal (Bij de Vaate et al., 1992), which is connected to the River Meuse by a canal. Also the two caddisfly species used to be present in the rivers Rhine and Meuse. Originally, H. angustipennis was widely distributed in small streams as well as in large rivers (Eddington and Hildrew, 1981), but nowadays this species is not found in the lower reaches of the rivers Rhine and Meuse. C. trimaculatus usually appears in the lower reaches of large rivers, but also occurs in ponds en lakes (Eddington and Hildrew, 1981). During the previous century, however, also this species was not found in the rivers Rhine and Meuse for a long period of time. Only since the last two decades of the previous century, specimens of C. trimaculatus were recorded again. Based on their past and present distribution, the three selected species are potential useful species as indicators for ecological recovery of river ecosystems.
OUTLINE OF THIS THESIS

To meet the objectives formulated in this thesis, a stepwise approach with increasing complexity and environmental relevance was chosen. This approach is reflected in the consecutive parts in which this thesis is divided:

Ecology and handling of riverine insects

In this part, methods were developed to start and maintain laboratory cultures of the caddisflies *H. angustipennis* and *C. trimaculatus*. For the mayfly *E. virgo*, a method was developed to collect fertilized eggs in the field, and keep them in the laboratory in an artificial diapause. For all three selected species, this ensured a continuous supply of young larvae, with a known history and age. Secondly, standardized short-term ecotoxicity tests were developed for all three species. With the development of cultures and tests, two basic requirements were fulfilled, providing the opportunity to gain insight into the sensitivity of the test-organisms and to determine the effects of toxicants (in single exposures or in combination with other toxicants or water quality parameters) and of field collected (pore)water samples in the laboratory.

Riverine insects coping with selected contaminants

This part reports on newly developed short-term survival tests with larvae of the three selected riverine insects, using two model toxicants, copper and diazinon. Copper and diazinon were selected as model toxicants based on their occurrence in large European rivers and differences in mode of toxicity. Copper represents a micro-nutrient which is essential for a variety of physiological processes within organisms, but may become toxic at higher concentrations. Diazinon is an organophosphorous insecticide used to control a wide variety of insects in agriculture. Inhibition of the enzyme acetylcholine-esterase (AChE) is considered to be the most important mode of toxicity.

In addition to the standardized experiments using mortality as the endpoint, a short-term behavioral test using larvae of the caddisfly *H. angustipennis* is developed. The behavioral responses were used to determine the adverse effects of ecological relevant doses of contaminants, below the acute lethal effect level. By testing the different model toxicants, using different endpoints, basic ecotoxicological information
for these riverine insects will become available. This information will be
compared with ecotoxicological data from literature, in order to deter-
mine the sensitivity of the selected species relative to other organisms.
In this way, the potential role of the newly developed ecotoxicity tests in
environmental risk assessments will be evaluated.

Survival of riverine insects under combined stressors

The extrapolation of results of single compound toxicity tests to natural
riverine ecosystems is speculative, since aquatic ecosystems are often
polluted with a large number of chemicals originating from many indus-
trial, agricultural and domestic activities. Therefore, in the last experi-
mental section of this thesis, attention is paid to the effects of mixtures
of chemicals. Since adverse effects are expected below individual effect
concentrations when compounds are present together, it is expected that
more insight is obtained in the potential effects of environmental
relevant concentrations of copper and diazinon, by determining the joint
effects of both compounds on larvae of the mayfly E. virgo. In addition, a
contribution is made to the theoretical field of mixture toxicity testing: a
method is proposed to gain insight in the influence of differences in the
shapes of dose response relationships of the separate compounds as well
as that of the mixture on the judgment of mixture toxicity, by testing for
additivity at different effect levels.

In standardized laboratory ecotoxicity tests, the adverse effects of
toxicants are generally determined under favorable experimental con-
ditions. In riverine ecosystems, however, environmental conditions are
often variable, therewith influencing the toxicity of contaminants. One of
the variables in the aquatic environment of considerable importance to
benthic communities is the oxygen concentration of the water and the
upper sediment layers and it can be expected that, besides its direct ad-
verse effects, low oxygen concentrations influence the response of
aquatic insects to environmental contaminants. Since temporary low
oxygen conditions still occur frequently on a regional scale in large
European rivers, and often coincide with elevated toxicant concen-
trations, the joint effect of low oxygen and the presence of toxicants is
investigated.
Concluding remarks

Finally, in the concluding remarks, it will be attempted to evaluate whether traditional ecological concepts on the distribution of species can be used to analyze the impacts of toxicants. The insight generated in this thesis on specialized river insects coping with large variations in water quality will be reviewed.

REFERENCES


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page 26


PART 2.

ECOLOGY AND HANDLING OF RIVERINE INSECTS

Based on:


2.1 DEVELOPMENT OF ECOTOXICITY TESTS USING LABORATORY REARED LARVAE OF THE RIVERINE CADDISFLIES HYDROPSYCHE ANGUSTIPENNIS AND CYRNUS TRIMACULATUS

SUMMARY

The diversity of aquatic insect species in large rivers in The Netherlands has been strongly reduced during the previous century. Therefore, aquatic insects can be used as indicators for ecological recovery in large rivers. However, there is a lack of ecological and ecotoxicological knowledge of riverine insect species. To provide this basic knowledge, laboratory cultures with two caddisfly species, Hydropsyche angustipennis and Cyrnus trimaculatus, were started, and standardized ecotoxicity tests were developed. A rearing method for both species is described, as well as reliable short-term ecotoxicity tests with first instars of the selected caddisflies.

INTRODUCTION

During the previous century, diversity of aquatic insect species has strongly declined in the large Dutch rivers (Van den Brink et al., 1990). A decrease in water quality together with a deterioration of natural habitats are considered to be major causes for this decline (Admiraal et al., 1993). Typical riverine insects, like caddisflies, mayflies and stoneflies (Ward, 1992) are nowadays hardly found in polluted river systems, like the lower part of the river Meuse (Bij de Vaate, 1995; Ketelaars and Frantzen, 1995). These insects could therefore play a key role in assessing the ecological status of aquatic communities and in indicating ecological recovery. However, the use of data on the distribution of aquatic insects is strongly limited by the lack of ecological and ecotoxicological knowledge.

Currently, two biological alarm systems are in use by the Institute for Inland Water Management and Waste Water Treatment (RIZA) to support the traditional physico-chemical monitoring of the water quality of the rivers Meuse and Rhine. One biological alarm system is performed with the golden ide (Leuciscus idus), and is based on the ability of this fish to swim upstream. The other system is based on changes in activity
of the water flea *Daphnia magna*. Over the years it has been shown that these warning systems, especially the *Daphnia*-system, give a good indication of the water quality for the intake of drinking water, but the indicative value for ecological rehabilitation may be limited. To this purpose, it seems more relevant to use species which have disappeared from the rivers Rhine and Meuse, since these species are representative for undisturbed river systems. However, riverine insects, like mayflies, stoneflies and caddisflies, have rarely been included in ecotoxicological test schemes and also the knowledge of the aut-ecology of these insects is superficial. The discerning of key factors limiting the distribution of aquatic insects in large rivers requires basic ecological knowledge (habitat conditions; oxygen demands) as well as insight in the sensitivity to toxicants of these insects. This project aims at providing this basic knowledge by starting laboratory cultures and developing ecotoxicity tests with the caddisflies *Hydropsyche angustipennis* and *Cyrnus trimaculatus*. Within this framework, rearing methods and standardized ecotoxicity tests for both species are described in this article.

Ecological background test species

Caddisflies (Trichoptera) are insects with a complete metamorphosis; the flying adult female usually deposits an egg mass on a submerged boulder. One egg mass contains a few hundred eggs. After hatching, the larva develops through a series of five instars. The fully developed larva constructs a stony pupal case which is firmly attached to hard substrate. When the pupa is fully developed, it swims to the water surface where it emerges from the pupal skin. The adult flies up from the surface, ready to mate. *Hydropsyche angustipennis* (Hydropsychidae) and *Cyrnus trimaculatus* (Polycentropodidae) are both caseless and net-spinning caddis larvae (figure 2.1). These species spin a net of silk material that is used to filter or trap food in. In order to filter particles out of the water *H. angustipennis* larvae spin a net in between hard substrates. The larvae are omnivorous; they eat algae, detritus and small invertebrates from their nets and occasionally it has been observed that they scrape periphyton off substrate. Nets spun by *C. trimaculatus* larvae consist of a silken tube, in which they hide, and has catching surfaces at both ends. They are usually described as carnivorous but it has been observed that they also feed on plant material.
Ecology and handling of riverine insects

Figure 2.1. The larvae of the caddisfly *Cynthus trimaculatus* (A) and *Hydropsyche angustipennis* (B). (from: Eddington and Hildrew, 1981)

*H. angustipennis* and *C. trimaculatus* play an important ecological role as decomposers of organic material and as a food source for fish and birds. Their utility to monitor ecological rehabilitation is based on the distribution of these two species in the rivers Rhine and Meuse during the previous century. Because of their ecological relevance and the possibility to culture them in the laboratory, the caddisfly species *H. angustipennis* and *C. trimaculatus* were selected for this study.

**Distribution test species**

*H. angustipennis* is widely distributed in small streams as well as in large rivers (Eddington and Hildrew, 1981). *C. trimaculatus* usually appears in the lower reaches of large rivers and also occurs in ponds and lakes (Eddington and Hildrew, 1981).

Around 1900, the rivers Meuse and Rhine had a species-rich caddisfly fauna, but during the previous century the diversity of caddisfly species declined (Van den Brink et al., 1990; Klink, 1985). In table 2.1, some important distribution data of the two caddisfly species in the rivers Rhine and Meuse are shown, demonstrating their value for indicating ecological recovery.

<table>
<thead>
<tr>
<th></th>
<th><em>C. trimaculatus</em></th>
<th></th>
<th><em>H. angustipennis</em></th>
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<tr>
<td></td>
<td>1900</td>
<td>recent</td>
<td>1900</td>
<td>recent</td>
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<tr>
<td>Meuse upstream</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>(Hastière)</td>
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<tr>
<td>Meuse border (B/NL)</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Meuse downstream</td>
<td>+</td>
<td>+ since '92</td>
<td>+</td>
<td>-</td>
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<tr>
<td>(NL)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Meuse tributaries</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rhine downstream</td>
<td>+</td>
<td>+ since '80s</td>
<td>+</td>
<td>-</td>
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<tr>
<td>(NL)</td>
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<td></td>
</tr>
<tr>
<td>Rhine tributaries</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2.1. Historic and recent distribution data of *H. angustipennis* and *C. trimaculatus* in the rivers Rhine and Meuse. Data from: Higler and Tolkamp, 1983; Ketelaars and Frantzen, 1995; Klink, 1985; Klink, 1989; Klink and Mulder, 1993; Van Urk et al., 1990; observations by authors.
LABORATORY CULTURES

Collecting and transport

Cultures were started by collecting larvae or egg masses in the river Erft, a tributary of the river Rhine, in West-Germany. Larvae were gathered by turning stones and put in plastic boxes containing wet tissues. Stones covered with egg masses were wrapped in wet tissues. The larvae and egg masses were transported to the laboratory under cooled conditions.

Rearing method

In the laboratory, the larvae or egg masses were placed in large rearing containers (figure 2.2). The rearing containers (40x60x20 cm) contained a stony substrate of gravel and stones and were filled with 30 L of Dutch Standard Water (DSW) (NEN 6503, 1980). DSW is a standardized synthetic analogue of common Dutch surface waters. A small internal pump and a flow-through system, using a reservoir, provided a continuous water flow inside the rearing containers.

Figure 2.2. Schematic view of the caddisfly laboratory culture and set-up for ecotoxicological experiments.
The 150 L of DSW in the reservoir were renewed every two weeks. The
temperature in the climate room was maintained at ± 20 °C, and a 16:7 h
light dark regime was applied, with 30 minutes twilight before and
after a light period. The larvae were fed five times a week with a
mixture of *Urtica* (3 g), two types of fish food (Trouvit and Tetrephyll;
respectively 1.5 g and 0.8 g), and fresh algae (*Scenedesmus* sp.; 50 mL). Additionally, water fleas (*Daphnia magna*) were given once a week as
living prey. In addition the larvae of *C. trimaculatus* were occasionally
fed with living midge larvae (*Chironomus riparius*). On top of the
rearing containers net cages (40x60x25 cm) were placed to collect the
adults after emerging from their pupal skin. The adults were caught
separately with a snap-cap to determine the sexes, and were released in
a cage where they could mate and deposit their egg masses. This mating
cage consisted of a plastic aquarium (18x35x20 cm) filled with a layer of
DSW (± 2 cm) and a few submerged stones. On top of the aquarium a net
cage (18x35x30 cm) was placed. Egg masses were deposited by the
females on the submerged stones. The stones were removed afterwards
and placed back in the rearing containers or used for experiments.

**Production**

The production of the laboratory cultures of both caddisfly species was
followed, and this gives an indication of the stability of these cultures.
As an example, the numbers of emerged males and females, and egg
masses produced during one year, in the *C. trimaculatus* culture are
presented in figure 2.3.
For both species more males than females emerged. The sex ratio (number of males/numbers of females) was 2.5 for *C. trimaculatus* and 1.7 for *H. angustipennis*. The percentage of females which produced an egg mass was 36% for *C. trimaculatus* and 73% for *H. angustipennis*. Apparently, most of the emerged *C. trimaculatus* females did not produce egg masses. Consequently, a difference was found in production of egg masses/week between *H. angustipennis* and *C. trimaculatus* (respectively 6.4 and 1.7 egg masses/week). A difference was observed in the length of the life-cycle as well. The life-cycle of *C. trimaculatus*, under rearing conditions, is completed in two months, while the life-cycle of *H. angustipennis* takes three months.

The stability of a culture is relevant for the planning of ecotoxicity tests. The set-up and maintenance of a laboratory culture will continuously provide larvae with a known history and age. This is essential for the reliability of ecotoxicity tests. Another advantage of laboratory cultures is that these give insight in the aut-ecology of the species. Maintaining a steady laboratory culture of these caddisflies, however, is laborious and time consuming.

