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A New Multi-cell Exposure System for Continuous Tracking of Daphnia Behavior for Toxicity Assessments

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Abstract

For several years, video tracking systems have been developed to analyze alterations in the swimming behavior of daphnia to provide early signals of chemical stress. However, these systems have limited testing abilities that do not allow for a systematic analysis of the robustness of behavioral endpoints. With recent advances in behavior tracking technology, we were able to develop a new behavioral analysis multi-cell exposure system named "Multi-DaphTrack" with a high-throughput testing capacity for assessing the behavioral response of *Daphnia magna*. The insecticide esfenvalerate was chosen as chemical model and tested on daphnid neonates at several concentrations for 48 h to (i) evaluate the performance of this new system and (ii) compare the sensitivity of our new multi-cell system with the standard immobilization assay and the Bbe®Daphnia Toximeter. Overall, the results demonstrated that our new "Multi-DaphTrack" system can detect significant behavioral effects of esfenvalerate at concentrations as low as 0.14 µg/L from a minimum of 1 h of exposure. Similar rapid behavioral effect trends were observed with the Bbe®Daphnia Toximeter. The behavior proved to be more sensitive than the standard immobilization endpoint. Significant behavioral changes were observed at the esfenvalerate concentrations that occur in contaminated rivers from agricultural areas in Europe and North America. Our results indicate that the "Multi-DaphTrack" system represents a powerful and convenient tool for the assessment of c and water quality.

Keywords: *Daphnia magna*; Behavioral analysis system; Water quality; Chemical toxicity; Risk assessment

Introduction

When assessing the risk of chemicals released into the environment, potential toxicity is typically assessed using standardized toxicity tests on representative organisms. The immobilization test on *Daphnia magna* is one of the most frequently used standardized tests [1,2] for assessing the hazardousness of chemicals and monitoring water quality. Indeed, this test is simple, fast and cost effective, and *Daphnia magna* is highly sensitive to a wide range of chemicals [3] and representative of freshwater organisms [4,5]. However, in many toxicity assessments of surface water or effluent, *Daphnia magna* immobilization is not sufficiently sensitive to measure water quality because sub-lethal effects can occur at considerably lower concentrations. Several indications of chemical stress produced by pollutants on organisms are overlooked by the standard acute toxicity test because this test only focuses on immobilization at two pre-defined exposure times (i.e., 24 and 48 h). Instead of measuring immobilization, tests that measure a reduction in the organisms' overall state of health can provide useful information on induced adverse effects and allow for the determination of a toxicity threshold. Significant impacts (e.g., altered behavior, growth and reproduction) can occur below the acute median effective concentration (EC_{50}) level [6]. Furthermore, monitoring the effects of toxicity over time is not a common practice even though the effects of chemical stressors are well-known to change over time and according to exposure duration [7]. Therefore, the detection of sub-lethal effects over time may be of great assistance in chemical toxicity and water quality assessments.

To protect the aquatic ecosystem, a rapid assessment of the toxic effects of environmental pollutants is required. Behavior is a sensitive indicator of acute/sub-lethal toxicity [8] that is particularly suitable for detecting stress induced by realistic environmental concentrations of pollutants. Therefore, behavioral monitoring of encaged organisms has become increasingly used for water quality assessments as an alternative or supplement to chemical monitoring [9]. Furthermore, behavioral tests provide early and intermediary responses prior to the death of organisms because toxic stress can induce rapid behavioral changes in exposed organisms [10]. Significant alterations of daphnia swimming behavior were previously reported after exposure to xenobiotics, such as metals [11,12], pesticides [13], and nanoparticles [14], surface water samples and cyanotoxins [15]. These alterations can impact the overall state of health and survival of organisms and may lead to long-term changes at the population and community levels [16]. With increases in average speed, the energy used for normal metabolic functions (e.g., growth, reproduction and locomotion) might have to be reallocated to locomotion, which might impact the fitness or the long-term survival of organisms [17]. Moreover, adaptive behavior, such as avoidance or alteration of mobility, under toxic stress can impact an organism's decision-making capabilities (e.g., location seeking) and make the organism more noticeable to predators.

There are currently two different types of systems that monitor daphnia behavior: the first type is dedicated to water monitoring and provides real-time signal processing, whereas the second type is designed to characterize the toxic effects of chemical compounds on

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swimming behavior. Systems that are dedicated to water monitoring often have a limited number of measuring cells with a flow-through system and automated image analysis algorithms to provide real-time signal processing. The main goal of these systems is to trigger alarms when abnormal behavior is observed so that water sampling or the shutdown of the water work inflows can be initiated. The behavioral analysis systems that are currently available on the market using daphnia under flow-through conditions (Multispecies Freshwater Biomonitor from LimCo®International GmbH, Germany, and Toximeter from Bbe®moldaenke, Germany) are used to detect toxic pollution peaks in effluents or rivers [18]. For instance, the Bbe® Daphnia Toximeter (Bbe®moldaenke, Germany) has been successfully used as an early warning system for chemical pollution in surface water from rivers throughout Northern Europe over the last 15 years.

