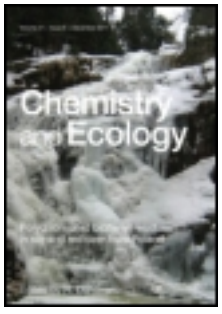

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Locomotory behaviour in the freshwater amphipod *Gammarus pulex* exposed to the pyrethroid cypermethrin

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SHORT NOTE

Locomotory behaviour in the freshwater amphipod *Gammarus pulex* exposed to the pyrethroid cypermethrin

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The use of pesticides in modern agriculture results in contamination of streams. Pyrethroid insecticides in particular may lead to long-term effects at the population and ecosystem levels. A key phenomenon observed upon pulse exposure to pyrethroids is drift, where freshwater invertebrates are carried along by the current and disappear from the contaminated stretch of the stream. The ecologically important freshwater amphipod *Gammarus pulex* is among the most sensitive species, and the aim of this study was to provide a detailed description of the locomotory behaviour displayed by this species during exposure to the pyrethroid cypermethrin, using an automated video tracking system. Marked changes in locomotory behaviour were observed within minutes of exposure to very low, environmentally realistic cypermethrin concentrations at which mortality was observed only after several days of continuous exposure. Exposure resulted in a biphasic behavioural response: at 0.01 $\mu g \cdot L^{-1}$ *G. pulex* displayed hyperactivity, which was maintained, until at higher exposure concentrations ($\geq 1\,\mu g \cdot L^{-1}$) immobilisation followed. The results indicate that the methodology constitutes a very powerful tool for detecting sublethal effects of pesticides on non-target stream invertebrates; effects that may be predictive of impacts at the population level.

Keywords: locomotory behaviour; *Gammarus pulex*; freshwater; invertebrates; pyrethroids; cypermethrin

1. Introduction

The application of pesticides in modern agriculture results in the contamination of freshwater ecosystems, including streams, and both exposure and effects in the field have been reviewed by Schulz [1]. Transport of pesticides from crop fields to adjacent streams typically occurs when field spraying is followed by significant amounts of precipitation. Under these circumstances, pesticides are transported directly to the stream by surface run-off or via drains, giving rise to transient pulses with elevated levels of pesticides. In addition, pesticides find their way into streams as a result of wind drift, atmospheric deposition and transport via groundwater.

For most pesticides, the concentrations caused by routine agricultural spraying practice, typically $<10\,\mu g \cdot L^{-1}$ in streams, cannot be expected to be acutely lethal. Studies have, however, documented that pulse exposure of stream invertebrates to especially pyrethroids, the most important group of insecticides, may lead to long-term effects at the population and ecosystem levels [1,2].

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A key phenomenon observed in streams during, and in many cases for a period after, a transient pesticide pulse is drift, where a range of freshwater invertebrates are carried along by the current and disappear from the contaminated stretch of the stream. In field studies, contamination with insecticides, including several pyrethroids, has caused drift in a number of invertebrate species [2–5].

Recently, Nørum et al. [6] documented the ability to extrapolate directly from pyrethroid-induced changes in locomotory behaviour of three invertebrate species, *Gammarus pulex*, *Leuctra nigra* and *Heptagenia sulphurea*, observed in the laboratory to drift in realistic stream microcosms. A distinct, biphasic behavioural response was displayed by *G. pulex*, with active avoidance behaviour and drift occurring at very low lambda-cyhalothrin concentrations ($\geq 0.01\ \mu g \cdot L^{-1}$), consistent with previous studies of *G. pulex* using fenvalerate [7], followed by immobilisation at higher concentrations ($\geq 1\ \mu g \cdot L^{-1}$).

The aims of this study were to explore in detail the locomotory behaviour of *G. pulex* exposed to the pyrethroid cypermethrin, and to compare the behavioural effect concentrations with lethal concentrations, in order to evaluate whether sublethal cypermethrin exposures are likely to induce behavioural changes that may be predictive of effects at the population or community level.

