Use of *Adenophlebia auriculata* Eaton 1881 and *Leptophlebia marginata* (L. 1767) nymphs (*Leptophlebiidae*, *Ephemeroptera*) in aquatic ecotoxicology

Uso de ninfas de *Adenophlebia auriculata* Eaton 1881 y *Leptophlebia marginata* (L. 1767) (*Leptophlebiidae*, *Ephemeroptera*) en ecotoxicología acuática

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ABSTRACT

Leptophlebiidae nymphs (Ephemeroptera) are good universal candidates as test organisms in aquatic ecotoxicology because of their wide distribution, large size, detritivorous feeding, long life cycles and robustness. Therefore, preliminary data on growth, development, reproduction, feeding and sensitivity to pollutants in the laboratory were collected from two species, *Leptophlebia marginata* (L., 1767) from the Northern hemisphere and *Adenophlebia auriculata* Eaton, 1881 from the Southern hemisphere. Nymphs and preadults of *A. auriculata* from a field population (Palmiet River, Cape Province, South Africa) could be kept in the laboratory for over 50 days with a maximum survival of 60%. Addition of fish food accelerated growth and allowed for maturation until emergence, compared to animals in natural detritus which did not survive so well and did not emerge. Reproduction in the laboratory and artificial fertilisation did not succeed. In ecotoxicological tests, *L. marginata* nymphs from a clean stream in South Sweden were exposed during 28 days to pH 7 and pH 4.5. Acidity did not affect survival, but decreased detritus feeding activity after 20 days and reduced the percentage of emergence.

RESUMEN

Las ninfas de Leptophlebiidae (Ephemeroptera) son buenos candidatos como organismos a usar en pruebas de ecotoxicología debido a su amplia distribución, gran tamaño, alimentación detritívora, ciclo de vida largo y robustez. De este modo, se tomaron datos preliminares del crecimiento, desarrollo, reproducción, alimentación y sensitividad a agentes contaminantes en laboratorio de dos especies, *Leptophlebia marginata* (L., 1767) del hemisferio Norte y *Adenophlebia auriculata* Eaton, 1881 del hemisferio Sur. Las ninfas y subimagos de *A. auriculata* de una población obtenida del campo (Palmiet River, Provincia de El Cabo, Sudáfrica) pudo ser mantenida en el laboratorio durante unos 50 días con un máximo de supervivencia del 60%. La adición de alimento para peces aceleró el crecimiento y permitió la maduración hasta la emergencia, en comparación a los animales alimentados con detritus naturales cuya supervivencia fue menor y no emergieron. La reproducción en el laboratorio y la fertilización artificial no tuvieron éxito. En las pruebas de ecotoxicología, las ninfas de *L. marginata* obtenidas de un arroyo limpio del Sur de Suecia fueron expuestas durante 28 días a pH 7 y pH 4.5. La acidez no afectó a la supervivencia, pero hizo disminuir la actividad de alimentación de detritus y redujo el porcentaje de emergencia.

INTRODUCTION

Ephemeroptera have been extensively used as bioindicators in aquatic biomonitoring programs (*e.g.*, Depiereux & Feytmans, 1985; Krieger *et al.*, 1996; Goetsch & Palmer, 1997), in biomarker studies (*e.g.*, Day & Scott, 1990) and in ecotoxicological studies (*e.g.*, Hamilton & Timmons, 1980). It is important to include these organisms in monitoring programs and bioassays in order to have some representatives of the insect fauna as important component of the food web (Thompson & Townsend, 1999). Therefore, species which could be reared in laboratory conditions should be identified.

The ephemeropteran family Leptophlebiidae can be found in high numbers both in the Northern (*e.g.*, Sweeney *et al.*, 1986) as well as in the Southern hemispheres (*e.g.*, Collier & Winterbourn, 1990; Palmer *et al.*, 1993), and comprises large species (Elliott *et al.*, 1988), more easy to handle than species of other ephemeropteran families. Leptophlebiidae are generally fine detritus feeders (Elliott *et al.*, 1988), which make them well-exposed to particle-bound pollutants. Their long life cycles add to their value as bioindicators for chronic exposure to pollution.