The other type of system designed to monitor the toxic effects of chemicals on daphnia behavior is typically developed in laboratories and under static conditions. These laboratory systems monitor one or several swimming parameters, such as mean velocity (most frequently used), activity/rest, distance travelled and path angle changes. Compared to commercialized *in situ* systems, laboratory systems are often hand-crafted systems developed to monitor the behavior of many daphnids simultaneously and often combine one or several cameras with inexpensive tracking software. To date, the system with the highest test capacity for the simultaneous observations of a group of organisms was developed [12], and the testing capacity reached a maximum of 6 measuring cells. Other systems have recently been developed to analyze the behavior of individual/small groups of daphnia. For instance, Jeon et al. [19] developed a system capable of individually monitoring 6 *Daphnia magna* moving simultaneously under static conditions. To date, recent technological advances and enhanced detection resolution and computer performance have increased the testing capacity and allowed for the tracking of up to 12 replicates of groups of organisms [14] and resulted in high-throughput screening assays of individuals with 24 replicates [13]. Most of these systems have focused on tracking individuals or small groups of organisms; the effects are often observed over a limited time (e.g., a single pre-defined time or a few hours), and the experimental conditions do not follow the standard acute toxicity test protocol.

The behavioral analysis systems that are currently available on the market offer limited testing capabilities that do not allow for a systematic analysis of the robustness of behavioral endpoints. Therefore, the aim of the study was to (i) improve behavioral analyses by developing and validating a new "Multi-DaphTrack" system with an optimized test capacity, (ii) establish the link between responses in daphnia behavior and exposure to a well-known neurotoxic insecticide, and (iii) compare the sensitivity of the behavioral effects detected in our new system with those of the standard immobilization endpoint and current online biomonitoring system, i.e., the Bbe® Daphnia Toximeter. Thus, in collaboration with the Viewpoint company (Life® Technologies, France), we developed new multi-cell exposure system named "Multi-DaphTrack" for the simultaneous video tracking and behavior analysis of a large number of groups of 10 daphnids dispatched in up to 20 cells for several hours or days. To perform comparisons of the behavioral endpoints with the standard immobilization endpoint, the "Multi-DaphTrack" system was adapted from standard acute toxicity test conditions. The neurotoxic insecticide esfenvalerate was selected as a model molecule to induce rapid behavioral effects, which have been reported in crustaceans for this insecticide. The neurotoxic insecticide esfenvalerate was tested in the "Multi-DaphTrack" system to validate the new behavioral monitoring system and tested in parallel in the Bbe® Daphnia Toximeter to provide a comparison between the behavior trends of these two behavioral analysis systems. All of the experiments were conducted over a period of 48 h at 20°C to allow for a direct comparison with the results of the standard acute toxicity test.

Materials and Methods

Design of the new behavioral multi-cell exposure system

The "Multi-DaphTrack" system was designed to simultaneously monitor swimming activity of a maximum number of groups of daphnids (group of 10 individuals) with exposure conditions similar to acute immobilization tests. A test capacity of up to 20 replicates (5 concentrations in triplicate and 5 control replicates) was considered the best option between the high-test capacity and detection efficiency. Twenty optical glass cells (50 ×50 × 10 mm) that were supplied by Hellma®were used as exposure cells and assembled in a vertical 4 × 5 rack. Infrared light-emitting diode (LED) strips with a wavelength of 850 nm were placed between each of the rack's vertical cell rows to ensure homogeneous cell illumination. After inserting the exposure cells, the rack was covered with an opaque polymer mask to block the light sources and cover the exposure cell walls to limit diffusive light and reflections. This "Multi-DaphTrack" platform was placed inside a 100 × 60 × 60 cm black box to exclude external illumination. A video of the experiment was recorded by an infrared digital HD camera with a high-resolution of 1600 × 1200 pixels that was operating at 25 frames and positioned squarely 54 cm from the rack containing the exposure cells. Figure 1 shows a synthetic schematic of the system. The animals reflected the infrared light and were thus detected as white silhouettes against a black background. The video was transferred directly to a computer and saved in AVI format. The raw data on the daphnid positions x, y were extracted from the video with high time resolution

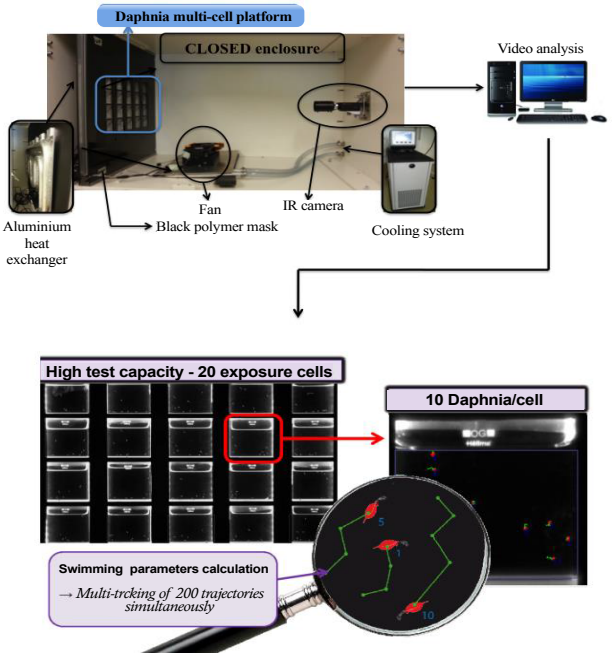

Figure 1: New behavioral multi-exposure cell test system named "Multi-DaphTrack" for behavioral analysis of *Daphnia magna* neonates with the Zebralab® software (Viewpoint® Life Technology, France). The exposure cell platform is placed in a closed chamber and can contain up to 20 cells that are illuminated by infrared light and kept at 20°C.