2. Materials and methods

2.1. *Artificial freshwater and chemicals*

In the experiments, artificial freshwater (AFW) was used to minimise between-experiment variability in water chemistry in order to keep the bioavailability of cypermethrin constant. The composition of the AFW equalled the ISO 6431 test water of the OECD test guidelines [8]: $CaCl_2 \cdot 2H_2O$: $294\ mg \cdot L^{-1}$, $MgSO_4 \cdot 7H_2O$: $123.25\ mg \cdot L^{-1}$, $NaHCO_3$: $64.75\ mg \cdot L^{-1}$ and KCl: $5.75\ mg \cdot L^{-1}$. Cypermethrin was obtained from Sigma-Aldrich (PESTANAL®). Cypermethrin concentrations were nominal. All chemicals used were of analytical grade.

2.2. *Experimental animals*

Freshwater amphipods *G. pulex* were caught in the stream Lindved Å, Funen, Denmark, which is an uncontaminated stream with a diverse invertebrate fauna. Adult individuals were obtained by straining the animals through a sieve and body lengths of a representative sample of the individuals were all >7.5 mm, as determined by image processing software. According to Welton [9] *G. pulex* >7.5 mm are adults. A few individuals were apparently infected by an acanthocephalan parasite, such as *Pomphorhynchus laevis*, as evident by a bright orange line along the dorsal carapace, and as this parasite is known to affect the behaviour of *G. pulex* [10] these individuals were discarded. The amphipods were acclimated in 10-L polyethylene aquaria in aerated AFW at $15 \pm 1\ ^\circ C$ under a 12:12 h light/dark regime for 1–2 weeks and were feed *ad libitum* with leaf litter collected at the site of capture. The AFW in the aquaria was changed twice per week.

2.3. *Experiment 1: effects on locomotory behaviour*

A fully automated video-tracking system, EthoVision Pro® (Noldus Information Technology, Holland), was utilised for quantifying the locomotory behaviour of *G. pulex*, and data on the distance moved, the velocity while moving (i.e. excluding the periods of inactivity) and the proportion of time spent swimming, crawling or being inactive were obtained. The differentiation between inactivity, crawling and swimming was based on velocity limits determined during the optimisation of the tracking system, which showed that a crawling event was initiated when

the velocity exceeded 0.5 cm·s^{-1}, whereas a swimming event was initiated when the velocity exceeded 2 cm·s^{-1}.

Prior to the experiment, the animals were acclimated overnight in AFW at 15 ± 1 °C in separate 100-mL glass Petri dishes with a diameter of ∼9 cm. For each exposure level, the background behaviour of 16 animals was recorded for 30 min in 80 mL of uncontaminated AFW, after which the video tracking was briefly interrupted. Cypermethrin, dissolved in 10 μL of ethanol in 20 mL of AFW, was added to eight of the Petri dishes (the exposed group). The remaining eight Petri dishes (the control group) were added 10 μL of ethanol in 20 mL of AFW. Consequently, a separate control group was used for each cypermethrin concentration tested. The 20 mL of liquid was added at a position as far away as possible from the animal, and the video tracking was resumed within 2 min of being halted. The volume of 20 mL was chosen to ensure rapid mixing. The behaviour of the control group and the exposed group was subsequently recorded during a 90-min exposure period, after which the experiment was terminated. The final concentration of ethanol was 100 μL·L^{-1}, which is in accordance with OECD test guidelines [11]. Furthermore, in a pilot study, no effect of this ethanol concentration on either behaviour or survival was observed. Cypermethrin concentrations of 0.003, 0.01, 0.3, 0.1, 0.3, 1, 3 and 10 μg·L^{-1} were used.

Immobility in the control period was observed in two individuals of apparent poor health, and these animals were excluded. A further two individuals were excluded due to poor tracking quality, i.e. when the number of scans without detection of the animals exceeded 1% of the total number of scans.

2.4. *Experiment 2: effect on survival*

A mortality study was performed to compare the lethal concentrations with the effect concentrations from the study of locomotory behaviour. Groups of 10 animals were transferred to 1.0-L Pyrex glass beakers containing 400 mL of AFW and acclimated overnight. At each of the cypermethrin concentrations of 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μg·L^{-1}, two groups of 10 individuals were used. At the start of exposure, cypermethrin dissolved in 50 μL of ethanol in 100 mL of AFW was carefully added giving a final volume of 500 mL. The control group was added 50 μL of ethanol in 100 mL of AFW only. The final concentration of ethanol was 100 μL·L^{-1}, as in Experiment 1.