In the present study, two species of Leptophlebiidae, *Adenophlebia auriculata* Eaton, 1881 and *Leptophlebia marginata* (L., 1767), were taken from two very different extreme environments in South Africa and Sweden respectively, and were tested in the laboratory for a number of parameters which contributes to the evaluation of their usefullness for ecotoxicological work. We specifically

analyzed: 1) the feasibility of maintaining laboratory cultures of *A. auriculata* by feeding the animals with an artificial diet; 2) whether this species can reproduce in laboratory conditions; and 3) the sensitivity of *L. marginata* to acid stress, which is known to create osmotic inbalances in aquatic insects (Vangenechten & Vanderborght, 1980) and to mitigate release of heavy metals in the environment (Wren & Stephenson, 1991).

MATERIALS AND METHODS

Test species

We used individuals of two species: *Leptophlebia marginata* L., from South Sweden (Skania), collected in a natural brownwater stream with low species diversity and varying pH (chemical stress), and *Adenophlebia auriculata* Eaton, from South Africa (Cape Province), collected in Palmiet River, a clean upland stream with discharge fluctuations throughout the year (physical stress) and equally a relatively low species diversity. The Palmiet River has 20-22°C summer water temperature and 10°C winter water temperature. The water regime is variable, with 5 to 10 high level peaks per year. Water chemistry of both streams is summarized in Table I.

Adenophlebia auriculata from the Southern hemisphere was characterized by Palmer *et al.* (1993) in South Africa as "brusher-collector". They are found under stones in the discharge shadow where sediments accumulate and in larger leaf packs. They have an omnivorous diet consisting of periphyton (diatoms, filamentous algae, bacteria), decomposed leaves and river detritus. They prefer slower flowing stretches of the river as habitat, as confirmed in the laboratory. They have a multivoltine life cycle: emergence peaks occur mainly in February-March, in May and in September, but recruitment is continuous throughout the year (Haigh, 1996; Haigh & Davies-Coleman, 1999).

Leptophlebia marginata from the Northern hemisphere is a univoltine species, with overwintering nymphs and emergence occurring in April-June.

Table I.—Water quality of Palmiet River and the Mullra stream.
Tabla I.—Calidad del agua de Palmiet River y del arroyo de Mullra.

River	*pH*	*Ca (mg l^{-1})*	*Cond. (S cm^{-1})*	*NO_3^- (mg l^{-1})*	*PO_4^- (mg l^{-1})*
Palmiet	6.7	1	16.3	0.01	0.008
Mullra	6.0-7.4	<30	100-235	0.1	0.015

It inhabits deeper stretches of slow flowing rivers and lakes and is a "collector-gatherer" of detritus (Brittain, 1972; Elliott *et al*., 1988), being a sprawler and climber on macrophytes. The flight period takes place from April to June (Elliott & Humpesch, 1983).

Feeding experiment

We tested the effects of an artificial diet on *A. auriculata.* The experiments were carried out at the end of May and in June, corresponding to the austral autumn, a period where small emergence peaks have been noted for *A. auriculata* in the field (Haigh, 1996). Six 3 l containers were used (numbered A to F). Each container was filled with filtered (85 μm) Palmiet River water and contained 20 nymphs; thus, a total of 120 *A. auriculata* nymphs were used for this experiment. A predried stone from the river was added in each container to facilitate emergence of preadults. The containers were aerated and covered with nylon mesh. The nymphs in the first 3 containers originated directly from a three days old sample from the Palmiet River (water quality, Table I) and were fed with predried detritus from Palmiet River *ad libidum*. The nymphs in the second set of 3 containers came from the same site but have been fed with detritus during the first 54 days in the laboratory, and then subjected to a food regime of Tetramin® *ad libidum* when they were 32 days older than nymphs used in the detritus treatment. In order to compare both experimental groups, only nymphs of the same absolute age were considered for the analysis. All experiments were conducted in a climate room at 20°C and 12h/12h photoperiod. Head width was measured after 1, 12, 26, 55 and 81 days up to 0.1 mm using the crossed lines method (Haigh, Rhodes University, pers. comm.). Replicates were homogenous throughout the experiment, except for container B which included 2 nymph outliers (Kruskal-Wallis, $H = 9.98$, $p = 0.007$, d.f. = 2, 24). Emerged preadults were counted daily. During head width measurements, nymphs were sexed (males have large eyes) and wingbud development was noted.