using Zebralab®software algorithms (Viewpoint®Life Technology, France). The trajectories were reconstructed, and several behavioral parameters were calculated for each measuring cell (each group of 10 daphnids), i.e., average speed, number of active organisms and change in path angle. Temporary averaged parameters for each daphnia group were then integrated into a time sequence of 1 s (25 images), and the averaged parameters were calculated and recorded within a predefined time bin (here, 30s) over 48 h and saved in an Excel file.

Simultaneous video tracking of 200 daphnia is challenging; therefore, numerous tests were conducted under control conditions to optimize the detection rate. Various numbers of organisms per cell (1,3,5,7,10, and 15) were also tested under control conditions in triplicate to estimate the impact of daphnia density on the behavioral parameters.

*Daphnia magna*culture and breeding and test organism preparation

Daphnia magna clones from parthenogenetic reproduction were reared in 1.5 L vessels containing artificial M4 culture medium at 20 ± 2°C with a photoperiod light/dark cycle of 16/8 h, which is consistent with the OECD test guideline 202 [1]. The M4 synthetic medium [1] was prepared from an M4 concentrated solution (Life®Technology) that was diluted 10 times with ultra-pure water. M4 synthetic medium was then stabilized at pH 8. *Daphnia magna* females were fed daily with a batch-cultured unicellular algae suspension of *Chlorellavulgaris* (3×10^5 cells/mL ~ 0.1 mg C/daphnia/day quantified by a particle counter (Coulter Z1, Beckman & Coulter®)). To maximize the neonate production, the vessels and M4 medium were replaced each week, 50% of the M4 medium was replaced twice a week, and filtration was performed each working day. To ensure consistency with the acute immobilization test and to reduce variability, the cultures from 2 to 4 weeks were filtered the evening before and on the day of the experiment to isolate neonates aged from 8 to 24 h within the same size range. The selected neonates were then acclimatized in ISO standard artificial reconstituted freshwater [1] at 20°C without feeding.

Chemicals

Esfenvalerate (CAS: 66230-04-4) was purchased as a solid 100 mg powder (99% purity) from VWR®, France. Because esfenvalerate has low solubility in water, the 250 mg/L stock solution was prepared by dissolving 25 mg of esfenvalerate powder in 100 mL of pure methanol. As recommended by OECD [20], the final concentration of methanol did not exceed 0.01% in the solvent control and different assays. All of the tested solutions were prepared with the artificial reconstituted freshwater recommended by ISO Standard 6341[2].

Esfenvalerate exposure

To define the concentration range for behavioral tests, an acute *Daphnia magna* toxicity test was first performed according to the OECD Test guideline 202 [1], and the results are available in the supplementary data file (a). Based on these results, 5 nominal concentrations (0.14, 0.35, 0.88, 2.2 and 5.5 µg/L) were selected for the test in the "Multi-DaphTrack" system, and the exposure conditions were designed to cover the concentrations below EC_5(48 H) up to EC_{100} (48 H) in the acute test. We used 3 replicates for each exposure treatment and 5 replicates for the controls (2 replicates of ISO water and 3 replicates of ISO water with 0.01% methanol). Each exposure cell was filled with 20 mL of the test solution, sealed with PARAFILM "M" ®and maintained at 20 ± 0.5°C (one additional exposure cell with ISO water was used to verify the temperature in the exposure cell over time). Ten neonates were carefully and randomly placed in each test exposure cell. In addition to the 48 h video tracking, immobilization of daphnia was determined at the end of the 48-h test. To compare the performance and sensitivity of the new test system, 4 concentrations (0.14, 0.35, 0.88 and 2.2 µg/L) were also tested in duplicate in the Daphnia Toximeter (Bbe® Moldaenke, Kiel, Germany), a commercially available online biomonitoring system. Ten neonates of less than 24 h were carefully placed in each 25 mL exposure cell. The specific conditions in the Bbe®Daphnia Toximeter are described in the supplementary data file (b).