**ECOTOXICITY TESTS**

*Development*

An ecotoxicity test has to be reliable (more than 80% survival under control conditions), reproducible and easy to perform. In general, young larvae are more sensitive to stress (physical or chemical) than older larvae. In order to assess potential risks for a population, a standardized test should be performed with instars as young as possible. Several experimental conditions were tested to develop an ecotoxicity test with first instars of the caddisfly larvae: different types of substrate, water, food, aeration and different ages of first instars. Larvae were exposed for 2, 4 and 7 days.

No differences were found between treatments in which different types of water (DSW/lake water) and substrate (sand/glass beads/no substrate) were tested. Because DSW is easy to obtain and has a standard composition, in contrast to lake water, and because the presence of substrate complicates the experimental set-up, a final set-up using DSW without substrate was chosen.

Factors that do influence the survival of first instars are: aeration, food
and age of the instars. Aeration seems to be necessary, probably to maintain a high oxygen concentration, as well as a constant water flow. The food type was relevant as well; it was observed that tests with food containing animal material resulted in a low survival of larvae (63 ± 6 % after 4 days), probably caused by the growth of bacteria and fungi. The best results were obtained with food containing 100 % plant material. Dried, grained Urtica was chosen as standard food because this is easy to obtain in high quantities. Additionally, different size fractions of Urtica were tested. It was found that larvae showed highest survival using the fraction >106 μm and the unsorted mix (respectively 95 % and 82 % survival after 7 days). In order to keep this test as simple as possible, the unsorted mix was preferred. In the test with Cyrrnus trimaculatus, a combination of Urtica, two types of dried fish food (Trouvit and Tetraphyll and fresh algae (Scenedesmus sp.) was found to be the optimal food source in the 96 h toxicity test. In addition, the age of the first instars appeared to be very important. For Hydropsyche angustipennis, the survival of young instars (0-9 days old) was low (65 ± 24 % (n=22)) and showed a high variation, caused by either a natural mortality during the first days or by mechanical damage due to handling. The use of 10-12 days old larvae showed higher survival and less variation (89 ± 7 % (n=8)), even after 7 days. For Cyrrnus trimaculatus, the age of larvae used for the toxicity test should be 20-25 days in order to prevent mortality due to handling.

Experimental set-up

After optimizing the conditions mentioned above, the following experimental set-up was obtained. For all treatments, glass jars (180 mL) were filled with 100 mL DSW. In the H. angustipennis test, 10 drops of a suspension of dried and ground Urtica (5 g/100 mL DSW) were added and twenty 12 days old first instar larvae from several egg masses were distributed randomly over the different treatments with a glass Pasteur pipette. In the test with C. trimaculatus, 14 drops of a suspension of an Urtica suspension (0.6 g), two types of ground fish food (Trouvit and Tetraphyll; respectively 0.3 g and 0.2 g), and fresh algae (Scenedesmus sp.; 10 mL) in 100 mL DSW, were added and ten 20-25 days old second instar larvae were placed in each jar. The larvae were exposed for 2, 4 or 7 days while a gentle aeration was applied. The experiments were carried out in a climate room under identical conditions as the cultures mentioned above. Survival was scored and the parameters growth, gut
content and development to second instar were determined.

**Validation**

The ecotoxicity test described above, was used to determine the effects of two model toxicants, copper (metal) and diazinon (insecticide). No effect was found on the parameters growth, gut content and development to second instar. Only survival was found to be a good parameter to measure effects after 2, 4 and 7 days exposure, an example is given in figure 2.4. The average survival in the controls after 2 days is $92 \pm 6 \%$ (n=10), after 4 days $92 \pm 6 \%$ (n=12) and after 7 days $88 \pm 8 \%$ (n=14). Also for *C. trimaculatus*, survival was a reliable endpoint after 4 days of exposure to the two different model toxicants (see also chapter 3.2).

![Dose-response relationship](image)

**Figure 2.4.** Dose-response relationships for laboratory reared first instars of *H. angustipennis* exposed to diazinon at different exposure times.

**CONCLUSIONS**

It can be concluded that the short-term ecotoxicity tests with first instars of *H. angustipennis* and *C. trimaculatus* are reliable, reproducible and easy to perform, when using the effect parameter survival. These tests are promising tools to determine the sensitivity of riverine caddisflies to toxicants. However, in order to use these species as indicators for ecological rehabilitation, more knowledge about sublethal effects of toxicants on these caddisfly species is required. Additionally, chronic experiments should be developed to determine the long-term effects of toxicants on the life-cycle of these species.
2.2 DEVELOPMENT AND VALIDATION OF AN ECOTOXICITY TEST USING FIELD COLLECTED EGGS OF THE RIVERINE MAYFLY EPHORON VIRGO

SUMMARY

The diversity of aquatic insects in large European rivers has been strongly reduced during the previous century. Therefore, aquatic insects can play a key role in indicating ecological recovery of large rivers. However, there is a lack of ecological and ecotoxicological knowledge of riverine insect species. To provide this basic knowledge, development of ecotoxicity tests with riverine insect species is necessary and therefore cultures or storage of field collected eggs of these species in the laboratory are needed. In this article we describe a method for collecting and storing eggs of the riverine mayfly Ephoron virgo and a reliable short-term ecotoxicity test using newly hatched larvae. Based on four different validation experiments, it is concluded that the newly developed ecotoxicity test using newly hatched larvae of the mayfly E. virgo is reliable, reproducible and easy to perform, when using the effect parameter survival after 96 h. This test can be used for determining dose-response relationships for toxicants as well as for testing river water samples.

INTRODUCTION

During the previous century, diversity of aquatic insects has strongly declined in most large European rivers. A decrease in water quality together with a deterioration of natural habitats are considered to be the major causes for this decline (Admiraal et al., 1993). Typical riverine insects, like caddisflies, mayflies and stoneflies are nowadays hardly found in disturbed river systems (Bij de Vaate, 1995; Ketelaars and Frantzen, 1995). They were among the first species that disappeared with the deterioration of the river systems and only some species returned after rehabilitation of rivers (Tittizer et al., 1994; Schöll et al., 1995). These insects could therefore play a key role in assessing the ecological status of aquatic communities and indicating ecological recovery. However, the use of data on distribution of these species is strongly limited by a lack of ecological and ecotoxicological knowledge. This project aims at providing this basic knowledge by development of ecotoxicity tests with the caddisflies Hydropsyche angustipennis and
Cyrrus trimaculatus (Greve et al., 1998) and the mayfly species Ephoron virgo. The mayfly Ephoron virgo is a typical riverine insect that disappeared during the previous century from the Rivers Rhine and Meuse. This species has recently returned to the River Rhine, probably due to improving water quality during the last decade (Tittizer et al., 1990; Bij de Vaate et al., 1992), although it is still absent from the middle regions of the River Meuse (Bij de Vaate et al., 1992). A method for collecting and storing eggs and the development of an ecotoxicity test with newly hatched larvae of the mayfly E. virgo are described in this article.

Ecology of the test species

Ephoron virgo Olivier 1791 (Ephemeroptera, Polymitarcidae) is one of the large mayfly species typical for large rivers and plays an important ecological role as filter feeder of fine organic material and as a food source for fish and birds (figure 2.5). The univoltine life-cycle of E. virgo in the River Rhine is described in detail by Kureck (1996). The eggs of E. virgo hatch in spring followed by a larval stage of 3-4 months. When the larvae reach the sub-imago stage they swim to the water surface where they emerge. E. virgo adults occur in mass swarms over the rivers just after twilight at the end of August and the beginning of September. The males emerge earlier than the females and land on the river banks where they molt their sub-imago exuviae after which they return to the river to fly horizontally above the water surface searching for emerging females. The females remain sub-imagoes during their adult lives and are fertilized in flight. After mating, the female deposits two egg masses containing in total 2000-3000 eggs on the water surface (Kureck, 1996). The adults die after the flight period, which last for approximately one hour. The eggs sink to the bottom of the river were they attach to the substrate with a sticky polar cap to prevent drifting. During winter the eggs are in diapause which is deactivated in spring by rising temperatures.

Figure 2.5. Fully developed larva and adult of Ephoron virgo (from Kureck, 1996).
The larvae of *E. virgo* live on and in the river sediment. The first instars do not have tracheal gills and live freely in the substrate. Later instars start burrowing U-tubes in the river sediment. By generating wave like movements with their feathered tracheal gills a water current passes through the U-tube providing oxygen and food, such as detritus and algae which are filtered from the water. 

There is little known about the habitat preferences of *E. virgo* larvae. Literature on required stream velocities and oxygen demands is not available, while data on the substrate requirements of *E. virgo* are divergent and all based on field observations. Schleuter (1989) observed that a combination of fine sediment and stones was the most favorable substrate in the River Main. In contrast, Bij de Vaate *et al.* (1992) concluded from a field survey that the river sediment from which larvae were collected mainly consisted of sand. Tobias (1996) reported stable layers of clay and Gysels (1991) loamy river banks as the most suitable substrate. Before they became extinct in the River Rhine, Schoenemund (1930) reported that *E. virgo* larvae could be found in muddy or sandy depositions and clay banks. In the River Rhine *E. virgo* larvae were found by Kureck (1996) in fine sediment between groins as well as in the main channel where fine sediment was obviously stabilized by stones. Based on all these different observations it can be concluded that the substrate preference of *E. virgo* larvae is not very strict. Therefore, the change in substrate composition during the previous century was probably not a major cause of the disappearance of this mayfly from the Rivers Rhine and Meuse. Also the recent mass development in the River Rhine is underlining this conclusion.

*Distribution of the test species*

Around 1900, the Rivers Meuse and Rhine had a species-rich mayfly fauna, but during the previous century the diversity of mayfly species declined (Van den Brink *et al.*, 1990). *E. virgo* was in the beginning of the previous century present in mass numbers in the Dutch rivers (Schoenemund, 1930; Albarda, 1889) but was observed for the last time in 1936 (Mol, 1985). It was extinct in The Netherlands for more than fifty years until Bij de Vaate *et al.* recorded some larvae near the German/Dutch border in 1991 (Bij de Vaate *et al.*, 1992). A survey afterwards concluded that the Rhine branches and a small part of the Meuse were already colonized by *E. virgo* (Bij de Vaate *et al.*, 1992) The colonization of the River Rhine took place in downstream direction, probably
Insect species in polluted rivers: an experimental analysis

starting from the River Main (Bathon, 1983). *E. virgo* is nowadays present in the River Rhine and some of its large tributaries (Mosel, Main and Neckar) downstream from Mannheim where the River Neckar flows in the River Rhine (Schöll, 1996). The colonization of the lower part of the River Meuse does probably not originate from upstream locations of the River Meuse, but from the River Waal (Bij de Vaate et al., 1992), which is connected to the River Meuse by a canal. In table 2.2, distribution data of *E. virgo* in the Rivers Rhine and Meuse are shown, demonstrating their value for indicating ecological recovery.

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<thead>
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<td>-</td>
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<tr>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Meuse (NL)</td>
<td>+</td>
<td>+ since 1991</td>
</tr>
<tr>
<td>Rhine (SW)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Rhine (G)</td>
<td>+</td>
<td>+ since 1986</td>
</tr>
<tr>
<td>Rhine (NL)</td>
<td>+</td>
<td>+ since 1991</td>
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</table>

**METHODS**

**Collection of eggs in the field**

*E. virgo* eggs were collected from a population in the River Waal, a branch of the River Rhine, on a location near the German-Dutch border (Hulhuizen) at the end of August 1997 (27/8/1997) by attracting adults with a light-trap during twilight. The flight period of the mayflies started half an hour after sunset, starting with male sub-imagoes molting on the river bank to imagoes. Fifteen minutes later, the first females appeared. The flight period of the females lasted half an hour, while at that time hardly any males were observed.

The light-trap (125 Watt Philips HPL-N lamp in front of a white cotton sheet) was placed 2-3 meters from the river on the river bank facing the water. Because eggs attach to the substrate after being deposited, they can be collected and stored on glass slides (76x26 mm) at which sand was glued with an inert epoxy resin (Araldit 2020, Vantico).
Approximately five hundred of these slides were placed on the bottom of 3 trays (1x1.5 m) which were filled with river water and placed beneath the light-trap. Each female attracted by the light deposited two egg masses immediately after touching the water surface in the trays. The egg masses sank to the bottom of the trays were they fell apart in thousands of eggs which stuck to the glass slides. After the flight period, which lasted approximately 45 minutes, the slides covered with eggs were transferred to polystyrene boxes that were placed in containers filled with river water and transported to the laboratory.

Storage of eggs

In the laboratory, the boxes containing the glass slides with eggs were placed in aquaria filled with Dutch Standard Water (DSW, NEN, 1980), a standardized synthetic analogue of common Dutch surface waters. The aquaria were covered with perforated plastic foil and stored at ± 20 °C. At this temperature the embryos developed and after 4 weeks the development stagnated and diapause was entered. Two weeks later the eggs were transferred to a refrigerator where the temperature was maintained at ± 4 °C. The DSW was renewed every month. In this way the eggs can be stored for at least 3 years.

Hatching of eggs

After a minimum of three months at ± 4 °C, the diapause was deactivated by transferring the eggs from ± 4 °C to a temperature of ± 20 °C. After 4 to 6 days at this temperature the larvae hatched.

TEST DEVELOPMENT

An ecotoxicity test has to be reliable (more than 80 % survival under control conditions), reproducible and easy to perform. In order to assess potential risks for a population, a standardized test should therefore be performed with larvae of the same age and origin. Several experimental conditions were tested to develop a short-term ecotoxicity test with
newly hatched larvae of *E. virgo*. Different types of water, substrate, aeration and food were tested.

No differences in survival were found between treatments in which different types and volumes of water (DSW/river water) and substrate (sand/glass beads/no substrate) were tested. Aeration seemed not to be necessary to maintain a high oxygen concentration and did not affect survival. Therefore, a final experimental set-up was chosen which consisted of DSW, without substrate and without aeration.

The only factor tested that did influenced the survival of larvae was the type and the amount of food. It was observed that tests performed with food containing animal material, like ground fish food, resulted in a low survival of larvae, probably caused by the growth of fungi and bacteria. The best results were obtained with food containing 100% plant material. Dried, ground *Urtica* given *ad libitum*, which had proven to be reliable in experiments with caddisflies (Greve *et al.*, 1998), was chosen as standard food.