Reproduction

We tested whether *A. auriculata* could reproduce in laboratory conditions as well as the feasibility of artificial fertilisation in this species. To test reproduction of *A. auriculata* in the laboratory, emerged preadults and adults from the growth experiment were used. They were transferred to a large glass aquarium (100 l) topped by a 1 m high cage of white mosquito

netting. The aquarium contained aerated Palmiet water with emerging stones, reed stems and a green netting set against one wall to allow preadults to crawl out of the water. Photoperiod and temperature were maintained as in the previous experiment.

To test feasibility of artificial fertilisation, preliminary trials demonstrated that it is essential to use freshly prepared Ringer solution instead of stock solution, as in the last an osmotic imbalance of the eggs (shrinking) was observed after 2 days. Haigh (1996) demonstrated that no differences in the number of fertilised eggs are obtained using either adult or subadult males or females for the artificial fertilisation. Because preliminary trials without aeration and with unfiltered water showed that Palmiet water brought a typical succession of undesirable organisms (Ciliata, fungi; green algae, and Oligochaeta), aeration of the containers and use of filtered water (85 μm) were used to diminish the growth of fungi and the presence of undesirable organisms and algae. Although Haigh (1996) used decapitation of female preadults to induce release of the eggs (see also Huff & McCafferty, 1974), however this method did not work in our trials, so we decided to dissect the gonads out of the female animals. Two trials were undertaken to artificially fecundate eggs obtained from dissected female preadults. 1) dissection of 2 adult male and 1 preadult female gonads in a Petri dish with freshly prepared Ringer solution, stirring during 5, 15, 30 and 40 minutes, followed by dispersion of eggs in triplicate sets of 100 ml beakers (ca. 160 eggs per beaker) with Palmiet water and aeration. Eggs were controlled under binocular microscope after 2, 5, 18 and 24 days. 2) idem, but in Yeager's solution (Giberson & Rosenberg, 1992), average of 80 eggs per beaker, and controls with a weekly frequency.

Ecotoxicological test

Two replicates of 50 specimens of *Leptophlebia marginata* were collected from the Mullra stream in South Sweden (water quality, Table I) and exposed in river water for 28 days to pH 7 and pH 4.5 in a static water system with individual exposure, weekly renewal of water and a 12h:12h photoperiod. Food was added *ad libidum* as fine detritus filtered on Whatman GFC glassfibre filters from a stream-sediment suspension (Gerhardt, 1992a). Survival, feeding and emergence were recorded twice a week. Recent feeding activity was evaluated by counting animals with the anterior half of the gut filled > 25%. The pH was monitored twice a day and adjusted in case of deviation > 0.1 unit by addition of 0.1 M H_2SO_4 or 0.1 M NaOH. Temperature was kept at 10 ± 2°C, typical for Swedish water temperatures in spring (April-May).

Statistical analysis

After testing for normality (Goodness of fit test), homogeinity of variance (Levene'test) and within-group differences (Kruskal-Wallis), one-way ANOVA was performed on the head widths in both feeding conditions (= independent factor). At individual days, t-tests were performed between the feeding conditions. Linear regressions between time and head width were performed. For survival the non-parametric Mann Witney U-test was performed between feeding conditions. Emergence data and proportions of males and females without, with small and with large wingbuds were tested after arcsine ($p^{1/2}$)-transformation ($p = x\%/100$) with repeated measures ANOVA (Sokal & Rohlf, 1987). For all the statistics (Statistica 4.5) the significance level was taken at $p < 0.05$.

RESULTS

Food experiment

There was a significant time effect for head width in both detritus ($F = 48.2$, $p < 0.0001$, d.f. = 4, 99; ANOVA), and Tetramin diets ($F = 68.4$, $p < 0.0001$, d.f. = 1, 39; Anova). At days 54 and 81 (t-tests, $p < 0.05$) Tetramin fed animals were significantly larger than detritus fed animals. The slopes of the regressions (growth rates, mm day^{-1}) between time and head width were 0.014 ± 0.0012 (SD) for detritus and 0.026 ± 0.0062 for Tetramin, and were significantly different ($t = -4.35$, $p = 0.01$). Tetramin caused an almost 2x increase on head width growth rate (Fig. 1A). Survival declined faster in the detritus fed animals than in the Tetramin fed ones, and the difference in survival became significant after 50 days ($z = -1.96$, $p < 0.05$, Mann-Whitney U-test; Fig. 1B). No differences in the proportion of males (ca. 47%) were found in the nymph population among the different days and diet. No differences in relative number of animals with small and large wingbuds between female and male nymphs were found among the days and food groups (repeated measures ANOVA). Only one animal emerged in the detritus diet after 26 days. In the Tetramin diet, emergence started after 47 days and lasted until day 76 to reach 30-60% emerged (pre)adults from the original nymph population (Fig. 1C). Males tended to emerge earlier.