Statistical analysis

For the acute toxicity test, concentration-response curves of immobilization were modeled using the Hill model in the Regtox®macro for Microsoft Excel. The effect concentrations (EC_x) and their confidence intervals were estimated using the non-parametric "bootstrap" method. Because the behavioral data provided by the "Multi-DaphTrack" system are more complex and time-dependent, all further statistical analyses were performed with customized scripts in the statistical software R (R 3.0.1). Because the signal was noisy, the average speed per condition and per hour together with the variability was calculatedto reduce the signal's noise and allow for comparisons between the different concentrations. After verifying the compliance of the variance homogeneity and normal distribution of data, a standard ANOVA model was performed with a simple Student's t-test through independent comparisons of each concentration to the control test (p=0.01) for each hour. Because of the test capacity constraints of the DaphToxI®system, only one replicate of control was performed in each experiment. To compare the results, 12 controls were combined and considered as a control reference. A linear mixed-effects model was then applied to account for the day-to-day variability in the average speed of *Daphnia magna*in the controls; and variability in the average speed was considered a random effect. This model was used to independently estimate the (fixed) effect of exposure with respect to the control conditions for each hour.

Results

Performance of the "Multi-DaphTrack" system

Optimization of the detection rate: Prior to optimization, the detection rates under the control conditions were below 50%, which was unacceptable. To increase the detection of the small daphnia neonates (size between 0.62 and 0.72 mm^2), the illumination was first optimized by placing the infrared LEDs in each vertical row to increase the light intensity on each side of the measuring cells and ensure the homogeneity of light dispersion to achieve optimal detection of daphnids. To avoid artefacts, the light reflection was reduced with black adhesive strips, a background refresh option was performed frequently (every 60 s) as a noise filtering operation and threshold detection parameters were determined for each exposure cell independently. The detection rate was approximately 93%, and was found to be consistent when measuring the different exposure cells and stable over time.

Control of the experimental conditions in the "Multi-DaphTrack" system: Because of the distinct geometry of the exposure cells and heat that was introduced by the light sources placed in close contact with the cells, substantial efforts were required to stabilize the temperature and limit evaporation. For the illumination, 300 infrared 0.1 WLEDs were placed close to the exposure cells, which required the dissipation of 30 W of thermal power to avoid a rise in temperature. The temperature in the cells after 48 h of exposure was equal to 26 ± 5.5°C. A cooling system that circulated water at 14°C and had an aluminum heat exchanger and two ventilators was added to the exposure cell close

to the LED strips, and a stable and fixed temperature of 20 ± 0.5°C was obtained. The measurements of weight loss in each exposure cells indicated that water evaporation was negligible (< 1%) during the 48 h exposure period. The oxygen level in each cell was equal to 91.5 ± 1.5% of the saturated oxygen concentration at the end of the exposure period, which is above the minimum value of 3mg/L required by the OECD [1] and indicates no depletion of dissolved oxygen compared with the conditions at the start of the experiment. Besides, reference tests with the $K_2Cr_2O_7$ were regularly performed on Daphnids and results indicate that the sensitivity of the strain remains unchanged.

"Normal" behavior under control conditions: To determine the "normal" behavior of *Daphnia magna* under static conditions, 65 replicates of 10 non-exposed individuals were gathered from 13 different experiments (5 replicates per experiment). Throughout their exposure, the daphnids swam homogeneously with a path angle changing from 0 to ± 45°. The average ± standard error over 48 h of the two parameters average speed and number of active organisms are presented in Figure 2 for *Daphnia magna*that were exposed to control conditions. The average speed over the entire 48 h of video tracking was equal to 2.55 ± 0.11 mm/s. Nevertheless, a slight decrease was observed at the end of the experiment (down to 2.24 mm/s). The number of active organisms initially detected by the system was approximately 9.3, which is consistent with the 10 daphnids introduced to each exposure cell. This slight under-detection was likely because of the loss of daphnids swimming close to the water surface or exposure cell walls or related to crossing trajectories that are not recognized by the software's detection algorithm. During exposure, the number of active organisms that were detected decreased constantly and dropped to a level of 6.6 at 48 h. No lethality was observed for daphnia under control conditions at the end of the 48 hours of exposure. Thus, the decrease of the number of active organisms is not linked to mortality but appears to reflect daphnia behavior in response to these exposure conditions. The number of active organisms was less uniform and exhibited higher variability between replicates compared with the average speed parameter, and the standard error increased at the end of exposure. Furthermore, the average speed remained stable when the number of active organisms tended to decrease between the 20th and 35th h. The resting time, which is defined as any period of inactivity, was also measured, but no significant pattern was demonstrated under the control conditions. No lethality was observed at 48 h after gentle agitation, meaning that neonates did not die during the experiment but was merely in a resting state. The experiments on the influence of daphnia density on the average speed indicated that except for the average speed of a single daphnia (averaged at 1.71 ± 0.13 mm/s over 48 h), there was no significant difference between the average speed of a given group with 3 to 15 individuals (averaged of 2.31 ± 0.06 mm/s). Therefore, density does not appear to modify the daphnia average speed except for isolated individuals.