**Experimental set-up**

After optimizing the conditions mentioned above, the following experimental setup was obtained. Glass jars (180 mL) were filled with 100 mL DSW, and 2 drops of an *Urtica* suspension (0.75 g/25 mL DSW) were added. Newly hatched (0-2 days old) larvae from eggs of several slides, were distributed randomly over the different jars with a glass Pasteur pipette until every jar contained 20 larvae. The jars were covered with perforated plastic foil and kept at ± 20 °C. A 16:7 h light dark regime was applied, with 30 minutes twilight before and after each light period. After an exposure time of 96 h, surviving larvae were counted.

**VALIDATION**

**Introduction**

In order to validate the newly developed ecotoxicity test, using field collected eggs of the riverine mayfly *Ephoron virgo*, four different sets of experiments were performed: 1) to determine the applicability of this test for single toxicant experiments, cadmium was chosen as a model toxicant. 2) To gain insight in the sensitivity of the newly developed test to environmentally relevant mixtures of chemicals and possible toxicity
enhancing or compensating factors, water and pore water samples from locations with different degrees of pollution were tested. 3) To gain insight in the reproducibility of the test, and applicability of the standard operating procedure, the test was performed simultaneously at the University of Amsterdam, at AquaSense BV and at the RIZA, using copper as a model toxicant and 4) to gain insight in the variation in sensitivity between test organisms originating from different field locations, 96 h copper and cadmium LC50 values were compared for larvae originating from eggs collected in two different rivers, the River Waal (The Netherlands) and the River Ebro (Spain).

_Cadmium_

Cadmium was added to DSW as CdCl₂ at the start of the experiment and nominal concentrations ranged from 0 to 2400 µg/L. A dose-response curve (figure 2.6) was obtained by plotting survival of newly hatched E. virgo larvae, expressed as a percentage of the corresponding controls, against the average actual cadmium concentration in the water. The LC50 value was calculated by a log-logistic curve-fitting procedure (Haanstra et al., 1985), being 367 (257-524) µg/L.

By determining the effect of the model toxicant cadmium, it is demonstrated that clear dose response relationships can be obtained when determining the effect of a single compounds on Ephoron virgo using the newly developed ecotoxicity test. This is also reflected in the relatively small confidence interval of the obtained LC50 value.

![Figure 2.6. Dose-response curve for newly hatched E. virgo larvae exposed to cadmium for 4 days.](image)

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**Bioassays**

Water samples were taken from two large rivers in the Netherlands, a branch from the River Rhine, the River Waal near Ochten, and the River Meuse. During the past decade, toxicant concentrations in the River Rhine have decreased drastically due to actions taken within the framework of 'The Rhine Action Program', whereas in the River Meuse average concentrations of most chemicals are still relatively high and elevated toxicant concentrations due to accidents still frequently occur. Also two small rivers have been selected, the Eindergatloop and the Tungelrooyse beek, known to be polluted with metals due to smelters. The Eindergatloop is a small river located near Neerpelt in the northern part of Belgium, which flows into the River Dommel, a tributary of the River Meuse. It contains high levels of metals, caused by runoff from soil surrounding a former zinc factory. The Tungelrooyse beek, a small river in the Northern part of the province of Limburg (The Netherlands) is also a tributary of the River Meuse. Samples were taken near Budel, where the river is contaminated with metals from the zinc factory Budelco. Furthermore, a sample from a Sewage Treatment Plant effluent in Boxtel (RWZI; Rieuol Water Zuivering Installatie), which discharges into the River Dommel, was taken. Finally, water samples were taken from the Avoca copper mine area, situated in south east Ireland approximately 60 km from Dublin city. The first sampling site (Meetings) is located downstream of the confluence of the Avonmore River and the Avonbeag River and served as the reference site. The next sampling site (Inflow) is located 300 m downstream from the reference site, on a point source stream discharging Acid Mine Drainage (AMD) from the abandoned Avoca copper mines into the Avoca river. AMD is a serious polluting effluent consisting of elevated metal concentrations coinciding with a low pH. A third sampling site (Avoca) is situated 2 km downstream of the inflow stream at Avoca village (Curran *et al*., submitted). Samples were taken in 1998 and 1999. Surface water samples were collected in clean acid washed polyethylene bottles and stored at 4 °C until further use. No filtration was applied.

Pore water samples were collected from the Eindergatloop, the Tungelrooyse beek and at two flood plain lakes of the River Waal near Ochten and Deest (Ochten 5 and Deest 4) in 1999. Ochten 5 is an exposed shallow lake (maximum depth ca. 2.5 m) with a firm unsorted sediment and is in open connection to the River Waal, while Deest 4 (a sheltered
shallow lake, maximum depth ca. 0.75 m, with a soft muddy sediment) is only flooded by the River Waal during extreme high water levels. At all four sites, samples of top sediment layer (ca. 1-15 cm depth) were collected using cores (5 cm diameter, 20 cm long) at several places in each sampling location in order to compensate for spatial heterogeneity. The sediment samples were transported in polyethylene containers to the laboratory where each sample was homogenized and then centrifuged (15 min at 3000 rpm) to separate the pore water from the matrix. Only for the Eindergatloop sediment, which consisted mainly of coarse sand, centrifugation was not applicable and pore water was collected by allowing the heavy particles to settle in the sampling container. All pore water samples were then filtered over a 1.2 μm acid-washed glass fiber filter (Whatman GF/C) and stored at 4 °C until further use. In 1998, different pore water samples were tested in the framework of the RIZA project “extractie, identificatie en karakterisering van onbekende stoffen”. These pore water samples originated from lake Drontemeer, lake Ketelmeer, the River Dommel and the River Oude Maas (Puttershoek).

In figure 2.7 and 2.8, survival of Ephoron virgo after 96 h exposure to surface- and pore water samples is plotted as percentage of the total number of recovered individuals. Under control conditions, survival of E. virgo was always 100 %. A significant decrease in survival (p<0.1) was observed after exposure to surface water from Eindergatloop (1999) and exposure to Outlet water even resulted in 100 % mortality. The RWZI-effluent and the pore water samples from Deest 4, Ketelmeer, the River Dommel and the River Oude Maas (Puttershoek) also affected survival of E. virgo (p<0.1). In all other samples, no significant decrease in survival was noted (p>0.1).

Attempts to explain the observed toxic effects remain however speculative, especially in samples taken from locations which suffer from complex pollution, like the rivers Maas and Waal (RIWA, 1993-1997) or the sewage treatment effluent. Also in other bioassay studies testing complexly polluted samples, it appeared impossible to attribute the observed toxicity to specific compounds, even when a relatively high number of compounds was measured (Hendriks et al. 1994; Stuijfzand 1999). Nevertheless, in the present study some relationships between toxic effects and metal concentrations (table 2.3) have been found for samples from the rivers in which metals were the dominant toxicants:
The high mortality after exposure to water from the inflow of the abandoned copper mines (100 % for both species) coincided with extremely high metal concentrations and a low pH.

The increased mortality due to exposure to water from the Eindergatloop, also coincided with high metal concentrations. Adverse effects of Eindergatloop water were previously demonstrated for the midge C. riparius (Groenendijk 1999). At the moment of sampling, metal concentrations in the pore water were much lower than in the river water (table 2.3). Consequently, exposure to the pore water did not affect survival.

The increased mortality of E. virgo after exposure to the RWZI sample and to pore water from the flood plain lake Deest 4 can not be explained by the measured metal concentrations. Most likely other compounds were present in the samples causing the adverse effects: at the moment of sampling, the biological filters of the sewage treatment plant in Boxtel, from which the RWZI sample was taken, was out of order (M. Mosink, personal communication) and hence high concentrations of especially organic contaminants could be expected. For the location Deest 4, it is expected that numerous chemicals that used to be present in the river Waal in the past (Admiraal et al. 1993; RIWA, 1993-1997) have accumulated in the sediment and are nowadays still present, because this flood plain lake is most of the time isolated from the river and no remediation by clean river water has occurred. This is in contrast to the flood plain lake Ochten 5, which is in open connection to the river, and in which (for example) metal concentrations are strongly reduced when compared to Deest 4. In the samples from all other locations, no extreme high metal concentrations were measured and no mortality of either species was observed in the bioassays.
Figure 2.7. Survival of newly hatched *E. virgo* larvae exposed for 4 days to water samples from various locations, taken in different years. * indicates 100% mortality.

Figure 2.8. Survival of newly hatched *E. virgo* larvae exposed for 4 days to pore water samples from various locations, taken in different years.
Table 2.3. Zn, Cd and Cu concentrations (μg/L) measured in the 1999 surface- and pore water samples from the selected locations. Standard deviations of AAS-measurements are given between parentheses.<dl is below detection limit.

<table>
<thead>
<tr>
<th>Surface water samples</th>
<th>Zn (μg/L)</th>
<th>Cd (μg/L)</th>
<th>Cu (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waal</td>
<td>8 (0.4)</td>
<td>&lt; dl</td>
<td>&lt; dl</td>
</tr>
<tr>
<td>Maas</td>
<td>16 (0.6)</td>
<td>&lt; dl</td>
<td>2 (0.5)</td>
</tr>
<tr>
<td>Eindergatloop</td>
<td>2412 (71.1)</td>
<td>102 (2.3)</td>
<td>15 (0.8)</td>
</tr>
<tr>
<td>Tungelooyse beek</td>
<td>33 (7.1)</td>
<td>0.03 (0.0)</td>
<td>7 (0.0)</td>
</tr>
<tr>
<td>RWZI Boxtel</td>
<td>17 (1.0)</td>
<td>&lt; dl</td>
<td>8 (0.1)</td>
</tr>
<tr>
<td>Ireland: Meetings</td>
<td>210 (26.5)</td>
<td>&lt; dl</td>
<td>2 (0.2)</td>
</tr>
<tr>
<td>Ireland: Inflow</td>
<td>60400 (2.1)</td>
<td>157 (6.6)</td>
<td>2825 (8.7)</td>
</tr>
<tr>
<td>Ireland: Avoca</td>
<td>501 (4.4)</td>
<td>1.28 (0.0)</td>
<td>44 (2.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pore water samples</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Deest 4</td>
<td>96 (1.7)</td>
<td>1.06 (0.1)</td>
<td>23 (0.3)</td>
</tr>
<tr>
<td>Ochten 5</td>
<td>21 (3.2)</td>
<td>0.3 (0.0)</td>
<td>8 (0.7)</td>
</tr>
<tr>
<td>Eindergatloop</td>
<td>292 (16.8)</td>
<td>37 (1.9)</td>
<td>9 (0.2)</td>
</tr>
<tr>
<td>Tungelooyse beek</td>
<td>501 (8.1)</td>
<td>3.53 (0.0)</td>
<td>4 (0.2)</td>
</tr>
</tbody>
</table>

Protocol testing

The survival of *E. virgo* after 96 h of exposure to different concentrations of copper was determined in three different laboratories (UvA, AquaSense and RIZA) simultaneously, using the standard operating procedure developed in this project. For these experiments, all larvae originated from the same egg batch. Copper was added to DSW as CuCl₂ at the start of the experiments and nominal concentrations ranged from 0 to 400 μg/L. Dose-response curves (figure 2.9) were obtained by plotting survival of newly hatched *E. virgo* larvae against the average measured copper concentrations in the water. The corresponding LC50 values are given in table 2.4.

Based on the comments from the different technicians who performed the test, it is concluded that the test is easy to perform and no alterations to the standard operating procedures were necessary. The LC50 values determined by the UvA and the RIZA were exactly the same, but the LC50 value determined by AquaSense was ca. 3 times lower. This deviation is, however, in the same order as for example for different LC50 values for the standard test organism *C. riparius* exposed
to copper, known from literature (ca. 3 times difference in 48 h LC50 values; Aquire 1999). In order to determine the reproducibility of the newly developed ecotoxicity test with *E. virgo* more accurately, however, more toxicity data is necessary.

![Figure 2.9](image)

**Figure 2.9.** Survival of newly hatched *Ephoron virgo* larvae after 96 h of exposure to different concentrations of copper, determined in different laboratories (lab1 is UvA; lab2 is RIZA; lab3 is AquaSense).

<table>
<thead>
<tr>
<th>lab</th>
<th>LC50 (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UvA</td>
<td>127 (108-149)</td>
</tr>
<tr>
<td>RIZA</td>
<td>127 (113-143)</td>
</tr>
<tr>
<td>AquaSense</td>
<td>38 (31-48)</td>
</tr>
</tbody>
</table>

**Table 2.4.** 96 h LC50 values for *Ephoron virgo* exposed to Cu, determined in different laboratories. 95 % confidence limits are given in parentheses.

Larvae originating from different populations

To gain insight in the difference in sensitivity between *E. virgo* larvae originating from different field populations, larvae originating from eggs collected in the River Waal, (The Netherlands) and larvae originating from eggs collected in the River Ebro (Spain) were exposed to concentrations series of both copper (figure 2.10) and cadmium (figure 2.11). By calculating the corresponding 96 h LC50 values (table 2.5) and applying a log likelihood ratio test, it was demonstrated that the larvae originating from the two different field locations are equally sensitive to cadmium. For copper, however, a significant difference was observed between the LC50 values. The observed difference is, however, smaller
than the difference between copper LC50 values determined in different laboratories (see above).

![Graph](image1)

**Figure 2.10.** Survival of newly hatched *Ephoron virgo* larvae, originating from the Rivers Ebro (Spain) and Waal (The Netherlands), after 96 h of exposure to different concentrations of copper (Ebro data from van Winsen, unpublished).

![Graph](image2)

**Figure 2.11.** Survival of newly hatched *Ephoron virgo* larvae, originating from the Rivers Ebro (Spain) and Waal (The Netherlands), after 96 h of exposure to different concentrations of cadmium (Ebro data from van Winsen, unpublished).

**Table 2.5.** 96 h LC50 values for *Ephoron virgo*, originating from the Rivers Ebro (Spain) and Waal (The Netherlands), exposed to Cu and Cd. 95% confidence limits are given in parentheses. (Ebro data from van Winsen, unpublished).

