ORIGINAL ARTICLE

Behavioural responses of juvenile *Daphnia magna* to two organophosphorus insecticides

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ABSTRACT

In this study, the behaviour of *Daphnia magna* was studied under equipotent and sub-lethal concentrations of two pesticides congeners: chlorpyrifos (CPF; 5 ng L^{-1} to 50 ng L^{-1}) and chlorpyrifos-methyl (CPF-m; 30 ng L^{-1} to 300 ng L^{-1}) with aims to assess and compare the behavioural swimming responses (BSRs) of the cladocerans elicited by both compounds at different concentrations and exposure times. A video tracking analysis after 24 h and 48 h of exposure allowed us to evaluate different behavioural responses (distance moved, average velocity, active time, and average acceleration). The results indicate that BSRs are sensitive indicators of sub-lethal stress. Highly concentration- and time-response changes for both compounds were observed during the experiments. In particular, in the first 24 h of exposure, both compounds elicited a similar decreasing trend in swimming behaviour, in which CPF induced the highest decline. Further, hypoactivity was associated with the narcotic effects of both compounds. Conversely, after 48 h of exposure, we observed an increasing tendency in the swimming parameters, particularly at the highest tested concentrations. However, the compounds did not exhibit the same trend. Rather, CPF-m induced high variations from the control groups. This reversal trend could be due to the activation of compensatory mechanisms, such as feeding, searching, or avoidance behaviours. These results suggest that BSRs are measurable active responses of organisms, which are controlled by time.

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INTRODUCTION

Aquatic systems are threatened by various anthropogenic chemical stressors, compromising the future provisioning of vital ecosystem services (Vörösmarty *et al*., 2010; Cardinale *et al*., 2012; Malaj *et al*., 2014), even in pristine areas (Villa *et al*., 2006; Ferrario *et al*., 2017; Morselli *et al*., 2018; Rizzi *et al*., 2019). Although these chemicals mostly occur at nonlethal concentrations, many have the potential to induce sub-lethal effects, such as behavioural disturbances in aquatic organisms. Behavioural disturbances, especially in key-species, can have severe consequences, thereby impacting ecosystem maintenance due to a cascade of indirect effects at various biological levels (Saaristo *et al*., 2018). In particular, organism disturbances induced at biochemical, cellular, or physiological levels can cause compensatory processes, including antioxidant and detoxifying processes that require an increase in energy consumption at the detriment of other functions, such as locomotion (Amiard-Triquet, 2009). A decrease in the locomotor ability of a species can affect population fitness, which in turn can have detrimental consequences at the community level as a result of changes in competition ability and prey/predation interactions (Saaristo *et al*., 2018).

For decades, animal behavioural swimming responses (BSRs), which are animal reactions to different stressors, have been used as a method of environmental monitoring (Cairns and Gruber, 1980, Gerhardt *et al*., 1998). However, lately, there is increasing interest in BSRs for ecotoxicology applications (Pyle and Ford, 2017; Parolini *et al*., 2018; Villa *et al*., 2020). This research trend was made possible by the development of technological tools available for quantifying behavioural changes (Chevalier et al., 2014). Thus, knowledge regarding the links between the responses of organisms to stress at different hierarchical organisation levels (biochemical, physiological, and behavioural) and their ecological consequences are improving (Amiard-Triquet, 2009; Sloman and McNeil, 2012; Ferrario *et al*., 2018; Villa *et al*., 2018; Di Nica *et al.,* 2020). According to Melvin and Wilson (2013), a BSR is at least one to two or-

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ders of magnitude more sensitive than the classical ecotoxicological endpoints (e.g., survival). Moreover, behavioural alteration signals can perceive even low levels of chemical pollution, making them an 'early warning signal' of environmental quality (Ägerstrand *et al*., 2020). For example, organism avoidance to contaminants, which is an adaptive behaviour that may reduce exposure to harmful conditions, is often one of the earliest responses of an exposed organism (Ren *et al*., 2009; Hellou, 2011).

Therefore, the use of behavioural endpoints (e.g., weight of evidence or higher tier assessments), in support of current standard tests, can increase the ecological significance of environmental risk assessment procedures. Currently, a consensus regarding the use of behavioural evidence has not been reached in the ecotoxicology field (Ägerstrand *et al*., 2020). Programmes such as the Registration, Evaluation, and Authorisation of Chemicals in the European Union and the Safe Drinking Water Act and the Food Quality Protection Act in the United States (Ankley *et al*., 2010) foster the consideration of a broader suite of toxic effects than those actually used in ecotoxicological risk assessment practices. However, efforts must be made to use BSR studies in regulatory assessments. Further, available behavioural studies, in general, cannot be compared because of a lack of test design and method standardisations (e.g., choice of exposure conditions and selection of behavioural endpoints) (Chevalier *et al*., 2015). In addition, a systematic characterisation or modelling of behavioural response mechanisms species must be developed. For instance, the hypothesis that compounds sharing that same mode of action (MoA) exert similar toxicological effects on organisms, which could elicit similar behavioural changes, requires robust scientific evidence, and further studies are therefore required.