Esfenvalerate exposure results

Test in the "Multi-DaphTrack" system: The parameters average speed, number of active organisms and change in path angle were measured for each exposure condition. However, because the number of active organisms and changes in path angle parameters did provide considerable useful information related to toxicity, the average speed parameter was selected to assess the behavioral effects induced by chemical exposures.Time courses of the average speed for the control and esfenvalerate-exposed *Daphnia magna* and a histogram of increasing and decreasing speed effects relative to the controls over time are shown in Figure 3 and 4. Overall, esfenvalerate induced a significant increase in swimming speed at all of the tested concentrations after the first hour of exposure (from + 23 to + 107 % above the control level). A slight but significant increase in average speed (+ 23 % relative to the controls, $p<0.01$) was observed for the lowest test concentration of 0.14 µg/L (below EC_5(48 h)) after the first hour of exposure and lasted for 16 h. The average speed then returned to the control level. The most pronounced increase in average speed was observed at 0.35 µg/L (near EC_{10} (48 h)), with the maximum speed occurring at 4 h (average speed increase was + 123 % ($p<0.01$) above the control level). This exposure concentration caused excitation of the daphnia for 21 h before the average speed returned to the control level (longest excitation effect duration observed in the experiment). A similar sharp increase was observed for concentrations of 0.88 µg/L (near EC_{50} (48 h)) and 2.2 µg/L (above EC_{70}(48 h)), with a maximum

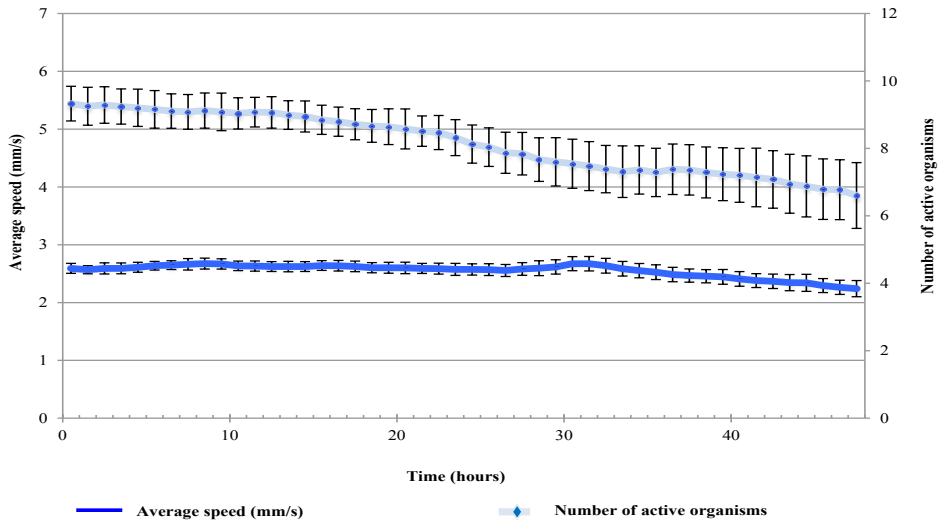

Figure 2: Behavior of *Daphnia magna* neonates under control conditions over 48 h in the "multi-DaphTrack" system at 20°C. Mean values of the average speed and the number of active organisms parameters of n=65 replicates (per h) with standard error - detection rate of approximately 93%.

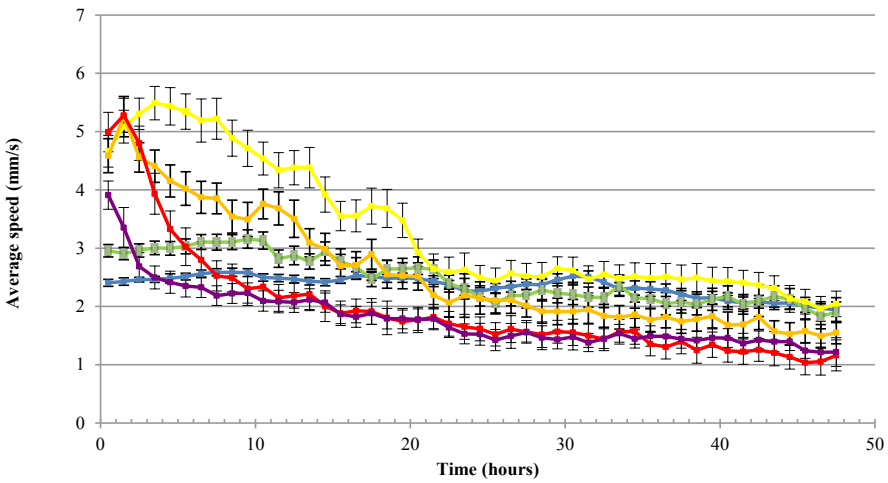

Figure 3: Average speed (per h) with standard error of *Daphnia magna* exposed to several concentrations of esfenvalerate for 48 h in the "Multi-DaphTrack" system. For controls, n=5 replicates and for each esfenvalerate treatment, n=3 replicates.**s**