<table>
<thead>
<tr>
<th></th>
<th>96h LC50 (μg/L)</th>
<th>larvae from River Waal</th>
<th>larvae from River Ebro</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>77 (71-84)</td>
<td></td>
<td>163 (149-177)</td>
<td>sign. (p&lt;0.05)</td>
</tr>
<tr>
<td>Cadmium</td>
<td>367 (257-524)</td>
<td></td>
<td>573 (383-858)</td>
<td>not sign. (p&gt;0.05)</td>
</tr>
</tbody>
</table>
CONCLUSIONS

Test organisms frequently used in present standardized test procedures were traditionally selected because of their ease in culturing, handling and testing (McCaHon and Pascoe, 1988; Watts and Pascoe, 1996). However, since rehabilitation programs have become more location specific, the representativity of the test species for the ecosystem of concern has become increasingly important. Therefore, in river water quality assessment studies, several other more representative species (for example mayflies Frick and Herrmann, 1990; Diamond et al., 1992) have been selected. In most of these studies, however, field-collected late instar individuals were used, because no culture methods were available. The major disadvantage of this approach is the relative insensitivity of late instars compared to young instars (for example Hutchinson et al., 1998; Williams et al., 1986; Stuijfzand, 1999). The newly developed bioassay used in the present study combine the representativity of the test species for river water and sediments with the availability of a continuous supply of young (and hence more sensitive) larvae with a known history and age. In contrast to other test species, like the midge Chironomus riparius and the caddisfly Hydropsyche angustipennis, no laborious and time consuming laboratory culture (Greve et al., 1998) is necessary, because fertilized eggs are easily collected and can be stored for at least 3 years.

Also, the newly developed ecotoxicity test using newly hatched larvae of the mayfly E. virgo is reliable, reproducible and easy to perform, when using the effect parameter survival after 96 h.

Based on these criteria and the observed sensitivities after exposure to field collected (pore) water samples (this study) and individual toxicants (Van der Geest et al., 2000), it is concluded that the newly developed bioassays are new useful tools in site-specific water and sediment quality studies.
REFERENCES


Insects in polluted rivers: an experimental analysis


PART 3.

RIVERINE INSECTS COPING WITH SELECTED CONTAMINANTS

Published as:


3.1 Survival and behavioral responses of larvae of the caddisfly Hydropsyche angustipennis to copper and diazinon

Summary

This study reports on newly developed short-term survival and behavioral tests with larvae of the caddisfly *H. angustipennis*, using two model toxicants, copper and diazinon. Mortality of first instar larvae has shown to be a reliable endpoint and it was demonstrated that *H. angustipennis* is among the more sensitive aquatic insects to both copper and diazinon. In addition, short-term behavioral responses were found to be indicative of adverse effects of ecological relevant low doses of copper. Hydropsychid species, using the tests developed in this study, are excellent tools for discerning effects of individual toxicants present in large European rivers and may help in defining the conditions for ecological rehabilitation.

Introduction

Biodiversity of river ecosystems in Western Europe has decreased dramatically during the previous century, as a result of both habitat loss and water quality deterioration. In particular the number of riverine insects, such as mayflies, stoneflies and caddisflies, has been greatly reduced (Bij de Vaate, 1995; Ketelaars and Frantzen, 1995). These insect groups could play a key role in assessing the ecological status of river communities and indicating ecological recovery once measures have been taken to rehabilitate disturbed river systems (Schultz, 1998; Petersen and Petersen, 1984; Resh, 1993; Vuori and Parkko, 1996). However, the lack of ecotoxicological data on individual toxicants makes an effective use of data on the distribution of these species very limited because the responsible environmental variables remain unknown.

The present project aims therefore to determine the sensitivity of three riverine insect species (the caddisflies *Hydropsyche angustipennis* and *Cynurus trimaculatus* and the mayfly *Ephoron virgo*) to several environmental key factors using standardized ecotoxicological tests. Within this framework, this study reports on newly developed short-term survival and behavioral tests with larvae of the caddisfly *H. angustipennis*, using
two model toxicants, copper and diazinon.

*H. angustipennis*, a case-less net-spinning caddisfly, plays an important ecological role as decomposer of organic material and as a food source for fish and birds and is widely distributed in small streams as well as large rivers (Higler and Tolkamp, 1983). In The Netherlands, however, this species has disappeared from the Rhine and Meuse, two rivers with a species-rich caddisfly fauna one century ago (Klink, 1989; Van den Brink et al., 1990), while it is still present in their tributaries (Engels, 1993). *H. angustipennis* is therefore likely to be an useful species for assessing water quality and analyzing the conditions for ecological recovery of large European rivers.

The present project has been started by developing a continuous laboratory culture with this species and standardized ecotoxicity tests (Greve et al., 1998). The reliability of the tests and the suitability of different endpoints (survival, growth, consumption and behavior) will be validated. Behavioral experiments, using the impedance conversion technique (Heinis and Swain, 1986), were included in the present test scheme because a wide variety of pollutants can influence the function of sensory organs and alter the behavior of aquatic organisms (Blaxter and Ten Hallers-Tjabbes, 1992). Such behavioral changes are often the most sensitive reaction to chemical stress (Rand, 1985) and are likely to reduce the fitness of organisms under field conditions (Blaxter and Ten Hallers-Tjabbes, 1992; Bayley et al., 1995).

Copper and diazinon were selected as model toxicants based on their occurrence in large European rivers 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 variety of aquatic organisms (AQUA/INFO, 1994), but no acute lethal effect concentrations for any hydropsychid species are available. Diazinon is an organophosphorus insecticide used to control a wide variety of insects in agriculture (Giddings et al., 1996). Inhibition of the enzyme acetylcholine-esterase (AChE) is considered to be the most important mode of toxicity. Organophosphorus insecticides are the most extensively used insecticides in the world and enter the aquatic environment via spray, drift, leaching, runoff or atmospheric deposition (Legierse, 1998). Direct adverse effects of organophosphorous insecticides have
been described for several aquatic organisms, including caddisflies (Carlson, 1966; Liess, 1994; Schulz and Liess, 1995) but not for H. angustipennis.

By incorporating different model toxicants, different biological endpoints and different exposure times in the present test-scheme, the reliability of the newly developed tests using H. angustipennis is validated and it is expected that basic ecotoxicological information for H. angustipennis will become available.

**MATERIALS AND METHODS**

*Survival tests*

All survival tests were performed with twelve day old first instar H. angustipennis larvae, originating from a laboratory culture (Greve et al., 1998), and lasted for 48, 96 and 168 h. Each experimental treatment consisted of twenty larvae placed in a glass vessel (180 mL) containing 100 mL Dutch Standard Water (DSW; (NEN, 1980)) using a binocular microscope and a pasteur-pipette. DSW is a standardized synthetic analogue of common Dutch surface waters, containing 200 mg CaCl$_2$·2H$_2$O, 180 mg MgSO$_4$·7H$_2$O, 100 mg NaHCO$_3$ and 20 mg KHCO$_3$ per liter demineralized water (pH 8.2, hardness 210 mg/L CaCO$_3$, alkalinity ca. 1.2 meq/L). To avoid mortality by starvation, the larvae were fed with 0.5 mL of a suspension of 1.25 g dried and ground Urtica in 25 mL DSW. During the experiments, all vessels were continuously but gently aerated and covered with plastic foil in order to avoid evaporation. A 16:8 h light dark regime was provided and the temperature was maintained at 20 °C.

Toxicants were added at the start of the experiments. Copper was added as CuCl$_2$ (Titrisol, Merck, Darmstadt, Germany) and nominal concentrations ranged from 0 to 10000 μg/L. Diazinon (derived from Luxan, Elst, The Netherlands, 99.7 % purity) was added using a generator column derived stock solution of 40 mg diazinon/L DSW and nominal concentrations ranged from 0 to 8 μg/L. For both compounds, the series of concentrations (including controls) was tested at least in duplicate. Water samples (3 x 1 mL in copper experiments; 1 x 10 mL in diazinon experiments) were taken after 1 h and at the end of each experiment in order
to determine actual toxicant concentrations in the water. At the start of each experiment the length of at least 10 additional larvae was measured and after 48, 96 or 168 h surviving larvae were counted and their length measured. In addition, the gut content (classified as full, half-full or empty) and the larval stage was determined for each surviving larvae.

**Behavioral tests**

Behavioral responses of field collected fifth instar *H. angustipennis* larvae were recorded using the impedance conversion technique in exactly the same experimental set-up as Heinis and Swain (1986). Prior to the measurements, larvae were placed individually in sealed nylon meshed tubes (7 cm length, 1.5 cm diameter) and a 24 h acclimation period in DSW, with *Urtica* powder as food, was provided. After the acclimation period, in which the larvae were able to construct their nets within the nylon tubes, they were exposed for 48 h in a 5 L aquarium to the following nominal concentrations ranges: 0-600 μg/L copper or 0-40 μg/L diazinon. For each concentration, including controls, behavioral patterns of ten fifth instar larvae were recorded. During the exposure time the test solutions were continuously aerated and renewed every 24 h. Water samples (3 x 1 mL in copper experiment; 1 x 10 mL in diazinon experiment) were taken before and after each renewal period in order to determine the actual average exposure concentration. No food was present, a 16:8 h light dark regime was provided and the temperature was maintained at 20 °C. At the end of the 48 h exposure period, activity patterns of all larvae were recorded during 1 h by placing the nylon tubes with the larvae in a measuring chamber (7x3x2.5 cm) which received re-circulating test water from the corresponding exposure aquarium at a flow rate of 5 mL/min. Changes in the impedance of the system caused by the movement of the larvae were detected by two stainless steel electrodes placed at either side of the measuring chamber connected to the impedance converter and assimilated with 53 msec time intervals with the computer program Aqualand™ on a MSDOS 486 micro-computer. From the activity signals, three different types of behavior were defined according to the relative frequencies and amplitudes: undulatory movements or ventilation (mono-frequent with a relative high and constant amplitude), inactivity (signals below background noise) and other activity (multi-frequent with different amplitudes) (examples are given in figure 3.1). For each larva, the time spent
Riverine insects coping with selected contaminants

on these different types of behavior were determined and expressed as percentages of the total registration time (1 h). In addition, for each treatment, the mean length of all undulation periods was calculated. By comparing behavioral responses with controls under the same experimental conditions, the influence of variables potentially influencing results of these tests (for example method and moment of testing, temperature or current velocity (Philipson, 1977; Gerhardt, 1996), is reduced.

![Graph showing different types of behavior](image)

**Figure 3.1.** Example of different types of behavior of fifth instar *H. angustipennis* larvae recorded with the impedance converter and the computer program Aqualand™ (mV plotted vs time).

**Analysis of copper samples**

Depending on the copper concentration, 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). No filtration was used therefore measured concentrations reflect total copper concentrations.

**Analysis of diazinon samples**

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 standard in hexane. Analysis was conducted on a Hewlett-Packard 5890 Gas Chromatograph equipped with a ‘cold-on-column’ injector, a Hewlett-
Packard 5970 series Mass Selective Detector and a J&W DB-5 (30 m long, 0.25 μm film thickness and 0.32 mm inner diameter) column. The following temperature program was used: initial temperature 80 °C, increased with 20 °C/min, final temperature 274 °C.

**Dose-effect relationships**

From the actual toxicant concentrations in the water at the start and the end of each survival test and before and after each renewal period of all behavioral tests, average exposure concentrations were calculated, assuming exponential decrease of toxicant concentration with time. Survival was expressed as percentage of the corresponding controls and plotted against the actual toxicant concentration. From the dose-response plots, LC50 values were calculated by a non-linear curve-fitting procedure with the computer program Kaleidagraph (Synergy Software, Reading, UK) using the logistic response model (Haanstra et al., 1985):

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

\( Y \) = survival ( %), \( c \) = survival in control (set to 100 %), \( a \) = log LC50 (µg/L), \( b \) = slope and \( X \) = log concentration (µg/L).

EC50 values based on changes in time spent on the different types of behavior were calculated by fitting the logistic model through dose-response plots in which percentages of time spent on ventilation, inactivity and other activities were plotted against the corresponding measured average exposure concentration.

**Statistics**

All results from behavioral tests were analyzed for outliers according to Anscombe (1960). Outlier analysis is a way to reduce the impact of an individual response with a much greater residual than the others, but carries the risk of over- or underestimating effect concentrations. The main pattern of behavioral changes due to exposure to copper or diazinon was in this study, however, not changed by performing outlier analysis. Differences in behavioral responses between the various exposure regimes were tested using one-way ANOVA followed by Tukey posthoc tests with the computer program Systat.
RESULTS

Survival tests

In the short-term survival tests, control mortality of first instar *H. angustipennis* larvae was on average <10% and never exceeded 15%. Clear dose-response relationships were observed for survival after 48, 96 and 168 h exposure to copper (figure 3.2). From these results, LC50 values based on measured total copper concentrations were calculated (table 3.1).

![Graph showing survival of first instar *H. angustipennis* larvae after 48, 96, and 168 h exposure to copper, plotted as percentages of the corresponding controls against the measured copper concentrations. Solid lines indicate curve-fits after Haanstra et al. (1985).]

**Figure 3.2.** Survival of first instar *H. angustipennis* larvae after 48, 96 and 168 h exposure to copper, plotted as percentages of the corresponding controls against the measured copper concentrations. Solid lines indicate curve-fits after Haanstra et al. (1985).
Copper toxicity strongly increased with increasing exposure time. The 96 h LC50 was 7 times lower than the 48 h LC50 but no differences were observed between the 96 h and the 168 h LC50 values (table 3.1). Copper had no significant effect on growth and larval development. After 48 h exposure, however, the gut content of surviving larvae decreased with increasing copper concentrations (figure 3.3). The 48 h consumption EC50 was significantly lower than the 48 h LC50 (table 3.1). After 96 and 168 h exposure, larvae with empty guts were only found at concentrations at which already a high mortality was observed and consequently, no 96 and 168 h effects concentrations based on gut content could be calculated.

Diazinon also clearly affected survival of first instar *H. angustipennis* larvae after 48, 96 and 168 h (figure 3.4). The increase in toxicity with increasing exposure time was smaller for diazinon than for copper: the 96 h LC50 was 2.2 times lower than the 48 h LC50 (table 3.1). In contrast to the copper tests, an increase in exposure time from 96 h to 168 h significantly increased diazinon toxicity. No effects on growth, larval development or gut content were found.

**Figure 3.3.** Number of first instar *H. angustipennis* larvae with a filled gut after 48 h exposure to different concentrations of copper, plotted as percentages of the number of surviving larvae against measured copper concentrations. The solid line indicates the logistic response model after Haanstra *et al.* (1985).
Figure 3.4. Survival of first instar *H. angustipennis* larvae after 48, 96 and 168 exposure to diazinon, plotted as percentages of the corresponding controls against the measured diazinon concentrations. Solid lines indicate the logistic response model after Haanstra *et al.* (1985).