To verify this hypothesis, two strictly congeners organophosphorus (OP) insecticides were selected as they are both acetylcholinesterase inhibitors. Particularly, behavioural tests were set up to investigate and compare the sub-lethal effects in juvenile *Daphnia magna* exposed to equitoxic concentrations of chlorpyrifos (CPF) and chlorpyrifos-methyl (CPF-m). These compounds are of high ecological concern as they are widely used worldwide. They are commonly detected in surface water bodies in concentrations ranging from 0.32 ng/L to 340 ng/L (Stamatis *et al*., 2013; Ccanccapa *et al*., 2016; Affum *et al*., 2018; Rizzi *et al*., 2019; De Souza *et al*., 2020).

Herein, the EC_{50} (48 h) of both compounds was set as one toxic unit (1 TU) and the BSRs were examined after 24 h and 48 h of exposure to five concentration gradients (0.05 TU, 0.1 TU, 0.25 TU, and 0.5 TU) of each compound. Finally, the behavioural effects (swimming velocity, acceleration speed, distance moved, and active-inactive time) were estimated using a video tracking system followed by image analysis. Moreover, in this paper we propose for the first time the use of behavioural concentration response curves (CRC) as a tool to compare qualitatively the overall changes (increasing or decreasing of monitored effects) induced by different chemicals.

METHODS

Test chemicals

CPF (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate; CAS n. 2921-88-2; purity = 98.0%), CPF-m (O,O-dimethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate; CAS n. 5598-13-0; purity ≥ 98.0%), and ethanol as the solvent used (analytical purity), were purchased from Merck Life Science S.R.L. (Milano, Italy). Stock solutions of CPF and CPF-m were in ethanol (1 mg mL^{-1}). The final concentrations of the carrier solvent (EtOH: ethanol) used in all the experimental exposure solutions were always below the level indicated by OECD guidelines (OECD, 2019).

Test species

The cladoceran *D. magna* Straus, which has been used as a freshwater model for behavioural studies, originated from a single clone derived from the Istituto Superiore di Sanità (Rome, Italy). The daphnids were cultured in 500 mL beakers (40 individual L^{-1}) of commercial mineral water (San Benedetto® - conductivity 415 µS cm^{-1} at 20°C; pH 7.42; 301 mg L^{-1} HCO^{-3}, 48.6 mg L^{-1} Ca^{2+}; 28.2 mg L^{-1} Mg^{2+}) under a 16:8 h (light:dark) photoperiod at a controlled temperature of 20.0±0.5°C in order to ensure continuous parthenogenetic reproduction (Frey, 1982). *D. magna* individuals were cultured under optimum conditions for maximum production of neonates (after 21 days, each animal produced a number of live offspring >60) and following the OECD Guideline 202 standard protocols (OECD, 2004). The validity criteria of the tests were fulfilled (not more than 10 percent of the control daphnids showed immobilisation or other signs of disease or stress).

The daphnids were fed three times a week with a suspension of the green alga *Raphidocelis subcapitata* (16 x 10^6 cells ind^{-1} day^{-1}) and the yeast *Saccharomyces cerevisiae* (100 mg L^{-1}). Half of the concentration of both suspensions was provided for individuals aged up to 8 days. The culture medium was renewed three times a week.

Culturing of the algae was performed in 2 L flasks filled with OECD TG 201 (OECD, 2006) medium at 20.0±2°C under continuous light and was aerated through gentle shaking. Once a week, during the exponential growth phase, algae were collected via centrifugation at 1500 rpm for 10 min (Hermle z 323 K, Labortechnik GmbH, Germany). The algal suspension density was measured using a Burker chamber under a bright field light microscope and stored at 4°C until use.

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Experimental setup

Behavioural experiments (test duration 48 h) were conducted under the same culture conditions described above, beginning with the third-generation neonates of *D. magna* (<24 h old). The selected exposure concentrations were obtained via serial dilutions starting from the EC_{50} (48 h) value of each chemical for *D. magna* (100 ng L^{-1} for the CPF and 600 ng L^{-1} for the CPF-m; EC_{50} values obtained from the Pesticide Properties Database; https://sitem.herts.ac.uk/aeru/ppdb/en/atoz.htm). Fixing the EC_{50} value for both chemicals as 1 TU, the dilutions were conducted at 0.5 TU, 0.25 TU, 0.1 TU, and 0.05 TU of each compound (50 ng L^{-1}, 25 ng L^{-1}, 10 ng L^{-1}, and 5 ng L^{-1} for the CPF; and 300 ng L^{-1}, 150 ng L^{-1}, 60 ng L^{-1}, and 30 ng L^{-1} for the CPF-m).

