Insects in polluted rivers: an experimental analysis
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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 *Cynurus 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 *Cynurus 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 *Cyrnus 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 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.

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<th><em>H. angustipennis</em></th>
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</tr>
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<td>-</td>
</tr>
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<td>+</td>
<td>+ since '80s</td>
</tr>
<tr>
<td>Rhine tributaries</td>
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<td>+</td>
</tr>
</tbody>
</table>

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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.

![Graph showing the production of males, females, and egg masses](https://via.placeholder.com/150)

**Figure 2.3.** The production of males, females and egg masses (total numbers/week) during one year, in the laboratory culture of *Cymnus trimaculatus.*

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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 they 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 Cyprinus 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 Cyprinus 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).

![Figure 2.4. Dose-response relationships for laboratory reared first instars of *H. angustipennis* exposed to diazinon at different exposure times.](image)

**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
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_Cyrnus 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 Kurcck (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
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.

**Table 2.2.** Historic and recent distribution data of *E. virgo* in the Rivers Rhine and Meuse. (B=Belgium; F=France; NL=Netherlands; SW=Switzerland; G=Germany). Data from: Albarda, 1889; Bij de Vaate *et al.*, 1992; Gysels, 1991; Ketelaars and Frantzen, 1995; Mol, 1987; Schoenemund, 1930; Schöll, 1996; Tittizer *et al.*, 1990.

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</tr>
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</tr>
<tr>
<td>Rhine (NL)</td>
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**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 ºC. 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 ºC. 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 ºC, the diapause was deactivated by transferring the eggs from ± 4 ºC to a temperature of ± 20 ºC. 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 set-up 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)
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; Riool Water Zuiverings 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 (Puttershoeck).

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 (Puttershoeck) 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:
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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 Eindergat-loop, 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 cannot 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>Tungelrooyse 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>Tungelrooyse 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_url)

**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 showing survival vs copper concentration for Ebro (Spain) and Waal (NL) larvae.]

**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 showing survival vs cadmium concentration for Ebro (Spain) and Waal (NL) larvae.]

**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>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>163 (149-177)</td>
<td>sign. (p&lt;0.05)</td>
</tr>
<tr>
<td>Cadmium</td>
<td>367 (257-524)</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 (McCa hon 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


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