**Behavioral tests**

All results of behavioral tests were characterized by a high variance in time spent on the different types of behavior. Consequently, 9% of all observations, randomly distributed over all treatments, were rejected by outlier analysis. Under control conditions, fifth instar *H. angustipennis* larvae (*n*=20) spent 18 ± 4 (SE) % of the time on ventilation, 29 ± 5 (SE) % on other activities and remained inactive for 53 ± 5 (SE) % of the time. In all copper treatments, time spent on ventilation was significantly lower than in the control (*p*<0.05) (figure 3.5). Based on time spent on ventilation, the EC50 for 5th instars was 150 times lower than the 48 h
LC50 for 1st instars (table 3.1). Furthermore, after an initial activity peak at low copper concentrations, larvae became more inactive at increasing copper concentrations with 100% inactivity at the highest test concentration. Consequently, time spent on other activities decreased at higher concentrations. Corresponding EC50 values based on changes in time spent on these types of behavior are also significantly lower than the 48 h LC50 value for 1st instars (table 3.1).

Figure 3.5. Time spent on different types of behavior (ventilation, other activities and inactivity) by fifth instar H. angustipennis larvae after 48 h exposure to different concentrations of copper, plotted as mean percentages against measured copper concentrations. The vertical bars indicate SE.
Figure 3.6 shows behavioral responses of fifth instar *H. angustipennis* larvae after 48 h exposure to different concentrations of diazinon. A significant increase in ‘other activities’ was observed at 3.4 μg diazinon/L DSW (p<0.05), but at all other concentrations no significant differences in the three types of behavior were found when compared to controls (p>0.05). The mean length of the ventilation periods, however, increased significantly at the highest diazinon concentration tested (figure 3.7) and also the variability in duration of these ventilation periods was highly increased.

**Figure 3.6.** Time spent on different types of behavior (ventilation, other activities and inactivity) by fifth instar *H. angustipennis* larvae after 48 h exposure to different concentrations of diazinon, plotted as mean percentages against measured diazinon concentrations. The vertical bars indicate SE.
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Figure 3.7. Mean length of ventilation periods (sec) during one h behavioral measurements of fifth instar *H. angustipennis* larvae after 48 h exposure to different concentrations of diazinon, plotted as mean percentages against measured diazinon concentrations. The vertical bars indicate SE.

Table 3.1. LC50 (survival) and EC50 values (consumption, behavior) for 1st and 5th instar *Hydropsyche angustipennis* larvae exposed to copper and diazinon at different exposure times. 95 % CI are given in parentheses. (x) indeterminable, (-) not determined.

<table>
<thead>
<tr>
<th>inst.</th>
<th>endpoint</th>
<th>LC50/EC50 (µg/L)</th>
<th>48 h</th>
<th>96 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>copper</td>
<td>survival</td>
<td>2510 (2100-3003)</td>
<td>350 (257-478)</td>
<td>502 (411-614)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>filled gut</td>
<td>977 (704-1355)</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ventilation</td>
<td>17 (x)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inactivity</td>
<td>160 (105-245)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>other activities</td>
<td>204 (95-439)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>diazinon</td>
<td>survival</td>
<td>2.9 (2.2-3.9)</td>
<td>1.3 (1.2-1.5)</td>
<td>1.0 (0.8-1.1)</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

*Lethal responses*

This study clearly demonstrated that short-term effects of toxicants on larvae of the caddisfly *Hydropsyche angustipennis* can easily be determined using the newly developed standardized survival test. Mortality of first instar larvae has shown to be an accurate and reliable
Riverine insects coping with selected contaminants

endpoint and it was demonstrated that *H. angustipennis* is among the more copper sensitive aquatic insects. Its 96 h LC50 (350 µg Cu/L) is at the bottom end of the 96 h LC50 range found in literature, ranging from 320 µg Cu/L for *Ephemera subvaria* and 540 µg Cu/L for *Chironomus tentans* to 4600 µg Cu/L for *Zygoptera* species (AQUA/INFO, 1994). LC50 values for *H. angustipennis* exposed to diazinon (96 h LC50 1.3 µg/L) are lower than for any other Trichoptera species in literature and is among the most sensitive aquatic insects (AQUA/INFO, 1994). The sensitivity to both copper and diazinon differs, however, strongly between taxonomically closely related macro-invertebrate species (AQUA/INFO, 1994; Van der Geest *et al.*, 1997). These differences make it difficult to classify sensitive and tolerant macro-fauna groups, and stress the importance of using test organisms in ecological risk assessment, that are representative for the ecosystem of concern.

*Exposure time*

Exposure time strongly influenced toxicity. The increase in toxicity of copper to *H. angustipennis*, after an increase in exposure time from 48 h to 96 h, was in the same order as found for other aquatic insect species (AQUA/INFO, 1994). A further increase in exposure time to 168 h had, however, no significant effect on toxicity. This may be explained by an increase in metal elimination rates at increasing exposure times, as has been described for accumulation of cadmium (Postma *et al.*, 1996) and copper (Timmermans *et al.*, 1992) in larvae of the midge *C. riparius*. The influence of exposure time on diazinon toxicity to *H. angustipennis* larvae was smaller than on copper toxicity, but still apparent after an increase of exposure time from 96 h to 168 h. This suggests that the presence of diazinon has a direct effect on survival which gradually increases with time. Legiersé (1998) demonstrated that such time dependent effects of organophosphorous insecticides are mainly caused by irreversible inhibition of AChE, and can not be explained by accumulation kinetics only.

*Behavioral responses*

Behavioral changes are often the first and most sensitive reactions to chemical stress (Rand, 1985). In this study, a strong behavioral response of fifth instar *H. angustipennis* larvae was found at copper concentrations 150 times lower than the 48 h LC50 for first instar larvae. Similar
activity responses in reaction to copper far below acute lethal concentrations have been previously reported for (for example) *Scrobicularia plana* (Akberali and Black, 1980), *Mytilus edulis, Dreissena polymorpha* (Slooff et al., 1983; Grace and Gainey, 1987) and *Gammarus pulex* (Taylor et al., 1994; Gerhardt, 1995). The strong decrease in time spent on ventilation and other activities of *H. angustipennis* observed in this study is in good agreement with the decreased locomotion of *H. angustipennis* after exposure to a complex industrial effluent (Gerhardt, 1996) and the decreased activity of the midge *Glyptotendipes pallens* exposed to cadmium (Heinis et al., 1990). This reaction may be a strategy of "waiting" until the pollution has passed by, as discussed by Gerhardt (1996). Besides effects on behavior, none of the tested sublethal effect parameters proved to be sensitive endpoints in copper toxicity tests with *H. angustipennis* larvae. Although it is known that sublethal copper concentrations can affect food uptake of aquatic organisms (Weeks, 1993), for *H. angustipennis* this was only observed in 48 h survival experiments. Sublethal effects of copper on larval growth have been described for (for example) *C. riparius* (Timmermans et al., 1992) and the mayfly *Epeorus latifolium* (Hatakeyama, 1989). The absence of effects on the growth of *H. angustipennis* larvae in this study is likely due to the length of the life-cycle of this species (3 months under laboratory conditions (Greve et al., 1998)) in relation to the relative short exposure time, resulting in larval growth below the detection limit of microscopic measurements.

For diazinon, the only sublethal effect observed in this study was an increase in duration and variability of ventilation periods of fifth instar *H. angustipennis* larvae, measured with the impedance converter technique. A similar response was also found for *Glyptotendipes pallens* exposed to cadmium (Heinis et al., 1990). For *H. angustipennis* this response was, however, only observed at the highest diazinon concentration tested (20 μg/L), which is 10 times higher than the 48 h LC50 for first instars. At 20 μg/L, altered behavior was also observed visually in short-term survival tests with fifth instar *H. angustipennis* larvae exposed to diazinon (Stuijfzand, 1999). With the impedance converter, no effects on time spent on ventilation, other activities or inactivity were observed. Since AChE inhibition suggests a direct mechanistic relationship between the nervous system and behavioral responses, it is assumed that low doses of diazinon do induce significant behavioral responses. A strong behavioral response was observed in experiments
with *G. lacustris* exposed to diazinon at a concentration far below the LC50 value (Morgan, 1976). Also for *Nereis diversicolor, Cerastoderma edule, Abra abra, Macoma balthica, Scoloplos armiger* (Moehlenberg and Kiorboe, 1983), *Crangon crangon* (Portman, 1972) and *Pterostichus cupreus* (Jensen et al., 1997), altered behavioral patterns were observed after exposure to sublethal insecticide concentrations. The absence of a clear response in the present study may be due to the resolution of the endpoint. Possible effects may be obscured by the crude classification of behavioral patterns. For example, it was not possible to discriminate between natural and toxicant induced locomotion. Also, by recording only 1 h at the end of a total exposure time of 48 h, effects that might have taken place shortly after the start of an experiment remained undetected. Although sublethal effects at ecological relevant concentrations were determined with the impedance converter technique for copper, it remains unknown if behavioral responses to specific toxicants will be detected by the registration method used. The absence of a clear response after exposure to diazinon demonstrates that results of such behavioral tests have to be interpreted with care.

**Environmental relevance**

To gain insight in the relevance of using *H. angustipennis* as a ecotoxicological test organism, the obtained toxicity data were compared with measured concentrations in the river Meuse, where a high mortality of hydropsychids have been observed (Stuijfzand, 1999). Since the sensitivity to toxicants under field conditions may be different from laboratory observations, for example by the presence of other stress factors, differences in water characteristics or inter- and intraspecific interactions, this extrapolation to the field-situation remains however speculative. A more or less continuing load of low copper (average of 5 μg/L, with incidental peaks of 60 μg/L) has been noted in the river Meuse over the five past years (RIWA, 1993-1997), thereby exceeding the Dutch environmental standard of 3.9 μg/L (Dutch Ministry of Transport, Public works and Water management, 1997). The highest copper concentrations (60 μg/L) did not reach concentrations at which acute lethal effects on first instar *H. angustipennis* larvae may occur (168 h LC50 502 μg/L), but exceeded the behavioral effect level (48 h EC50 ventilation 17 μg/L). Bayley et al. (Bayley et al., 1995) demonstrated that altered behavior of the adult carabid beetle *Pterostichus cupreus* is associated with copper-induced internal structural damage during larval development and
therefore expresses a prolonged or permanent effect. Because it reduces the fitness of organisms (Blaxter and Ten Hallers-Tjabbes, 1992), changes in behavior may predict potential life-cycle effects. Indeed, the low 48 h EC50 value for altered activity patterns presented here for *H. angustipennis* (17 μg/L) may be indicative of chronic effects, because in life-cycle tests with the caddisfly *Clistoronia magnifica*, Nebeker *et al.* (1984) demonstrated a significant reduction in adult emergence at 13 μg Cu/L and a prevented life-cycle completion at 17 μg Cu/L. From monitoring studies it is known that concentrations as low as 12-32 μg Cu/L strongly reduce the abundance and diversity of aquatic insect species (Clements *et al.*, 1988; Schulteis *et al.*, 1997). In addition, the isopod *Asellus aquaticus* appeared to be unable to survive during chronic exposure to 5 μg Cu/L (De Nicola Giudici *et al.*, 1988) and the midge *Polyphemus nubifer*, exhibited a decreased emergence at 10 μg Cu/L (Hatakeyama, 1988). Sublethal copper doses may even have prolonged ecological consequences, as demonstrated in microcosm experiments in which benthic macro-faunal recovery took up to 1 year after contamination with copper stopped (Hall and Frid, 1995). Based on these literature findings and results of the present study, life-cycle effects on this caddisfly species may be expected after chronic exposure to the average load of copper in the River Meuse (5 μg/L).

In contrast to copper, incidental peak concentrations of diazinon in the River Meuse (1 μg/L) do reach levels at which acute lethal effects on first instar *H. angustipennis* larvae occur (96h LC50 1 μg/L). Peak concentrations of diazinon are caused by direct discharges and runoff from agricultural land and usually occur in combination with other organophosphorous insecticides (RIWA, 1993-1996; RIZA, 1996) which results in additive toxicity (Bailey *et al.*, 1997). In the River Meuse, pesticides are present in more than 15 % of all measurements (RIZA, 1996). Aquatic species, that require stable environmental conditions because of their long life-cycle, are likely not able to maintain populations in such frequently disturbed rivers. This may explain the absence of *H. angustipennis* in the River Meuse (Bij de Vaate, 1995; Ketelaars and Frantzen, 1995). Sublethal parameters gave no indication for adverse effects of low doses of diazinon to *H. angustipennis* larvae after chronic exposure. Therefore, long-term exposure to ecologically relevant background concentrations of diazinon (< 0.1 μg/L; (RIWA, 1993-1996)) have to be studied in more detail.
Conclusions

It is concluded that hydropsychid species, using the standardized short-term survival and behavioral tests developed in this study, are useful tools for analyzing effects of individual toxicants present in the large European rivers. Although maintaining a steady laboratory culture is laborious (Greve et al., 1998), the low short-term LC50 values stress the importance of incorporating such autochthonous species in a risk assessment. In this way, different stress factors can be discerned and the water quality conditions for ecological rehabilitation can be defined.
3.2 Sensitivity of characteristic riverine insects, the caddisfly *Cyrnus trimaculatus* and the mayfly *Ephoron virgo*, to copper and diazinon

**Summary**

This study reports the effects of two model toxicants, copper and diazinon, on two characteristic riverine insect species, the caddisfly *Cyrnus trimaculatus* and the mayfly *Ephoron virgo*. It was demonstrated that these species are very sensitive to both compounds in comparison with aquatic insects traditionally used in ecotoxicity tests. For diazinon, the 96 h LC50 value of *Cyrnus trimaculatus* (1.1 µg/L) is lower than for any other insect species known from literature and for copper it was demonstrated that *Ephoron virgo* is among the most sensitive aquatic insect species. The observed low LC50 values stress the importance of using these indigenous species in assessing the risk of environmental contaminants in large European rivers and in defining conditions for ecological recovery.

