DEVELOPMENT AND VALIDATION OF AN ECOTOXICITY TEST USING FIELD COLLECTED EGGS OF THE RIVERINE MAYFLY *EPHORON VIRGO*

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Summary

The diversity of aquatic insects in large European rivers has been strongly reduced during the past 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. This test can be used for determining dose-response relationships for toxicants as well as for testing river water samples.

INTRODUCTION

During the last 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 & 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 *Cyrnus trimaculatus* (Greve *et al*., 1998) and the mayfly species *Ephoron virgo*. The mayfly *Ephoron virgo* is a typical riverine insect that disappeared during this 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. The test was validated by determining a dose-response relationship for cadmium and by testing water samples from several field locations.

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 1). 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 subimago 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 moult their subimago exuviae after which they return to the river to fly horizontally above the water surface searching for emerging females. The females remain subimagoes 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.

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 favourable substrate in the River Main. In contrast, Bij de Vaate (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. Therefore, the change in substrate composition during this 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.

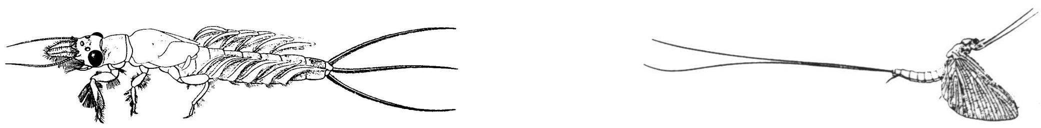

Figure 1. *Fully developed larva and adult of Ephoron virgo (from Kureck, 1996).*

Distribution of the test species

Around 1900, the Rivers Meuse and Rhine had a species-rich mayfly fauna, but during the last century the diversity of mayfly species declined (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 and Oosterbroek 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 1, distribution data of *E. virgo* in the Rivers Rhine and Meuse are shown, demonstrating their value for indicating ecological recovery.

Table 1. *Historical and recent distribution data of E. virgo in the Rivers Rhine and Meuse. (B=Belgium; F=France; NL=Netherlands; SW=Switzerland; G=Germany).*

	circa 1900	recent
Meuse (B/F)	+ (c)	rare (e,b,d)
Meuse (B)	+ (c)	- (b,d)
Meuse (border B/NL)	+ (a)	- (b)
Meuse (NL)	+ (a)	+ since 1991 (b)
Rhine (SW)	- (g)	- (g)
Rhine (G)	+ (f)	+ since 1986 (h)
Rhine (NL)	+ (a,f)	+ since 1991 (b)

Data from: a) Albarda, 1889; b) Bij de Vaate et al., 1992; c) Gysels, 1991; d) Ketelaars & Frantzen, 1995; e) Mol, 1987; f) Schoenemund, 1930; g) Schöll, 1996; h) Tittizer et al., 1990.

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 subimagoes moulting 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 metres 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, Ciba-Geigy). 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 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 hours light : 7 hours dark regime was applied, with 30 minutes twilight before and after each light period. After an exposure time of 96 hours, surviving larvae were counted.

Validation

In order to validate the newly developed ecotoxicity test a model toxicant was tested as well as water samples from several field locations. The metal cadmium was selected as model toxicant . Cadmium was added to DSW as $CdCl_2$ at the start of the experiment and nominal concentrations ranged from 0 to 2400 µg/L. A dose-response curve (Figure 2) 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 LC_{50} value was calculated by a log-logistic curve fitting procedure (Haanstra, 1985), being 375 (254-553) µg/L.

The test was also used as a bioassay for testing water samples from several field locations. The effects of contaminated water from some small and large rivers in The Netherlands and Ireland were determined (Figure 3). Water from the Dutch Rivers Meuse (Eijsden), Rhine (Lobith), Dommel and the Irish Avoca River as well as water from an outlet of an old copper mine (Mine outlet) in Ireland were filtered and tested in triplicate using DSW as a control. In addition a pore water sample from river sediment (Puttershoek, Oude Maas) was tested. The survival in water samples of different locations shown in Figure 3 ranges from 0 to 100 % with small standard deviations demonstrating the discriminating power of this bioassay.

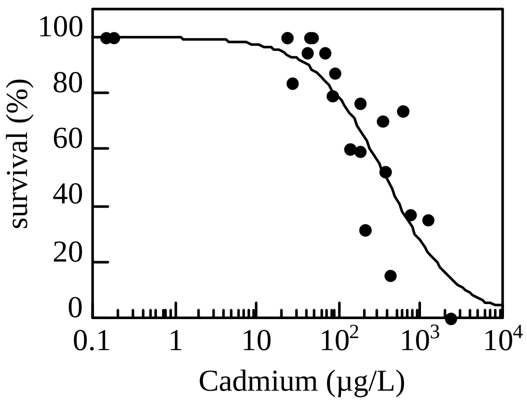

Figure 2. *Dose-response curve for newly hatched E. virgo larvae exposed to cadmium for 4 days.*

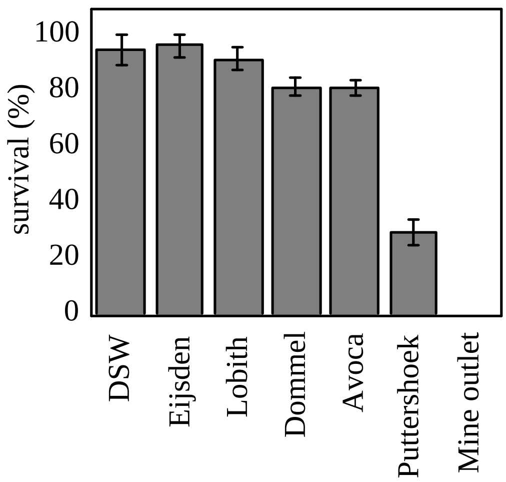

Figure 3. *Effect on survival of newly hatched larvae of E. virgo exposed to water samples from several field locations for 4 days. Error bars indicate standard deviations.*

DISCUSSION

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 hours. The test can be used as a bioassay as well as determining dose-response relationships for toxicants. The advantage of using this test species for determining short-term effects is not only the ecological relevance of *E. virgo*, but also the availability of larvae. 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.

This test is a promising tool to determine the sensitivity of this riverine mayfly to toxicants. In future experiments it will be used for testing the model toxicants copper and diazinon. It is shown that this test can also be used as a bioassay to determine actual risks of contaminated effluents, waste water and pore water. However, in order to use this species as indicator for ecological rehabilitation, more knowledge about sublethal effects of toxicants on this species as well as more insight in the habitat requirements are needed. Therefore, further research is necessary on oxygen, temperature and velocity demands. Additionally, chronic experiments have to be developed to determine long term effects of toxicants on the life cycle of *E. virgo*. Kureck (1996) developed a method to rear the larvae in the laboratory, which may provide the opportunity to develop chronic experiments in the future.

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