After 24, 48, 72 and 96 h, the animals were counted and scored as being either alive or dead. Individuals were identified as being alive if they were visually active during the observation or following gentle mechanical stimulation using a needle, or if they were observed to move limbs such as antennae or legs when observed in a stereo dissection microscope. Dead animals did not react to stimulation or show any movement and were removed. Following the observation at 24, 48 and 72 h, ∼400 mL of AFW was carefully removed and an equal volume of AFW containing ethanol and cypermethrin was carefully added. This procedure of water renewal was chosen to minimise stress to the animals due to handling.

2.5. *Data analysis*

An important issue in studies of locomotory behaviour is the choice of time window when analysing the results. If behavioural parameters are averaged over to long a time window a detailed description of rapid changes in behaviour is not possible. If, by contrast, a very short time window is chosen the interindividual variability increases dramatically since not all individuals are synchronous in their behaviour. For *G. pulex*, a time window of 5 min has previously been found to provide a satisfactory compromise between obtaining a sufficient resolution in describing the behavioural changes while minimising the interindividual variability [6].

Data on distance moved and velocity while moving were evaluated using two-way repeated measures analysis of variance. At each cypermethrin concentration, the 30-min control period and 90-min exposure period were analysed separately in order to evaluate potential differences between the two groups in background behaviour and during exposure, respectively. When necessary, data were transformed to obtain normality and homogeneity of variances. Data for mortality were evaluated using logistic regression analysis. The statistical analyses were performed using SigmaStat® (version 2.03, SPSS Inc., Chicago, IL, USA). In all cases a significance level of $\alpha = 0.05$ was used.

3. Results

The effect of cypermethrin on the distance moved by *G. pulex* is presented in Figure 1. Exposed individuals displayed a biphasic response, while the control group maintained a relatively constant