**Introduction**

During the previous century, biodiversity of large river ecosystems in Western Europe has declined strongly as a result of both habitat loss and water quality deterioration. Rehabilitation of these rivers is nowadays a major concern of environmental management and to this purpose several action programs have been started (like the 'Rhine Action Program', which was set up in 1987) or are being initiated. To support such programs test organisms are needed that indicate the progress of recovery. The selection of these test organisms should be based on both the sensitivity to stressors and the representativity for the ecosystem of concern. For large rivers, in particular the number of typical riverine insect species, such as mayflies, stoneflies and caddisflies, has been greatly reduced (Nijboer and Verdonschot, 1997; Bij de Vaate, 1995; Ketelaars and Frantzen, 1995) and therefore such species play a key role in assessing the ecological status of river communities. However, the use of such indigenous insect species in standardized monitoring programs is still hampered by a lack of ecotoxicological knowledge, limiting the possibility to explain why they...
Riverine insects coping with selected contaminants are present or absent. The present project aims therefore to determine the sensitivity of three riverine insect species (the caddisflies *Hydropsyche angustipennis* and *Cyrrus trimaculatus* and the mayfly *Ephoron virgo*) to various environmental stressors. Within this framework, the present study describes the short-term effects of two model toxicants, copper and diazinon, on the caddisfly *C. trimaculatus* and the mayfly *E. virgo*, following a previous study on *Hydropsyche angustipennis* (Van der Geest et al., 1999).

*E. virgo* is a mayfly species typical for large rivers (Kureck, 1996). *C. trimaculatus* usually appears in the lower reaches of large rivers, but also occurs in ponds and lakes (Edington and Hildrew, 1981). In undisturbed river systems, both species play an important ecological role, for example in decomposing organic material or as predator and as a food source for fish and birds. In polluted rivers, like the River Meuse at the Dutch-Belgian border, however, these species have disappeared during the previous century and not yet returned (Bij de Vaate, 1995). Based on their past and present distribution, these insects are likely to be useful test species for indicating ecological recovery.

In order to gain insight in the sensitivity of these species to environmental contaminants, the effects of copper and diazinon were determined in newly developed standardized short-term ecotoxicity tests (Greve et al., 1998; Greve et al., 1999). These compounds were selected as model toxicants based on their occurrence in large European rivers (RIWA, 1993-1997) and differences in mode of toxicity. By testing these different model toxicants it is expected that basic ecotoxicological information for *E. virgo* and *C. trimaculatus* will become available. This information will be compared with ecotoxicological data from literature, in order to determine the sensitivity of both species relative to other organisms. In this way, the potential role of the newly developed ecotoxicity tests in environmental risk assessments will be evaluated.

**MATERIALS AND METHODS**

*Toxicity tests*

The effects of copper and diazinon on larvae of the caddisfly *C. trimaculatus* and the mayfly *E. virgo* were estimated by determining the sur-
vival after 96 h exposure to different concentrations of both compounds in standardized laboratory tests (Greve et al., 1998; Greve et al., 1999). Each experimental treatment consisted of a glass vessel (180 mL) filled with 100 mL Dutch Standard Water (DSW; (NEN, 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). For C. trimaculatus, ten 20-25 day old second instar larvae, originating from a laboratory culture (Greve et al., 1998), were placed in each vessel and fed with 1 mL of a suspension of 150 mg dried and ground Urtica, 75 mg Trouvit, 40 mg Tetraphyll and 2.5 mL fresh algae (Scenedesmus sp.) in 25 mL DSW to avoid mortality by starvation. During the experiments with C. trimaculatus, all vessels were continuously but gently aerated. For E. virgo, twenty 2 day old larvae, originating from field collected egg masses (Greve et al., 1999), were placed in each vessel and fed with 0.1 mL of a suspension of 750 mg dried and ground Urtica in 25 mL DSW. In these tests, no aeration was supplied. In all experiments, vessels were closed with perforated plastic foil in order to avoid evaporation, a 16:8 h light dark regime was provided and the temperature was maintained at 20 °C.

Toxicants were added at the start of the experiments. Copper was added as CuCl₂ (Titrisol, Merck) and diazinon (derived from Luxan Inc.; 99.7 % purity) was added using a generator column derived stock solution (Bleecker et al., 1998). For both compounds, a series of concentrations (including controls) was tested at least in duplicate.

**Chemical analysis**

Water samples (3 x 1 mL in copper experiments; 1 x 10 mL in diazinon experiments) were taken after 1 h and at the end of each experiment in order to determine 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 (Perkin-Elmer 1100B equipped with an impact bead and Perkin-Elmer 5100PC/HGA600/AS60 equipped with Zeeman background correction respectively) after acidification with 20 μL 65 % nitric acid (Merck; P.A.). Since no filtration 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.
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. Analysis of samples from the *E. virgo* experiments was conducted on a GC-MS according to (Van der Geest *et al.*, 1999). For the analysis of samples from the *C. trimaculatus* experiments, this method was replaced by GC-NPD measurements which are equally effective but less expensive and time consuming. Samples were measured 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 the start and the end of each survival test, average exposure concentrations were calculated assuming an exponential decrease of the toxicant concentration over time.

*Data analysis*

Survival was expressed as percentage of the corresponding controls and plotted against the actual toxicant concentration in the water. From the obtained dose-response plots, LC10 and 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):

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

in which \(Y\) = survival ( %), \(c\) = survival in control (set to 100 %), \(a = \log\) LC50 (µg/L), \(b\) = slope and \(X = \log\) concentration (µg/L).
RESULTS AND DISCUSSION

Comparative ecotoxicity

The mayfly *Ephoron virgo* and the caddisfly *Cygnus trimaculatus* appeared to be suitable organisms for short-term toxicity testing of individual toxicants. Control survival was within acceptable limits (average after 96 h respectively 98 and 78 %) and clear dose-response relationships were observed for survival after 96 h exposure to both diazinon (figure 3.8) and copper (figure 3.9). From these results, LC10 and LC50 values and the corresponding 95 % confidence limits based on measured toxicant concentrations were calculated (table 3.2).

![Figure 3.8](image)

**Figure 3.8.** Survival of 20-25 day old *Cygnus trimaculatus* and 1-2 day old *Ephoron virgo* larvae after 96 h exposure to different concentrations of diazinon, plotted as percentages of the corresponding controls against measured diazinon concentrations in μg/L. The lines represent curve-fits after Haanstra *et al.* (1985).
Riverine insects coping with selected contaminants

Figure 3.9. Survival of 20-25 day old *Cymnus trimaculatus* and 1-2 day old *Ephoron virgo* larvae after 96 h exposure to different concentrations of copper, plotted as percentages of the corresponding controls against measured total copper concentrations in µg/L. The lines represent curve-fits after Haanstra *et al.* (1985).

Table 3.2. 96 h LC10 and LC50 values for diazinon and copper. 95 % confidence limits are given in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>96 h effect concentrations (µg/L)</th>
<th><em>Cymnus trimaculatus</em></th>
<th><em>Ephoron virgo</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diazinon</strong></td>
<td>LC10: 0.2 (0.1-0.6)</td>
<td>5.3 (3.0-9.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LC50: 1.1 (0.7-1.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Copper</strong></td>
<td>LC10: 38 (6-225)</td>
<td>61 (49-77)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LC50: 759 (402-1433)</td>
<td>77 (71-84)</td>
<td></td>
</tr>
</tbody>
</table>

The 96h diazinon LC50 value for *C. trimaculatus* is ca. 10 times lower than for *E. virgo*, but for copper the reverse was found and *E. virgo* appeared to be ca. 10 times more sensitive than *C. trimaculatus*. Such species and toxicant specific variation in sensitivity is, however, even described for taxonomically closely related species. Pantani *et al.*, (1997) assessed the acute toxicity of 26 chemicals towards two amphipod...
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species, G. italicus and E. tibaldii. It was demonstrated that for 15 compounds E. tibaldii was the most sensitive species and that for 8 compounds G. italicus was the most sensitive species. This variation demonstrates that a ranking of sensitive and tolerant species is highly toxicant dependent and emphasizes the need of incorporating more than one model toxicant when the sensitivity of species is compared. Therefore, for both copper and diazinon a comparison with effect concentrations from literature is made to gain insight in the sensitivity of both species relative to other organisms. Figure 3.10 shows the 96 h diazinon LC50 values for all freshwater macro-invertebrate and fish taxa listed in the U.S. EPA aquatic toxicity database AQUIRE (Aqua/Info, 1997). Large differences in sensitivity of different species to diazinon are apparent: the 96h LC50 of diazinon for the most tolerant species listed, the fish Carassius carassius, and for the most sensitive species, the amphipod Gammarus fasciatus, are respectively 12267 and 0.2 μg/L (Bathe et al., 1975; Johnson and Finley, 1980). In general, insects and crustaceans are the most sensitive taxa and fish, molluscs and oligochetes are the least sensitive taxa to this insecticide. This ranking of sensitivities based on laboratory derived short-term LC50 values is generally confirmed by mesocosm studies in which the effects of organophosphorous insecticides on aquatic communities have been studied: in experimental ditches treated with diazinon (Arthur et al., 1983) and chlorpyrifos (Van den Brink et al., 1996) it was demonstrated that indeed arthropods suffer most from the presence of organophosphorous insecticides. The higher sensitivity of arthropods to diazinon is as expected since the individual species of this group are related to the target organisms of this insecticide. However, also between taxonomically closely related species large differences exist: for example, Gammarus fasciatus is almost 1000 times more sensitive to diazinon than Gammarus lacustris (Johnson and Finley, 1980; Sanders, 1969). Within the aquatic insects, a difference of two orders of magnitude in LC50 values is noted between the most sensitive and the most tolerant species. The three insect species selected in the present project, the mayfly E. virgo, the caddisfly C. trimaculatus (this study) and the caddisfly Hydropsyche angustipennis (Van der Geest et al., 1999), indicated in figure 3.10 by arrows, are among the most sensitive species to diazinon. The 96 h LC50 for Cyrrus trimaculatus is even lower than for any other insect species known from literature.
Riverine insects coping with selected contaminants

Gammarus fasciatus
Daphnia magna
Cymnus trimaculatus
Gammarus pseudolimnaeus
Chironomus tentans
Hyalella azteca
Ephoron virgo
Hydropsyche angustipennis
Acronoeuria ruralis
Asellus communis
Baetis intermedius
Pteronarcys californica
Paraleptophlebia pallipes
Physa gyrina
Lestes congener
Anguilla anguilla
Chironomus riparius
Leuciscus idus
Gammarus lacustris
Lepomis macrochirus
Notemigonus crysoleucas
Salmo trutta lacustris
Salvelinus namaycush
Salvelinus fontinali
Oncorhynchus mykiss
Carassius auratus
Channa punctatus
Jordanella floridana
Poecilia reticulata
Heteropneustes fossilis
Oncorhynchus clarki
Brachydanio rerio
Cyprinus carpio
Pimephales promelas
Lumbriculus variegatus
Ameiurus melas
Ictalurus sp
Salmonidae
Gillia altiss
Carassius carassius

Figure 3.10. Acute toxicity of diazinon (96 h LC50 values in µg/L) to several aquatic taxa. Data from Aqua/Info, 1997. If more than one LC50 value was listed in Aqua/Info for a specific species, the average value is presented and error bars indicate the lowest and the highest reported value. The arrows indicate test organisms used in this study.
Figure 3.11. Acute toxicity of copper (96 h LC50 values in μg/L) to several aquatic organisms. Data from Aqua/Info, 1997. If more than one LC50 value was listed in Aqua/Info for a specific species, the average value is presented and error bars indicate the lowest and the highest reported value. The arrows indicate test organisms used in this study.
Also for copper a comparison with 96 h LC50 values from literature for other aquatic taxa is made (figure 3.11) and again a wide variety of effect concentrations, ranging from 0.16-13560 μg/L for respectively the oligochete *Tubifex tubifex* and the fish *Pimephales promelas*, is noted (Das *et al.*, 1993; Brungs *et al.*, 1976). In contrast to diazinon, however, no general classification of sensitive and tolerant taxa can be made: in all taxa, both sensitive and tolerant representatives are found. The three species selected in this study (indicated again by arrows in figure 3.11) are among the most sensitive insects, and in the middle range of all copper toxicity data.

Although comparative toxicity data as presented above provide important information on variation in responses of aquatic organisms, one must keep in mind that differences in sensitivity between species can be strongly influenced by several (a)biotic factors and that such a ranking is therefore to some extend speculative. One major factor influencing toxicity, for example, is the size or age of the test organism (for example Hutchinson *et al.*, 1998; Legierse, 1998). For diazinon, it is demonstrated that differences between first and last instar of one species are often even bigger than differences between species (Stuijfs, 1999). But also other factors like the exposure regime (Kallander *et al.*, 1997), water characteristics (Bailey *et al.*, 1997) and (bio)transformation of the parent compound (Keizer *et al.*, 1993) can strongly influence the response of individual species to diazinon. For copper, physico-chemical and environmental water characteristics like pH (Meador, 1991), hardness (Winner and Gauss, 1986) and presence of organic matter (Stuijfs, 1999; Meador, 1991; Pantani *et al.*, 1995) can possibly have an even stronger impact on toxicity. These modifying factors not only complicate the ranking of sensitive and tolerant species (groups) as presented in figures 3.10 and 3.11, but also limits the applicability of using standard test organisms as substitutes for sensitive indigenous species in for example bioassays. Since these responses are species specific, standard (substitute) test species may react in another way to natural environmental characteristics than indigenous species. It is therefore argued to use species that are representative for the ecosystem of concern and that risk assessments should be based on the sensitivity of these species.
Environmental relevance

The insect species selected in the present project were among the first who disappeared from contaminated rivers like the River Meuse and among the last who return after recovery takes place. Although only two model toxicants were tested, indications were found that indeed water quality hampers the distribution of these species: in the River Meuse, a more or less continuing load of low copper (average of 5 µg/L, with incidental peaks of 60 µg/L) has been noted over the five past years (RIWA, 1993-1997), thereby exceeding the Dutch environmental standard of 3.9 µg/L (Ministry of transport, public works and water management, 1997). The highest peak concentrations are within the effect concentration range of both species (LC10 of 38 and 61 µg/L copper for C. trimaculatus and E. virgo respectively). For diazinon, incidental peak concentrations (ca. 1 µg/L) in the River Meuse also exceed the Dutch environmental standard (0.04 µg/L; Ministry of transport, public works and water management, 1997) and reach levels at which acute lethal effects on first instar C. trimaculatus larvae may occur (96h LC10 0.2 µg/L). In more than 15 % of all measurements in the River Meuse pesticides are present (RIZA, 1996) and since peak concentrations of diazinon usually occur in combination with other organophosphorous insecticides (RIWA, 1993-1997; RIZA, 1996) an even higher impact may be expected that based on diazinon toxicity only.