Reproduction

Reproduction in the large cage did not occur. Although the preadults

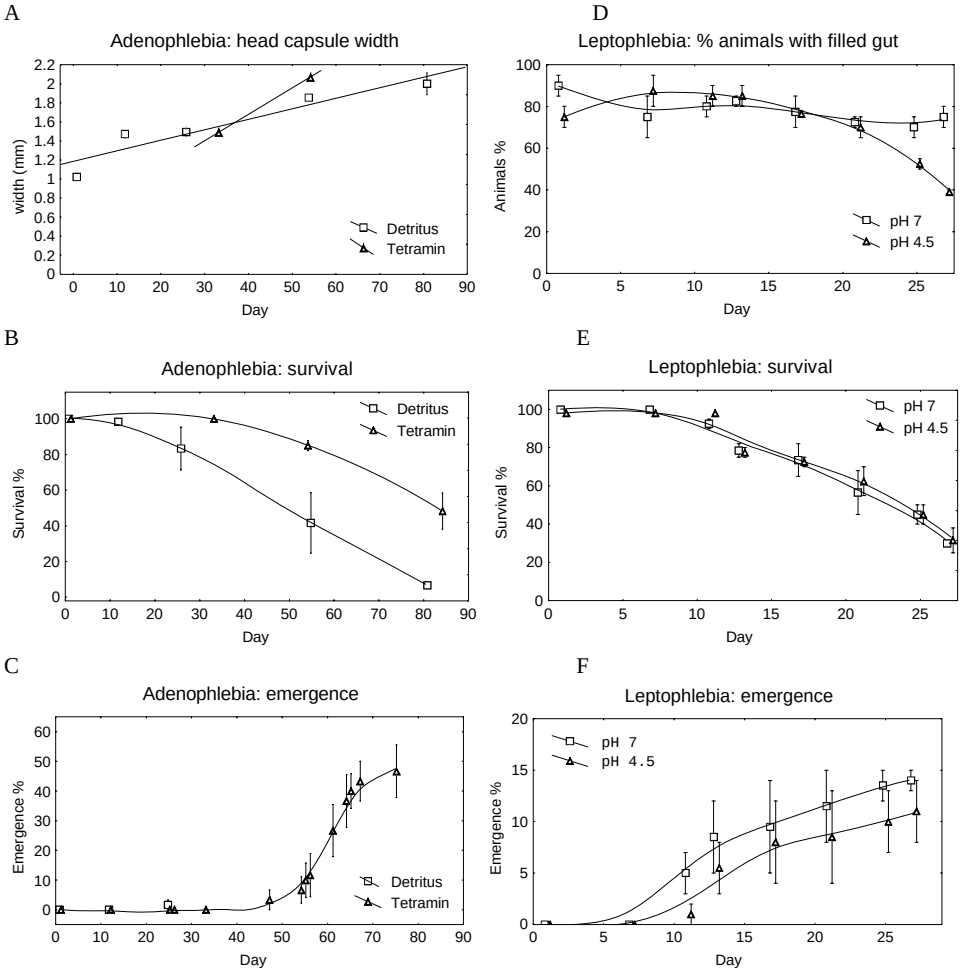

Fig. 1. A, B, C.— comparison between detritus and Tetramin as food source of *A. auriculata* as a function of time (least squares fit, except when stated otherwise). A, Head width (linear fit, mean ± SE, N = 60); B, survival (mean % ± SE, N = 3); and C, emergence (cumulative % ± SE, N = 3). D, E, F: comparison of *L. marginata* in pH 7 and in pH 4.5 as a function of time. D, % animals with > 25% anterior half of gut filled (mean ± SE, N = 2); E, survival (mean % ± SE, N = 2); and F, emergence (cumulative % ± SE, N = 2).