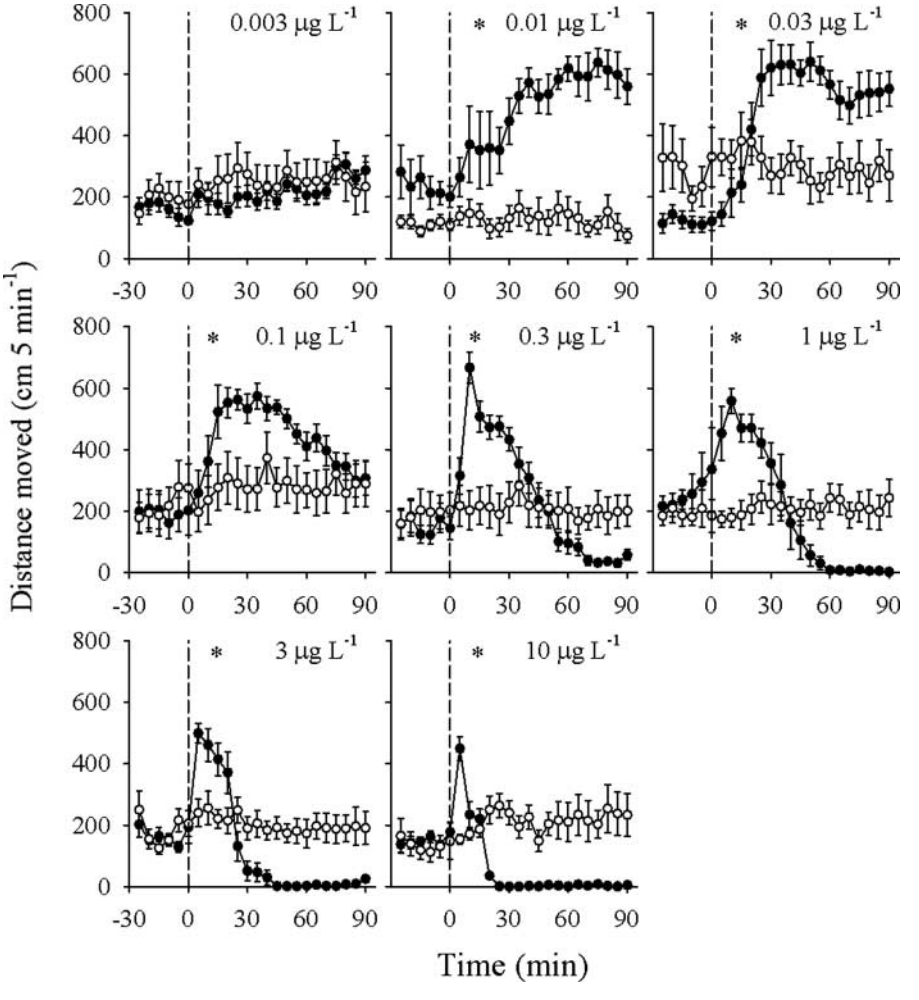

Figure 1. Effect of cypermethrin on the distance moved by *Gammarus pulex*. The background behaviour of *G. pulex* in uncontaminated water was recorded for 30 min. Subsequently, the behaviour in cypermethrin-contaminated water was recorded for a further 90 min. The dashed line indicates the start of exposure. Open circles, control group; solid circles, exposed group. An asterisk (*) indicates a significant difference between the control and the exposed group (average ± SEM, $n = 7$–8).

level of activity of ~200 cm·5 min^{-1}. At lower concentrations (≥0.01 μg·L^{-1}) a marked increase in the distance moved was observed upon exposure, and a constant maximum activity level of ~600 cm·5 min^{-1} was reached after ~60 min of exposure at 0.01 μg·L^{-1}. With increasing exposure concentration, this hyperactive state was observed sooner, while the duration of the maximum level decreased. At higher concentrations (≥0.1 μg·L^{-1}) the hyperactivity was followed by a marked decrease in activity, and immobilisation was observed sooner with increasing concentrations ≥1 μg·L^{-1}. At 10 μg·L^{-1}, the hyperactivity was maintained in the first 5-min interval only, and immobilisation occurred 25 min after the start of exposure.

The results for the changes in velocity while moving (Figure 2) agreed with the observed changes in distance moved. Significant differences between the control and the exposed group were observed at concentrations ≥0.01 μg·L^{-1}. The velocity while moving changed from ~1 cm·s^{-1} in uncontaminated water to ~4 cm·s^{-1} in the hyperactive state during exposure.