The low short-term LC50 values of the three species selected in this study, illustrate the sensitivity of these species to environmental contaminants and are in agreement with their field observed distribution pattern. This stresses the importance of using these species in assessing the risk of environmental contaminants in large rivers.
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PART 4.

SURVIVAL OF RIVERINE INSECTS UNDER COMBINED STRESSORS

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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).
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 CaCl_2\cdot2H_2O, 180 mg MgSO_4\cdot7H_2O, 100 mg NaHCO_3 and 20 mg KHCO_3 per liter demineralized water (pH 8.2, hardness 210 mg/L CaCO_3, 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 CuCl_2 (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 Kaleidagraphe (Synergy Software) using the logistic response model after Haanstra et al. (1985):
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\[ Y = \frac{c}{1 + e^{b(X - a)}} \]  
(equation 1)

in which \( Y \) = survival (\%), \( c \) = survival in control (set to 100%), \( a \) = log LC50 (μg/L), \( b \) = slope and \( X \) = log concentration (μg/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_{MIX} was calculated following the method described above. The joint toxic effect of copper and diazinon was defined as concentration additive (LC50_{MIX} = 1 TU), or as more or less than additive (LC50_{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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**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).

**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>
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<tr>
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<th>96 h LC50 values (µg/L)</th>
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<td>diazinon</td>
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<tr>
<td>previously</td>
<td>11.7 (9.7-14.1)</td>
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<td>this study</td>
<td>6.9 (4.7-10.1)</td>
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<td>together</td>
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Figure 4.3. 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 based on re-calculated LC50 values for the individual compounds.

**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
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:

\[
\text{LC}_i = \frac{\ln\left(\frac{100}{100 - i} \right) - 1}{b} + a \quad \text{(equation 2)}
\]

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 \(\text{LC}_i\) 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
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.

**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.

**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\(_{10}\); 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\(_{10}\); 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
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commonly observed (Deneer et al., 1998; Kö nemann, 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.
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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
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.

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 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 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.
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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 (\(\mu\)g/L), \(b\) = slope and \(X\) = log concentration (\(\mu\)g/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](image-url)
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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
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](image)

**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.
Environment 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.

![Graph showing minimum oxygen concentration, 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).](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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Understanding the distribution and abundance of species is one of the cornerstones of ecological science. Therefore, insight is required in the resource requirements of species, their life history, intra- and inter-specific interactions and their response to environmental conditions (Townsend et al., 2000). Similarly, understanding the distribution of species as affected by pollution requires a specific analysis of the parameters determining the persistence of species in disturbed environments. Here it will be attempted to evaluate whether traditional ecological concepts on the distribution of species can be used to analyze the impacts of toxicants. The insight generated in this thesis on specialized river insects coping with large variations in water quality will be reviewed.

In many studies, physical characteristics shaping differences in riverine habitats, are often recognized as major factors governing the distribution
of species (for example Allen, 1995; Ward, 1992; Leland and Fend, 1998; Rempel et al., 2000). Riverine ecosystems consist of a diverse array of habitats along the longitudinal axis of a river, varying profoundly in many physical factors (Allen, 1995; Ward, 1992). In undisturbed lotic ecosystems aquatic insects (and other aquatic species) are (more or less) predictably structured according to longitudinal gradients in, for example, current velocity, substrate composition or oxygen regime (for example Hynes, 1970; Statzner et al., 1988; Charvet et al., 2000). Also resource requirements of riverine organisms predict the shifts in the relative abundance of feeding guilds over the longitudinal profile of a river, as described by the river continuum concept (Vannote et al., 1980).

The ability of a species to maintain a viable population in its specific river habitat, however, depends on its response to the prevalent environmental conditions (Townsend et al., 2000). The ways in which tolerances and requirements of species interact and match the conditions and resources provided by certain habitats are addressed in the niche concept: numerous environmental conditions together build a multidimensional hypervolume within which a certain species can maintain a viable population (Hutchinson, 1959). On the large longitudinal scale of rivers ranging from groundwater-fed streams to low-land rivers, temperature is, for example, considered to be a factor of major importance in determining the distribution of species by influencing various life-cycle aspects (Ward, 1992). Insects can maintain viable populations only within certain temperature limits, as demonstrated for caddisflies by Lowe and Hauer (1999). On the other hand, an increase in the temperature, caused by human influences such as cooling water discharges, has been shown to enable the invasion of (exotic) species from other climate zones (Rajagopal et al., 1999). Thus, the habitat characteristics and (complex interactions between) the numerous environmental conditions add up to a set of locally defined selective forces which act as 'filters' selecting certain (indigenous) species from a much larger pool of potential inhabitants.

Can the presence of contaminants also be thought of as being dimensions in the multidimensional niche? Consequently, can the impact of toxicants be analyzed analogously to the analysis of natural conditions? Since species specific responses of aquatic insects to different degrees of pollution are apparent (chapter 2), toxicants could act analogous to other dimensions, like for example temperature, in defining conditions that discriminate the distribution of species. The absence of species in the
severely polluted large European rivers in the 1960/70s and the mortality of aquatic organisms after the Sandoz accident in 1986 (chapter 1), clearly illustrate that the temporal persistence of species could indeed be affected by the presence of toxicants in the field. Therefore we argue that a disturbance by environmental contaminants, due to anthropogenic activities, can cause niche dimensions to vary outside of the normal range or introduce previously non existing dimensions. Subsequent shifts in the abundance of species could be expected, therewith impairing the structure or functions of the riverine ecosystem. However, whereas changes in for example current velocity, temperature, and light result in a (more or less) predictable annual cycle of seasonal succession of species (Patrick, 1975; Allan, 1995), the run-off of environmental contaminants superimposes a large proportion of unpredictability on this regularity: minute changes in concentrations of chemicals, due to for example accidental spills, could result in large changes in niches and therefore in the dominance of opportunistic versus specialized species. Indeed in the present thesis it was demonstrated that the maximum peak concentrations of organophosphorous insecticides in the field are in the same order as the observed effect levels for sensitive juvenile river insects determined in the laboratory (chapter 2). Riverine insects, that require stable environmental conditions because of their relative long life-cycle, are likely not able to maintain populations in rivers which are frequently disturbed by such accidental peak concentrations of contaminants. Simultaneously, this example shows that spatial and temporal aspects of pollution in relation to the life-cycle characteristics of the organisms, determine the extend to which contaminants impair the persistence of species. For example, not only the concentration and kind of chemical spilled are determining the effects on biota, but also the frequency and seasonal timing at which the organisms are exposed to it. Considering this relationship between chemical stressors and life-cycle characteristics of the exposed riverine species, also chemicals present during longer periods at concentrations below acute lethal effect levels could prevent species to persist in certain habitats. For copper, maximum field concentrations do not reach acute effect levels (chapter 2), but the average load in for example the river Meuse is in the same order as the observed behavioral effect levels for *H. angustipennis* (chapter 2). These behavioral responses are likely to reduce the fitness of organisms (Blaxter and Ten Hallers-Tjabbes, 1992) and therewith indicative for potential life-cycle effects. Conditions that are steadily sub-optimal such as a continuous exposure to low concentrations of copper, as well as peak
Concluding remarks

exposures such as pesticide spills, may therefore limit the distribution of species. Especially the species with a sensitivity above average and/or species with relative long life-cycles are prone to local extinction.

It can be expected that chemicals interact with each other as well as with other environmental conditions. Mixture toxicity is an example of such an interaction: in the present thesis, experiments revealed that the tested model toxicants contribute to mixture toxicity below their individual effect levels (chapter 4.1). Adverse effects in the field are, therefore, not determined by the effects of (incidental discharges of) individual compounds only. In the field, mixture toxicity has indeed been demonstrated to be responsible for adverse effects on riverine invertebrates (Stuijtzand, 1999). Similarly, interactions between environmental contaminants and other limiting conditions can be expected. Considering the large differences in response types to these different dimensions, such interactions can be expected to appear in a variety of ways: in chapter 4.2 it was demonstrated that the combined effect of copper and low oxygen is much higher than can be expected based on the effects of both factors separately. Since in the field the lowest oxygen concentrations often coincide with the highest toxicant concentrations, it was concluded that ‘multiple stress’ actually occur in large rivers carrying complex pollution and that under such conditions adverse effects of chemicals on riverine biota can be unexpectedly high. Simultaneously it was shown, however, that such interactions between multiple stressors are compound specific: no synergistic effects of a combination of lowered oxygen and diazinon were found (chapter 4.2). Similar contrasting observations have been reported for the effects of temperature, salinity and food availability on the toxicity of chemicals (Heugens et al., in press). On the other hand, there are also variables in complexly polluted rivers, such as elevated concentrations of nutrients or organic waste, that may mask or (over-) compensate the response of certain organisms to toxicants (De Ruiter and Hendriks, 1996; Dubé and Culp, 1996; Stuijtzand, 1999).

Based on the results of the experiments presented in this thesis, we argue that in rivers suffering from a complex pollution, chemicals and other environmental factors act together in re-structuring the niches of benthic invertebrates. This is consistent with the manifold observations on changing biodiversity in polluted rivers (for example Doledec et al., 1999; Delong and Brusven, 1998). When general ecological principles are
used to put our limited understanding of the impact of pollution on specialized river insects in perspective, it becomes clear that spatio-temporal aspects of environmental contaminants in relation to life-cycle characteristics of the organisms are critical in regulating the persistence of species.

REFERENCES

Concluding remarks


PART 6.

SUMMARY, SAMENVATTING AND ACKNOWLEDGEMENTS

SUMMARY

A century ago the large river ecosystems in western Europe were still characterized by a high biodiversity, especially of the benthic communities. Due to pollution and habitat deterioration, many large rivers ecosystems show nowadays a low biodiversity and opportunistic species are dominant. Especially the number of riverine insects (like mayflies and caddisflies) has been strongly reduced, and they could therefore play a key role in assessing the ecological status of aquatic communities and in indicating ecological recovery. The lack of basic ecotoxicological and ecological data on these specific insects is, however, limiting the use of these species as sensitive tools for the ecological management of large rivers. Since ecological recovery and rehabilitation of these aquatic ecosystems have become major objectives of many governmental institutes, industries, drinking water companies and NGOs, it becomes increasingly important to know how more specialized species can benefit
from improving environmental conditions and what environmental standards have to be fulfilled to restore some of the original biodiversity.

It has been demonstrated that the currently used methods to measure the water quality of large rivers, do not always detect the possible risks of contaminants for river ecosystem communities. Therefore, there is a need for test organisms and bioassays which can be used for ecological water quality assessments other than the traditional test organisms. To this purpose, three different riverine insects (the caddisflies *Hydropsyche angustipennis* and *Cyrnus trimaculatus* and the mayfly *Ephoron virgo*) were selected as model species (chapter 1). These selected model species are to be developed as sensitive tools for the ecological management of rivers. Before these species can be used to as new tools in ecotoxicological water quality assessment studies, investments in rearing and test development are necessary. Therefore, in chapter 2 methods for the culturing of the two caddisfly species and a method for collecting and storing eggs of the mayfly were developed. Therewith, a continuous supply of young larvae with a known history and age was ensured. Secondly, standardized ecotoxicity tests were developed for the three selected species. Based on different validation experiments, it is concluded that the newly developed ecotoxicity tests using newly hatched larvae of the selected riverine insects are reliable, reproducible and easy to perform, when using the effect parameter survival after 96 hours.

Using these newly developed ecotoxicity tests, this thesis aimed to generate insight in how specialized river insects cope with large variations in water quality.

Chapter 3 reports on the sensitivity of the three selected species to environmental contaminants. To this purpose, two model toxicants (copper and diazinon) were selected. Mortality of first instar larvae has been shown to be a reliable endpoint and it was demonstrated that the three selected insects are among the species most sensitive to diazinon. The 96 hour LC50 for *C. trimaculatus* is even lower than for any other insect species known from literature. The concentrations at which effects on the selected insect species were detected in the laboratory, were also relevant to the maximum concentrations of organophosphorous insecticides in (for example) the River Meuse, illustrating that such accidental peak concentrations are limiting the distribution of riverine insects in
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the field. For copper, it was shown that the three selected insect species are among the most sensitive insects, and in the middle range of all copper toxicity data. Maximum field concentrations of copper, however, do not reach levels at which acute effects are observed. Therefore, a behavioral test, using the impedance conversion technique, was developed to determine sublethal effects of toxicants. Using this technique, behavioral effects of low doses of copper were indeed demonstrated for *H. angustipennis*: the 48h EC50, based on a changed behavioral pattern, determined using 5th instar larvae was ca. 150 times lower than the 48h LC50 value for 1st instar larvae. These short-term behavioral responses were found to be indicative of adverse effects of chronic exposure to ecological relevant low doses of copper.

To determine the potential impact of environmental contaminants in nature, single substance laboratory tests under favorable conditions (optimal temperature, oxygen regime etc.) are, however, no longer sufficient: the presence of mixtures of chemicals and multiple stressors in the complexly polluted large rivers hampers the translation of results from standard laboratory tests to field situations, and insight in combined effects is therefore required. It was hypothesized that the joint adverse effects of mixtures of chemical and multiple stressors may be a steering factor in the distribution of riverine insect species. To validate this hypothesis, in chapter 4.1 the effects of a mixture of copper and diazinon on larvae of the mayfly *E. virgo* were determined. It was 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 was higher than that of the individual compounds. At the lowest calculated effect level in the mixture (LC10), the concentrations of copper and diazinon were relevant to the concentrations of both compounds as measured in for example the River Meuse. In this chapter, also a contribution has been made to the theoretical field of mixture toxicity testing: a method is proposed to gain insight in the influence of differences in the shapes of dose response relationships of the separate compounds and that of the mixture on mixture toxicity, by judging additivity at different effect levels.

The aim of chapter 4.2 was to determine the combined effects of toxicants and oxygen depletion. To this purpose, larvae of the mayfly *E. virgo* were exposed to the two model toxicants, (copper and diazinon)
under normoxia and hypoxia (50 % air saturation). Based on the results of these experiments it is concluded that the combination of toxicants and lowered oxygen may have a stronger impact than can be expected based on the adverse effects of the individual factors. Also it was demonstrated 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 this chapter.