Fig. 1. A, B, C.— comparación entre detritus y Tetramin como fuente de alimento de *A. auriculata* en función del tiempo (ajuste mediante mínimos cuadrados, excepto si se dice lo contrario). A, anchura de la cabeza (ajuste lineal, media ± E.E., N = 60); B, supervivencia (media del % ± E.E., N = 3); y C, emergencia (% acululado ± E.E., N = 3). D, E, F: comparación de *L. marginata* en pH 7 y pH 4,5 en función del tiempo. D, % de animales con > 25% de la mitad anterior del tubo digestivo lleno (media ± E.E., N = 2); E, supervivencia (% medio ± E.E., N = 2); y F, emergencia (% acumulado ± E.E., N = 2).

moulted to adults within two days, the adults did not show any mating behaviour nor any oviposition and died after another two days.

The artificial fertilisation could only produce < 5 hatchlings, and most eggs died in the Ringer solution within 18-25 days. The eggs did not survive more than 15 days in the Yeager's solution. Either the fertilised eggs died because of an unsuitable environment, or the eggs were not fertilised. Egg morphology showed the following sequence of changes during development: 1) transluscent white, 2) membrane becomes more visible (= periviteline space, meaning that the egg is fertilised (Haigh, 1996), 3) granular appearance, 4) faint longitudinal medial stripe, 5) faint dark distal spot. The duration of stirring the gametes in the respective media did not seem to affect egg survival. Considerable differences in fecundity existed among males (visual qualitative observation of seminal cloud) and among the females (2027 ± 647.5 eggs/female, N = 3).

Ecotoxicological test

Up to day 20 more than 75% of the animals in both treatments contained detrital food in their anterior gut. Thereafter, pH-stressed animals decreased in feeding activity down to 40%, compared to 75% in the pH 7 group nymphs (Fig. 1D). When the whole dataset was compared, no differences between both pH groups were found (repeated measures ANOVA, $p > 0.05$). However, the last two datapoints in the series differed significantly between the pH groups (ANOVA, $p < 0.05$). Survival was similar in both pH groups and declined from day 10 on quite linearly until day 28 down to ca. 30% (Fig. 1E). The low survival can probably be attributed to suboptimal feeding conditions. The emergence curves for both pH groups were parallel with large variance, with a generally higher emergence success at pH 7 compared to pH 4.5 (ANOVA, $p < 0.05$) (Fig. 1F).

Discussion

Diet

Individuals of *A. auriculata* fed with Tetramin were larger than individuals fed with detritus after ca. 20 days. Unfortunately, emergence and mortality prevented a third head width measurement in the Tetramin fed animals, which could further support this finding, but these results are in complete agreement with earlier results (Haigh, 1996). The obtained head capsule widths and

growth rates fell within the range of those obtained by Haigh (1996) on the same Palmiet population. The proportion of males showed a small decrease (not significant) because of faster emergence of males than females. We suspect that an inadequate quality and quantity of detritus was the cause of the stop in development and the mortality. Also, for *Leptophlebia marginata* (ecotoxicological test) detritus as food was not optimal for keeping high survival rates during the longterm experiment. Dieterich & Anderson (1995) mention that detritivorous Ephemeroptera are very sensitive to food quality. Addition of high quality food can increase growth rates by a factor 2 to 7 (Söderström, 1988). The reason for that may lie in the higher rate of assimilation of the Tetramin, due to a lack of cellulose, combined with a high caloric value (9% fat), protein value (48%) and addition of several vitamines. The experiment shows that *Adenophlebia* nymphs can be kept in culture for a few months on the basis of artificial fish food until partial successful emergence and > 50% survival.

Reproduction

Results showed that reproduction in the laboratory was very unsuccessful, although not very high rates of reproductive success were obtained by other authors either: similar trials produced a 15% hatching rate at 25°C (Haigh, 1996). Hatching occurred after 16-22 days and hatchlings reached the second instar. As stated by Brittain & Campbell (1991), hatching rate in Ephemeroptera is strongly dependent on water temperature (which is species-specific) and does not appear to be dependent on photoperiod, artificial fertilisation usually yielding hatching rates < 10%. However, Rowe *et al*. (1988) managed to obtain a 94% hatching rate from eggs issued after force-copulating of imagoes of the mayfly *Stenonema femoratum* (Say, 1823). The eggs were placed on nets in such a manner that aerated water circulated above them. Based on the above results, we recommend to try either force-copulating or, when this does not work, to mix the dissected gametes in a small volume of freshly prepared Ringer solution, stir gently for ca. 15 minutes and transfer to a 250 ml glass beaker with gentle aeration, filtered circulating river water and fine sand substrate. Water evaporation has to be controlled by adding distilled water or by covering the beakers. Optimal water temperature still has to be determined. The fact that the eggs died after ca. 18-25 days (= hatching time) means that the hatching process is the sensitive point in artificial reproduction. The reproduction in laboratory cultures of this species remains unresolved (Haigh & Davies-Coleman, 1999).