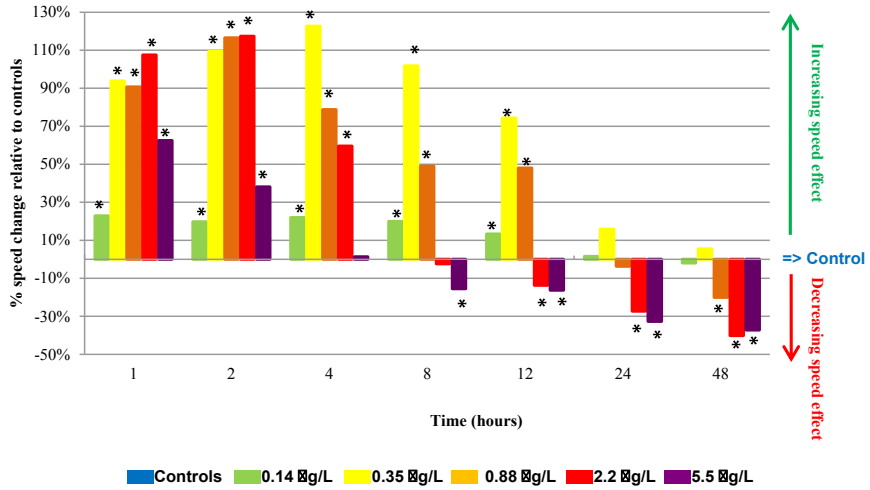

Figure 4: Histograms of increasing and decreasing speed effects relative to the control levels for *Daphnia magna* exposed to different concentrations of esfenvalerate in the "Multi-DaphTrack" system. The average speed change relative to controls is calculated by dividing the average speed difference between exposed-Daphnia and controls by the average speed value of the controls at 7-fixed times (i.e., 1, 2, 4, 8, 12, 24 and 48h). * Significantly different from the control level (Pvalue<0.01).

increase of + 117% reached at 2 h. The excitation effects lasted for 15 h at the concentration of 0.88 µg/L and 6 h at the concentration of 2.2 µg/L until the swimming speed dropped significantly below the control level starting at 28 h and 15 h, respectively. A smaller but significant increase in average speed compared to the control level occurred at 5.5 µg/L of esfenvalerate (maximum of + 63 % attained at 1 h ($p<0.01$)), but it did not last more than 3 h. A significant decrease in average speed was then observed for this same concentration after 8 h in the experiment compared to the control level (approximately 45%, $p<0.01$), and after 48 h of exposure, 100% immobilization of the daphnids was observed. The calculated 48 h EC_{50} for *Daphnia magna* immobilization exposed to esfenvalerate was 1.04 ± 0.01 µg/L in the "Multi-DaphTrack" system, which is consistent with the results of the standard acute toxicity test (EC_{50} (48 h) = 0.89 ± 0.12 µg/L).

Test in the Bbe® Daphnia Toximeter: The time course of the average speed of the control and esfenvalerate-exposed *Daphnia magna* over time in the Bbe® Daphnia Toximeter is shown in Figure 5 (a). In the Bbe® Daphnia Toximeter, an average speed of 3.65 ± 0.13 mm/s was observed for the control conditions over a period of 48 hours, with the daphnids swimming 1.5 times faster in the Bbe® Daphnia Toximeter than in the "Multi-DaphTrack" system (2.35 ± 0.18 mm/s). Although the average speed decreased slightly over time in the "Multi-DaphTrack" system, the average speed remained stable in the Bbe® Daphnia Toximeter. No significant effect on average speed was observed for the lowest tested concentration of esfenvalerate at 0.14 µg/L (below EC_5(48 h)). A significant increase in average speed ($p<0.01$) was observed from the first hour for daphnia exposed to 0.35 µg/L (below EC_{10} (48 h)), and it lasted for 14 h. A slight but significant increase in average speed ($p<0.01$) was measured at the concentration of 0.88 µg/L (near EC_{50} (48 h)), and it lasted for 3 h. The highest significant increase in average

speed was observed for the highest concentration of 2.2 µg/L (above EC_{70} (48 h)), and it lasted for 10 h.

Comparison of the "Multi-DaphTrack" system and the Bbe® Daphnia Toximeter: The relative speeds (i.e., ratio of the average swimming speed of daphnids exposed to esfenvalerate divided by the average swimming speed of the controls) were calculated for each concentration and used to compare the "Multi-DaphTrack" system and the Bbe® Daphnia Toximeter, which are presented in Figure 5 (b). Overall, the relative speeds followed the same trends (i.e., rapid peak increase) except for the first tested concentration of 0.14 µg/L. The most pronounced increase was observed for the tested concentration of 0.35 µg/L (below EC_{10} (48 h)).

Discussion

Validation of the new "Multi-DaphTrack" system

Several biotic and abiotic factors, such as age, temperature and light, may have a significant impact on daphnia behavior; therefore, a significant effort was therefore made to control the experimental conditions inside the new "Multi-DaphTrack" system. For instance, infrared light, which is not visible to daphnia, was chosen to prevent any light-induced behavior disturbances. Moreover, temperature was tightly controlled because it is one of the most important abiotic factors and capable of influencing several physiological and biological processes in all organisms [21] was tightly controlled. For instance,