The newly developed ecotoxicity tests with three selected riverine insects combine the representativity of the test species for pristine riverine ecosystems with a relative high sensitivity, observed after exposure to field collected (pore) water samples (chapter 2), individual toxicants (chapter 3), mixtures of toxicants (chapter 4.1) and toxicants in combination with low oxygen (chapter 4.2). Based on these criteria, and on the newly developed practical provisions, such as the availability of a continuous supply of young (and hence more sensitive) larvae with a known history and age (chapter 2), it is concluded that these bioassays are useful tools in site-specific water quality studies. The low short term LC50 values for the three species selected in this thesis, illustrate the sensitivity of these species to environmental contaminants and are in agreement with their field observed distribution pattern. This stresses the importance of using species which are representative for pristine rivers, such as the selected insect species, in assessing the risk of environmental contamination in large rivers. Based on the results of chapters 4.1 and 4.2, we also argue that attention needs to be paid to more environmental realistic non-optimal conditions in toxicity testing, such as mixture toxicity and joint effects of multiple stressors, to adequately fulfill the needs of ecological recovery programs.

In the concluding remarks (chapter 5), it was attempted to evaluate whether traditional ecological concepts on the distribution of species can be used to analyze the impacts of toxicants. Environmental contaminants were thought of as being dimensions in a multidimensional niche, within which a certain species can maintain a viable population. Subsequently, the impact of toxicants was analyzed analogously to the analysis of natural conditions, like for example temperature. Based on the results of the toxicity tests in this thesis, it was argued that a disturbance by environmental contaminants can cause niche dimensions to vary outside of the normal range or introduce previously non-existing dimensions. In complexly polluted rivers, chemicals and other environ-
mental factors act together in re-structuring the niches of benthic invertebrates. Subsequent shifts in the abundance of species could be expected, therewith impairing the structure or functions of the riverine ecosystem.

It was illustrated that the spatial and temporal aspects of pollution in relation to the life-cycle characteristics of the organisms, determine the extend to which contaminants impair the persistence of species: conditions that are steadily sub-optimal such as a continuous exposure to low concentrations of copper, as well as peak exposures such as pesticide spills, may limit the distribution of species. Moreover, especially the species with a sensitivity above average and/or species with relative long life-cycles are prone to local extinction.
Ongestoorde levensgemeenschappen, zoals die van nature voorkomen in stromend water, herbergen een grote soortenrijkdom aan aquatische organismen. Vooral insecten als haften, kokerjuffers en steenvliegen zijn met talloze soorten vertegenwoordigd. In Nederland was een dergelijke insectefauna van oorsprong te vinden in bijvoorbeeld de Maas en de Rijn. Gedurende de vorige eeuw is in veel van de grote rivieren in west Europa het aantal soorten en de diversiteit van aquatische insecten echter sterk terug gelopen. Als gevolg van de bevolkingsgroei, verstedelijking en industrialisatie en de daarmee gepaard gaande lozingen van afvalproducten zijn de flora en fauna in de grote rivieren onder grote druk komen te staan. Daarnaast zijn ten gevolge van de steeds hogere eisen die gesteld werden aan de infrastructuur van deze rivieren (denk hierbij aan grindwinning, oeververharding, het aanleggen van stuwen en kanaliseren ten behoeve van de beroepsvaart) vele natuurlijke habitats verdwenen.

Het herstel van rivierecosystemen is in gang gezet door beleid van diverse overheidsinstellingen, drinkwaterbedrijven en NGO's. Voor het nemen van concreet maatregelen is het echter noodzakelijk om te weten hoe representatieve organismen kunnen profiteren van de verbeterende omstandigheden en aan welke eisen moet worden voldaan om het herstel van natuurlijke ecosystemen mogelijk te maken. Omdat aquatische insecten (zoals bijvoorbeeld kokerjuffers en haften) van oorsprong in rivierecosystemen in een grote verscheidenheid voorkwamen maar tegenwoordig in belaste rivieren niet of nauwelijks meer gevonden worden, kunnen zij een sleutelrol spelen bij het vaststellen van de ecologische status en het sturen en volgen van ecologisch herstel. Om insecten in te kunnen zetten in gestandaardiseerde programma’s moet echter eerst basale ecologische en ecotoxicologische kennis beschikbaar komen.

Eerder onderzoek naar de effecten van waterkwaliteit op levensgemeenschappen in de grote rivieren heeft aangetoond dat de methoden die nu gebruikt worden voor de waterkwaliteitsbeoordeling niet altijd alle risico’s voor het rivier ecosysteem opmerken. Er is dus een behoefte aan nieuwe testorganismen en standaardtests die gebruikt kunnen worden voor een locatie specifieke ecologische beoordeling van de waterkwaliteit. Daarom zijn in dit onderzoek drie verschillende rivier insecten (de kokerjuffers *Hydropsyche angustipennis* en *Cynurus trima-
culatus en de haft Ephoron virgo) geselecteerd als model soorten (hoofdstuk 1). Voordat deze geselecteerde soorten gebruikt kunnen worden in nieuw te ontwikkelen standaard ecotoxiciteitstesten is het ontwikkelen van kweekmethoden voor deze insecten echter essentieel. Daarom zijn in hoofdstuk 2 methoden ontwikkeld voor het kweken van de twee kokerjuffer soorten en voor het vangen en bewaren van bevruchte eieren van de haft. Met de ontwikkeling van deze technieken is een continue aanvoer van jonge larven, met een bekende achtergrond en leeftijd gegarandeerd. Vervolgens zijn nieuwe standaard toxiciteitstesten ontwikkeld die gebruik maken van jonge larven van de drie geselecteerde soorten. Op basis van verschillende validatie experimenten wordt geconcludeerd dat deze nieuwe kortdurende testen (met sterfte als eindpunt) betrouwbare, reproduceerbare en gemakkelijk uit te voeren testen zijn.

Het doel van het in dit proefschrift beschreven onderzoek was om, gebruik makend van deze nieuwe testen, inzicht te verkrijgen in hoe meer gespecialiseerde insecten soorten reageren op de grote variaties in waterkwaliteit van de grote rivieren.

In hoofdstuk 3 is gekeken naar de gevoeligheid van de drie geselecteerde insecten soorten voor twee verschillende toxicanten, koper en diazinon. Koper is een metaal dat als micronutriënt van belang is voor veel organismen, maar in hoge concentraties toxisch kan zijn. Koper wordt in een groot aantal industriële lozingen aangetroffen en het is een component van veel pesticiden. In de grote rivieren is koper continue aanwezig in relatief lage concentraties. Diazinon is een organofosfor-insecticide dat wordt gebruikt tegen een grote verscheidenheid aan insecten. Door het gebruik van diazinon in de landbouw kan de concentratie in omringende wateren tijdelijk sterk toenemen, door directe lozingen en run-off van het land. In Nederland worden van alle vervuilende stoffen vooral bestrijdingsmiddelen, en in het bijzonder de organofosfor-verbindingen, veelvuldig aangetoond in de oppervlaktewateren. De toxische werking van diazinon is een gevolg van de remmende werking op het enzym acetylcholine-esterase, wat leidt tot verkramping van de spieren en uiteindelijk tot sterfte.

Met behulp van deze nieuw ontwikkelde toxiciteits-experimenten zijn de acute letale effecten van deze twee modelstoffen op de drie testorganismen vastgesteld. De uit de experimenten berekende 96 uurs LC50 waarden zijn in vergelijking met andere aquatische insecten uit de
literatuur relatief laag. De 96 uurs LC50 waarde voor \textit{C. trimaculatus} blootgesteld aan diazinon is zelfs lager dan voor enig ander insect beschreven in de literatuur. De concentraties van diazinon waarbij in het laboratorium schadelijke effecten op de geselecteerde insecten werden gemeten zijn relevant voor de piek concentraties van organofosfor-bestrijdingsmiddelen in het veld. Daarom wordt geconcludeerd dat pieken van dergelijke bestrijdingsmiddelen een directe invloed hebben op de verspreiding van representatieve insecten soorten in de grote rivieren. De totale koperconcentraties (gemeten in de periode 1992-1995 in de Rijn en de Maas) overschrijden de in dit onderzoek gemeten LC50- waarden voor de drie testorganismen echter niet. Er zijn dus geen direct negatieve gevolgen van koper voor deze soorten. Om het effect van lage (niet letale) concentraties toxicanten vast te stellen is daarom een methode ontwikkelt om het gedrag van de kokerjuffer \textit{H. angustipennis} onder invloed van toxicanten te registreren. Hiermee is aangetoond dat subletale effecten van koper al optreden bij concentraties die ca. 150 x lager liggen dan letale concentraties. Deze lage effect concentraties van koper liggen in dezelfde orde grootte als de gemeten concentraties in het veld, en zijn indicatief voor mogelijke schadelijke effecten na chronische blootstelling.

Om de schadelijke effecten van stoffen in het veld vast te stellen zijn laboratorium testen met één stof onder optimale condities (optimale temperatuur, zuurstof regime etc.) echter niet toereikend: de aanwezigheid van mengsels van stoffen in combinatie met andere sub-optimale condities (zoals bijvoorbeeld lage zuurstof concentraties) in de grote rivieren bemoeilijken de vertaling van de resultaten van laboratorium studies naar de veld situatie. Op basis van de huidige waterkwaliteits- gegevens en de soortensamenstelling in de grote rivieren verwachten we dat mogelijke schadelijke effecten ten gevolge van mengseltoxiciteit en multi-stress de terugkeer van representatieve rivier insecten kunnen belemmeren. Om deze hypothese te toetsen zijn de hoofdstuk 4.1 de effecten van een mengsel van de twee modelstoffen (koper en diazinon) op \textit{Ephoron} larven onderzocht. In dit hoofdstuk is aangetoond dat beide stoffen een bijdrage leveren aan de totale mengsel toxiciteit onder hun individuele LC10 waarden. In de laagste berekenende concentratie waarbij nog negatieve effecten te meten waren (LC10) waren de concentraties van beide stoffen relevant voor gemeten concentraties in bijvoorbeeld de rivier de Maas. Tegelijkertijd is in hoofdstuk 4.1 een theoretische bijdrage geleverd aan mengseltoxiciteits-
onderzoek: er is een methode voorgesteld om inzicht te verkrijgen in de invloed van verschillen in de hellingen van de dosis-respons relaties van de individuele stoffen en van het mengsel op de beoordeling van mengseleffecten, en daarmee in de toepasbaarheid van het toxic unit concept.

In hoofdstuk 4.2 is gekeken naar de gecombineerde effecten van toxicanten en een verlaagd zuurstof concentratie. Hiervoor zijn jonge Ephorong larven blootgesteld aan de twee modelstoffen (koper en diazinon) onder optimale condities (100 % luchtverzadiging) en sub-optimale condities (50 % zuurstofverzadiging). Op basis van de resultaten van deze experimenten is geconcludeerd dat de gecombineerde effecten veel schadelijker kunnen zijn dan verwacht wordt op basis van de effecten van de factoren afzonderlijk. Omdat ook is aangetoond dat lage zuurstof concentraties in het veld vaak samengaan met verhoogde toxicant concentraties (hoofdstuk 4.2) zijn multi-stress effecten (zoals die aangetoond zijn in dit proefschrift) relevant voor de veld situatie en daarmee mogelijk een belangrijke factor die de verspreiding van rivier fauna kunnen belemmeren.

In de nieuw ontwikkelde ecotoxiciteit experiments met de drie geselecteerde rivier insecten wordt de representativiteit van de testorganismen gecombineerd met een relatief hoge gevoeligheid voor veldverzamelde (porie) watermonster (hoofdstuk 2), individuele toxicanten (hoofdstuk 3), mengsels van toxicanten (hoofdstuk 4.1) en combinaties van toxicanten en verlaagde zuurstof concentraties (hoofdstuk 4.2). Op basis van deze criteria en de nieuw ontwikkelde technieken en producten, zoals laboratorium kweken die zorgen voor een continue aanvoer van jonge (en dus gevoelige) larven met een bekende achtergrond en leefijd (hoofdstuk 2), wordt geconcludeerd dat deze nieuwe testen belangrijke instrumenten zijn voor de locatie specifieke beoordeling van de waterkwaliteit. De lage LC50 waarden illustreren de gevoeligheid van deze soorten en zijn in overeenstemming met het verspreidingspatroon van deze insecten in de grote rivieren. Dit onderstreep het belang van testorganismen die representatief zijn voor onverstoorde systemen (zoals de geselecteerde rivierinsecten) in de waterkwaliteitbeoordeling. De resultaten van de mengseltoxiciteit- and multi-stress experimenten (hoofdstuk 4) benadrukken ook het belang van realistische (suboptimale) condities bij het vaststellen van de schadelijke effecten van stoffen in laboratorium studies.
In hoofdstuk 5 is nagegaan in hoeverre traditionele ecologische concepten over de verspreiding van soorten gebruikt kunnen worden om de effecten van toxicanten op de verspreiding organismen in perspectief te kunnen plaatsen. Toxicanten werden beschouwd als een van de fysisch-chemische randvoorwaarden waarbinnen soorten een populatie in stand kunnen houden. Op basis van de resultaten van de toxiciteits experiments in dit proefschrift word beargumenteerd dat de aanwezigheid van toxicanten nieuwe dimensies in een multi-dimensionale niche kunnen introduceren of bestaande dimensies kunnen veranderen. In complex vervuilde rivieren kunnen (combinaties van) toxicanten en andere omgevingsfactoren leiden tot veranderingen in de niches van benthische organismen, en daarmee tot veranderingen in de soorten-samenstelling, de structuur en de functies van het rivier ecosysteem.

Geconcludeerd wordt dat naast de concentratie van toxicanten en de gevoeligheid van organismen, de mate waarin toxicanten het voorkomen van soorten kunnen beïnvloeden mede bepaald wordt door de ruimtelijke- en tijdsaspecten van de vervuiling in relatie tot de levenscyclus kenmerken van de blootgestelde organismen: zowel condities die gedurende langere tijd suboptimaal zijn (zoals bijvoorbeeld een chronische blootstelling aan lage koper concentraties) als kortdurende gebeurtenissen (zoals kortdurende piek concentraties van bestrijdingsmiddelen) kunnen de verspreiding van rivieren organismen belemmeren. Soorten met een lange levenscyclus en een meer dan gemiddelde gevoeligheid voor toxicanten (zoals de geselecteerde rivier insecten), worden door langdurige of herhaalde vervuiling negatief beïnvloed.
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