Ecotoxicological test

Emergence analysis for aquatic insects is a very integrative tool to assess the effects of various stressors on life history and is sensitive to sublethal pollution stress, *e.g.* for Chironomidae (Janssens de Bisthoven *et al.*, 1998). The integrative power of an emergence curve lies in the fact that it reflects both the survival and the ability of a fraction of the population per unit of time to reach (pre)reproductive maturity. For example, Hatakeyama (1989) found that copper and zinc reduced emergence of *Epeorus latifolium* (Ephemeroptera) by doubling the moult interval. In the present study, pH 4.5 had a negative effect on the rate of emergence compared to pH 7. It has been demonstrated that low pH resulted in decreased abundances and productivity of leptophlebiid species in New Zealand (Collier & Winterbourn, 1990). Recently, Gerhardt *et al.* (submitted) found 30-40% mortality for *Choroterpes picteti* (Leptophlebiidae) exposed to acid mine drainage with pH = 3.3.

As feeding rate is the basis for energy uptake, it could be a very interesting functional parameter in ecotoxicology too. Collier & Winterbourn (1990) found that leptophlebiid nymphs from acidic streams had a higher grazing rate than nymphs from alcaline streams. They attributed it to a poorer quality of the epilithic food in acid streams, inducing a higher grazing effort. *Leptophlebia marginata* has earlier been reported to be acid-tolerant down to pH 4.0 (Okland & Okland, 1986), and relatively metal-tolerant as well (Cd: $LC_{50\text{-}120\ h}$ = 5.0 mg/l; Gerhardt 1992b, 1995). Acidity-dependent differences in metal toxicity were found for *L. marginata* exposed to Fe ($LC_{50\text{-}96h}$ = 106.3 mg/l at pH 7; 89.5 mg/l at pH 4.5) and Pb ($LC_{50\text{-}96h}$ = 5 mg/l at pH 7; 1.1 mg/l at pH 4.5) (Gerhardt, 1994). During a 28 days exposure of *L. marginata* to Fe in combination with low pH 4.5, feeding activity and motility decreased from day 15 on and animals were constipated by Fe precipitation in the gut, whereas no effects were found at pH 7 and Fe exposure (Gerhardt, 1992a, 1995). Increasing inactivity of *L. marginata* was found at 1 mg Cd/l and at 1 mg Cd/l combined with 16 mg Fe/l (Gerhardt, 1995). Decreased activity and feeding activity was found at Fe = 10 mg/l at pH 4.5 (Gerhardt, 1992a).

Concerning the South African species *Adenephlebia auriculata*, Gerhardt & Palmer (1998) demonstrated behavioural sensitivity to sublethal concentrations of copper. The nymphs showed escape and ventilation behaviour at concentrations higher than 0.23 ppm Cu, but were somewhat less sensitive in terms of LC_{50} than a limpet (Mollusca), also part of the local fauna.

CONCLUSIONS

Although nymphs of *A. auriculata* and *L. marginata* seem to be relatively easily kept in culture, reproduction in the laboratory (tested on *A. auriculata* only) had low success and therefore remains a major bottleneck. Their relative robustness and large size make them potentially interesting bioindicator organisms. However, their relative metal- and acid-tolerance (Okland & Okland, 1986; Gerhardt, 1992b, 1995) indicate relatively low sensitivity to toxic substances. Hence these species cannot be considered as optimal ecotoxicological test organisms. Being representatives of the detritus-processing epibenthic or epilithic insect community, and having the capacity to show intermediate sublethal responses to stress, their inclusion in batteries of bioassays still may be recommended, provided enough field nymphs can be collected and other more sensitive organisms are included.

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