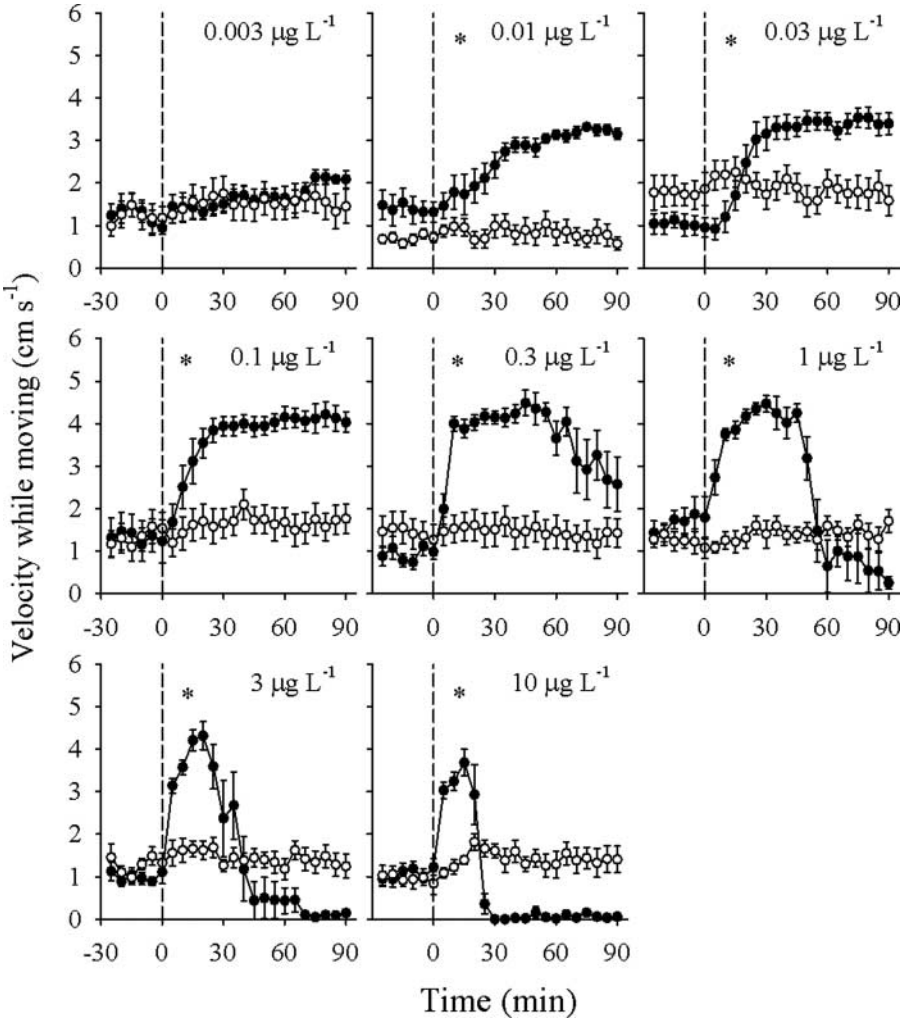

Figure 2. Effect of cypermethrin on the velocity while moving by *Gammarus pulex*. The background behaviour of *G. pulex* in uncontaminated water was recorded for 30 min. Subsequently, the behaviour in cypermethrin-contaminated water was recorded for a further 90 min. The dashed line indicates the start of exposure. Open circles, control group; solid circles, exposed group. An asterisk (*) indicates a significant difference between the control and the exposed group (average ± SEM, $n = 7$–8).

The locomotory behaviour of *G. pulex* was divided into swimming behaviour, crawling behaviour and inactivity, and the average proportion of these three types of behaviour in the exposed groups is presented in Figure 3. The control groups all had nearly constant proportions of swimming, crawling and inactivity, which were very similar to the group exposed to 0.003 $\mu g \cdot L^{-1}$, and are consequently not presented. The proportion of swimming behaviour (Figure 3) was highly correlated with the distance moved (Figure 1), and *G. pulex* consequently moved farther and faster at low concentrations ($\geq$0.01 $\mu g \cdot L^{-1}$) because a larger proportion of the time was spent swimming, whereas the time spent crawling remained relatively constant and inactivity decreased (Figure 3). A detailed analysis of the swimming behaviour revealed that the number of swimming events increased from $\sim$10 to 40 per 5 min, while the duration of a single swimming event remained