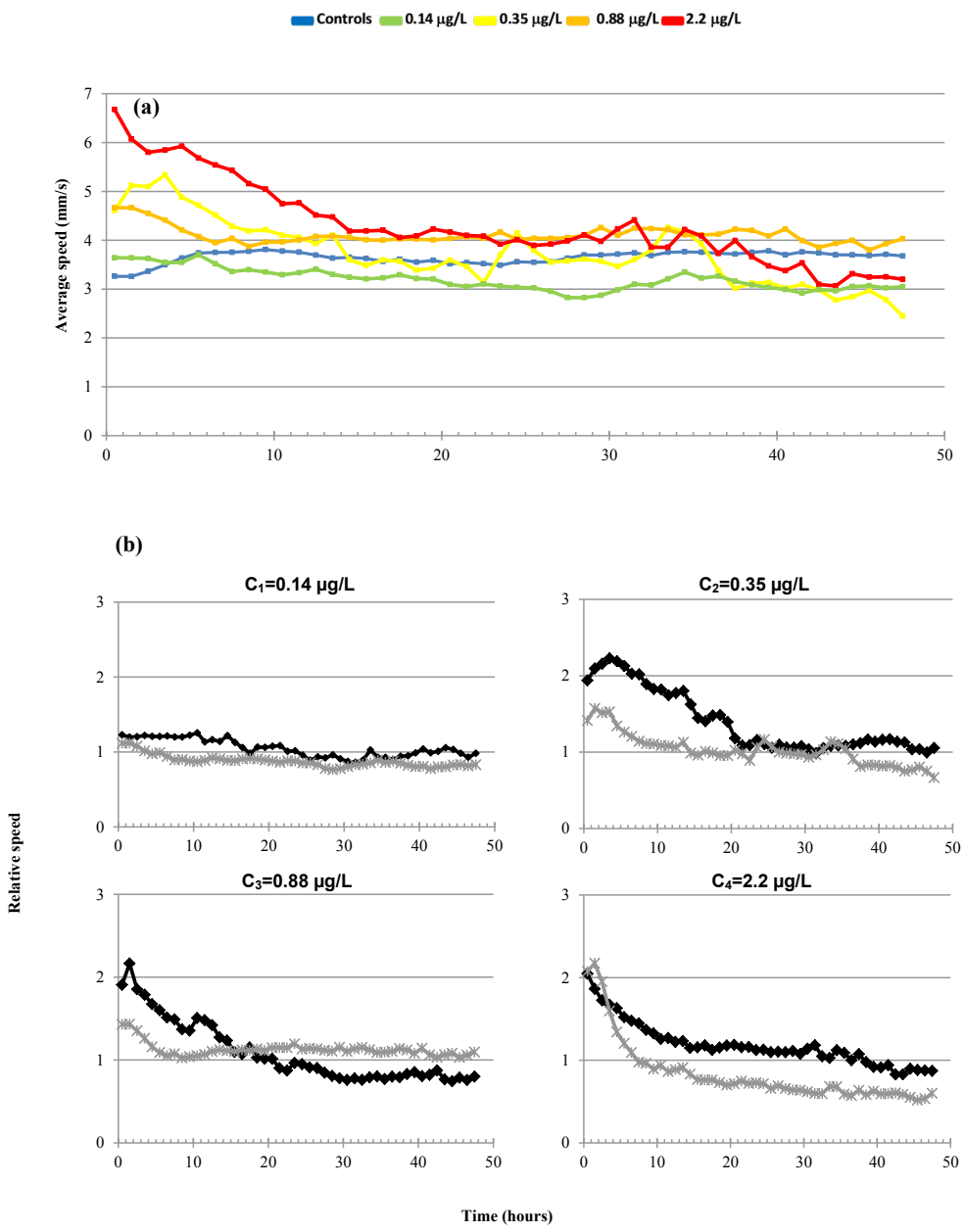

Figure 5: (a) Average speed (per h) of *Daphnia magna* exposed to several concentrations of esfenvalerate for 48 h on a Bbe® Daphnia Toximeter, (b) Comparison between relative average speeds (per h) of *Daphnia magna* exposed to several concentrations of esfenvalerate during 48 h in the Bbe® Daphnia Toximeter and in the "Multi-DaphTrack" system, legend: Black: relative speed in the "Multi-DaphTrack" system, Grey: relative speed in the Bbe® Daphnia Toximeter. For controls, n=12 replicates and for each concentration of esfenvalerate, n=3 replicates.

when temperatures increase, animals swim up more frequently [22]. On the other hand, low temperatures (e.g., 0 to 5°C) can greatly decrease the swimming ability of *Daphnia magna* [23]. Temperature is also a potential stressor that may modify toxic effects in daphnids and other species [24-26]. Furthermore, although detection errors cannot entirely be avoided (e.g., cross-over swimming, detection loss), our optimization of illumination conditions and adjustments to the software parameters resulted in an acceptable detection rate of 93 % for the new "Multi-DaphTrack" system.

Results for the "normal" behavior indicated that daphnids swam homogeneously over time and exhibited stability in the parameters average speed and number of active organisms, although there was a decrease in both parameters at the end of the experiment. This decrease can be explained by the exposure conditions because static conditions do not stimulate daphnia movement and neonates were not fed during the experiment; thus, they were likely tired and in need of rest. By watching the video track during the experiment, we can confirm that neonates alternated between swimming and periods of rest at the end of exposure. This decrease in daphnia activity was not caused by a decrease in the detection rate. Furthermore, the software was unable of detecting small movements, such as turns and gyres along the main body axis, for numerous active organisms. As a result, the parameter 'number of active organisms' cannot be considered to be entirely similar to the standard parameter 'immobilization' over time. The organisms may interact with each other, and the density of organisms inside the measuring cell may affect behavioral parameters; however, our results indicated that density did not modify the daphnia swimming speed expect for isolated individuals. Therefore, our results showed that when mortality occurred for some individuals during exposure to an acute toxic concentration of chemicals, it did not significantly influence the swimming speed of the remaining living individuals.