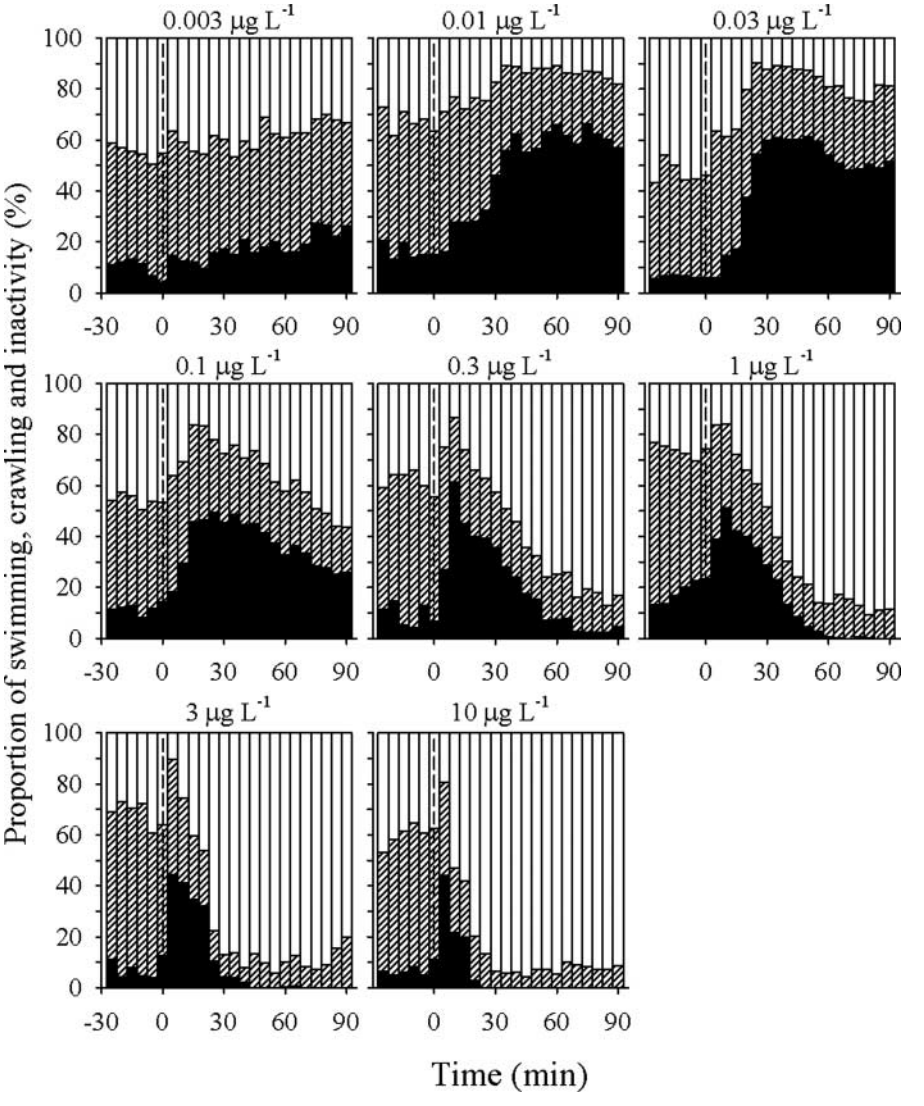

Figure 3. Effect of cypermethrin on the proportion of swimming, crawling and inactivity in the exposed groups of *Gammarus pulex*. Background behaviour of *G. pulex* in uncontaminated water was recorded for 30 min. Subsequently the behaviour in cypermethrin-contaminated water was recorded for a further 90 min. The dashed line indicates the start of exposure. Black bars, swimming; hatched bars, crawling; open bars, inactivity (average, $n = 7$–8).

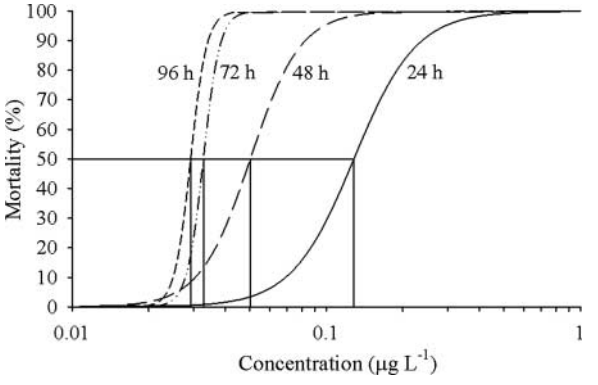

Figure 4. Concentration–response relationship between cypermethrin exposure and mortality in *Gammarus pulex*. Logistic regression curves and LC_{50} values for continuous exposure for 24, 48, 72 and 96 h are shown ($n = 20$ in each of 11 groups exposed to concentrations ranging from 0 to 100 $\mu g \cdot L^{-1}$).

comparatively constant at ~2–4 s (results not presented). At higher concentrations ($\geq 1\ \mu g \cdot L^{-1}$) the ability to display coordinated swimming behaviour was lost, and the animals were immobilised.

The relationship between cypermethrin exposure and mortality is given in Figure 4. The concentration–response curves became increasingly steep with time, and the LC_{50} values decreased markedly: LC_{50}-24 h: 0.128 $\mu g \cdot L^{-1}$, LC_{50}-48 h: 0.050 $\mu g \cdot L^{-1}$, LC_{50}-72 h: 0.033 $\mu g \cdot L^{-1}$, and LC_{50}-96 h: 0.029 $\mu g \cdot L^{-1}$. No mortality was observed in the control group.

4. Discussion

The main aim of this study was to provide a detailed description of the changes in locomotory behaviour associated with cypermethrin exposure in *G. pulex*. The results clearly demonstrate that marked behavioural changes were observed within minutes of exposure to low, environmentally realistic cypermethrin concentrations; concentrations at which mortality was observed only after several days of continuous exposure.