Effects of esfenvalerate on Daphnia magna behavior

Behavior disturbances can be considered as a sequence of neuro-sensorial, muscular and metabolic alterations [10,12,27]. A number of pesticides are known to be extremely toxic to aquatic organisms and although esfenvalerate is generally applied at low doses, non-target aquatic organisms are highly sensitive to even low levels of this insecticide [28]. For instance, significant alterations of the behavior were previously reported for the fathead minnow *Pimephalespromelas* [29] and arthropods Black margined Aphid, Black Pecan Aphid, and Yellow Pecan Aphid [30] exposed to esfenvalerate. Our results showed that the toxic effects of esfenvalerate on behavior increased in a dose-dependent manner. A rapid increase in the average speed of *Daphnia magna* occurred from the first hour of esfenvalerate exposure; this result was consistent with the mode of action of esfenvalerate, which modulates sodium channel activity by inducing the reversible blocking of sodium channels in the neurons, leading to an impaired action potential along the axon and hyper-excitation that is followed by muscle paralysis and death by respiratory arrest [31]. Another hypothesis is that daphnids increase their swimming speed (hyperactivity) as a protective avoidance response to escape from polluted areas [10]. In the "Multi-DaphTrack" system, sub-lethal concentrations up to 0.35 µg/L (below EC_{10} (48 h)) resulted in speed increases that were followed by decreases back to the control level. For concentrations of 0.88 µg/L (near EC_{50} (48 h)) and above, the swimming speed declined over time to levels two-fold lower than the control levels. The recovery at low concentrations was likely related to esfenvalerate's reversible mechanism of action and the low acute effects it exerts at those concentrations. Detoxification mechanisms may also be involved and produce a rapid elimination of the toxin and lead to a return to normal behavior.The speed decrease for acute concentrations (≥0.88 µg/L) suggests a loss of energy for muscle activity or locomotion and may result in tiredness and/or the first signs of harmful effects [11]. These results corroborate previous studies in which significant alterations in *Daphnia magna*behavior were detected for higher concentrations of esfenvalerate [32] and similar concentrations of cypermethrin, a chemical that shares the same mode of action as esfenvalerate [33].

Overall, the behavioral effects that were induced by esfenvalerate exposure and measured in the "Multi-DaphTrack" system followed the same trends as the behavioral effects in the Bbe® Daphnia Toximeter, which showed a similar increase in swimming speed from the first hour of exposure. However, although the average speed in the Bbe®Daphnia Toximeter was higher under the control conditions, the esfenvalerate effects were less pronounced in the Bbe®Daphnia Toximeter compared with that of the "Multi-DaphTrack" system. Moreover, the duration of the effects was shorter in the Bbe®Daphnia Toximeter.

Sensitivity of the "Multi-DaphTrack" system

The organisms reacted shortly after exposure to esfenvalerate and exhibited a significant increase in average speed from the first hour of exposure at the concentration of 0.14 µg/L of esfenvalerate. The results showed that this increase in average speed was a highly sensitive and early behavioral response to multi-stresses, including pollutant exposure. Behavioral disturbances have been reported in daphnids that were exposed to sub-lethal concentrations of xenobiotics, such as cadmium and copper [12,34,35]. Furthermore, this system could be sufficiently sensitive to detect the effects induced by environmental concentrations of esfenvalerate, such as concentrations of 0.66 µg/L, which were measured in Danish streams [36], or concentrations of approximately 0.15 µg/L, which were measured in rivers from California [37]. With this possibility in mind, tests on substances with different modes of action can be performed to determine whether the system is equally sensitive to a wide range of chemicals. This system offers the potential to monitor effects over a period of at least 48 h, which is of high relevance for substances that have delayed toxic effects.

Conclusion

The "Multi-DaphTrack" system developed for this study shows a higher degree of sensitivity compared with the conventional immobilization assay and provides significant responses detected below EC_5 (48 h). The "Multi-DaphTrack" system is also more sensitive than the Bbe®Daphnia Toximeter because the number of replicates in the "Multi-DaphTrack" system provides more robust data and decreases variability. The average speed of *Daphnia magna* can likely be considered as a rapid and sensitive indicator of toxic stress. One interesting aspect of the average speed parameter is that it is not only sensitive relative to the traditional acute toxicity test but also responds rapidly after exposure, whereas lethal effects are obviously delayed. Overall, the "Multi-DaphTrack" system has the potential to successfully and simultaneously record the behavior of 200 daphnids in different chambers. This new system can be used to detect subtle and early behavioral changes in *Daphnia magna*induced by sub-lethal concentrations of pollutants.

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