The video-tracking methodology proved to be very powerful, with rapid and unambiguous detection of even minor changes in behaviour, as well as low interindividual variability within the experimental groups for the different behavioural parameters measured. The experimental design was optimised to obtain a constant level of background behaviour in uncontaminated water, in order to enable the detection of both an increase and a decrease in the level of activity. The experimental set-up, in which animals placed in Petri dishes without food or cover were exposed to comparatively bright light, obviously did not provide the animals with natural conditions. Consequently, the background behaviour of the animals in the laboratory may deviate from normal behaviour in the wild. The study by Nørum et al. [6] did, however, clearly show that the changes in locomotory behaviour observed in this study were predictive of the active avoidance behaviour of drifting *G. pulex*.

At cypermethrin concentrations as low as 0.01 $\mu g \cdot L^{-1}$ *G. pulex* displayed maximum hyperactivity, which was maintained as long as possible, until at higher exposure concentrations ($\geq 1\ \mu g \cdot L^{-1}$) immobilisation followed. These effects concentrations for cypermethrin agree well with those reported by Nørum et al. [6] for *G. pulex* exposed to the pyrethroid lambda-cyhalothrin. Lambda-cyhalothrin and cypermethrin have very similar physicochemical and toxicological properties, and in the study by Nørum et al. [6] the actual concentrations of lambda-cyhalothrin at the end of the 90-min exposure period were typically >70% of the nominal concentrations. Some loss of

the pyrethroids from the water phase is expected due to adsorption to the arenas and due to breakdown by photolysis or hydrolysis. Nørum et al. [6] reported hyperactivity in *G. pulex* exposed to a lambda-cyhalothrin concentration of 0.011 $\mu g \cdot L^{-1}$ and immobilisation at 0.58 $\mu g \cdot L^{-1}$. Similar changes in behaviour have been observed for the stonefly *L. nigra* and the mayfly *H. sulphurea* exposed to lambda-cyhalothrin [6], and for backswimmers (Notonectidae) and whirligig beetles (Gyrinidae) exposed to cypermethrin and lambda-cyhalothrin in a mesocosm study by Farmer et al. [12].

Qualitative changes in the locomotory behaviour were also noted. In *G. pulex*, the marked increase in distance moved during exposure was caused by an increase in the proportion of time spent swimming. Whereas control animals primarily moved along the bottom of the Petri dishes, the exposed, hyperactive individuals were frequently observed to swim at the water surface. In a previous study, exposure of *G. pulex* to 0.1 $\mu g \cdot L^{-1}$ of fenvalerate in laboratory channels resulted in a marked increase in drift, with the majority of the individuals changing behaviour and leaving the substrate by actively swimming up into the water column and heading downstream at a speed exceeding the current velocity [7].

At higher concentrations, the hyperactive state is followed by a decrease in the distance moved, as *G. pulex* gradually loses the ability to swim coordinately, and immobilisation follows. In the wolf spider *Pardosa amentata*, cypermethrin exposure induces paralysis of the hind limbs, caused by an effect on the flexor muscles [13]. Although this was not evident in *G. pulex*, a similar effect might explain the loss of ability to display coordinated swimming behaviour.

Although widely used, the pyrethroids have not been included in the standard methods used for monitoring pesticides in Danish surface waters and no data for cypermethrin concentrations are available. In a major Danish study, however, esfenvalerate concentrations up to 0.66 $\mu g \cdot L^{-1}$ in streams were determined, with 20% of all the water samples containing $>0.1\ \mu g \cdot L^{-1}$ [14]. Given that the toxicity of the α-cyano-pyrethroids esfenvalerate and cypermethrin to freshwater invertebrates is similar [15–17], it is likely that these concentrations found in natural streams would cause hyperactivity in *G. pulex*. Because the hyperactivity in *G. pulex* occurs as active swimming at the water surface, it is highly probable that these behavioural changes would result in drift in a stream. For the pyrethroid lambda-cyhalothrin, the ability to extrapolate effect concentrations from laboratory studies of locomotory behaviour to studies of drift in stream microcosms has been documented for *G. pulex*, *L. nigra* and *H. sulphurea* [6].

Pronounced drift of invertebrates, as well as direct mortality, induced by pyrethroids will, obviously, alter the population size and potentially the community structure of the affected stretch of a stream [2,3,5]. Furthermore, the observed changes in locomotory behaviour in this study might affect the ability of the remaining animals to obtain food, avoid predators or reproduce successfully. Studies are underway in our laboratory to elucidate these sublethal effects.

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