Eighteen replicates were performed for each treatment group. Particularly, each replicate contained four individuals exposed in glass vessels filled with 40 mL of the exposure solution. Each exposure concentration was tested in the same day (replicates plus CTRLs).

Moreover, eighteen replicates for each control group (CTRL = control commercial mineral water treated) and controls with the same percentage of carrier solvent ($CTRL_{EtOH}$ = control ethanol treated) were included during the test period to verify the carrier solvent effects.

Every 24 h, the test solutions were renewed and daphnids were not fed during the experiment. The measured concentrations of the highest and lowest test solutions at the start and after 24 h (renewal time) ranged from 90% to 102% of the nominal values. These results indicate that the prepared test concentrations correspond to nominal concentrations and that the test substances were stable during the study. Details regarding the analytical method are provided in paragraph S1 and Tab. S1 of the Supplementary Material.

Behavioural analysis

After 24 h and 48 h of exposure, the daphnids were randomly distributed (without any identifier) into 12-well plates designed for the video tracking analysis. Each well contained one individual and 2 mL of the exposure medium. The well plates of both congeners were analysed in sequence (total recording time: 2 hours). However, in the same run a CTRL was added for each treated group.

The plates were placed on a light panel into the thermostatic chamber (20.0±0.5 °C), and a digital high-definition camera (Raspberry Pi 3 with Camera Module v2) at high resolution (1920–1080 pixels) that worked remotely via a PC was positioned 35 cm above the plate. After 30 min of acclimation, a video record of 120 s was performed for each plate. FFmpeg software (http://www.ffmpeg.org) was used to convert 60 s sections of the videos to .avi files (1500 frames; 25 frames $second^{-1}$), which were successively analysed using LoliTrack v.4 (Loligo Systems, Tjele, Denmark) software. Then, LoliTrack v.4 assigned an arena to each well of the plate and located objects (daphnids) that were in contrast with the background (exposure medium) by adapting the RGB (red/green/blu) thresholds. When all the daphnids identified as similarly coloured pixels were contrasted for all 1500 frames from the water medium, the software assigned a pair of x,y coordinates to their centroid in order to enable the simultaneous tracking of the daphnid swimming parameters in each well plate. The software was calibrated to measure the following BSRs: distance moved (cm), active/inactive time (%), average (AVG) swimming velocity (cm s^{-1}), and AVG acceleration (cm s^{-2}). After the 24 h-test, individuals were relocated into the same glass from which were taken for further recording after 48 h.

Statistical analysis

A statistical analysis of the behavioural results of *D. magna* for each treatment group was performed using GraphPad Prism 6 software (version 6.01; 2012). The robust regression and outlier removal method was used to investigate the presence of significant outliers in the dataset. A two-way analysis of variance (two-way ANOVA) was applied to compare the results from each treatment group with the controls and to detect differences between congeners of corresponding treatment level by using the Sidak's multiple comparisons test. The significance level was set at $p \leq 0.05$. Results of statistical analysis are reported in Tab. S4 A-D.

The non-parametrical statistical analysis (U Mann-Whitney test) was applied to compare responsiveness between Distance moved and AVG velocity after 24 h and 48 h of exposure to each pesticide (Tab. S5). To perform this comparison, measured data for each endpoint was normalised on CTRL.

The Pearson correlation coefficients for the coupled endpoints (active time *vs* distance moved; and AVG velocity *vs* AVG acceleration) for the CPF and CPF-m after 24 h and 48 h of exposure were calculated to investigate the existence of a significant linear correlation (Tab. S6 A-B).

Cumulative response curves

Cumulative response curves (CRCs) represent the cumulative sum of responses from lower to higher compound concentrations for a given time of exposure. In this study, the CRCs were derived using the absolute value of BSR changes independently from the sign (positive if an increased swimming activity is recorded, negative if there is a decrease). The steps for the calculation of the CRCs are reported in Tab. S7. In particular, the percentage of the organism stress responses obtained at a given concentration was added to those of the preceding ones, and so forth, independent of the direction of the response (increase or de-

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crease in swimming parameters). The obtained cumulative sum of the response data for each swimming parameter were fitted to linear regression models (Tab. S8). The CRCs were obtained using GraphPad Prism 6 software (version 6.01; 2012).

RESULTS

After 24 h and 48 h of exposure, no significant mortality (<10%) was found in the CTRLs or $CTRL_{EtOH}$ groups, showing that the data met the requirements of OECD 202 (OECD, 2004). In addition, for both pesticides and all the considered behavioural endpoints, no statistically significant differences were found between the CTRL and $CTRL_{EtOH}$ groups up to a concentration of ethanol present in daphnids treated with 0.25 TU. Consequently, we compared the effects of CPF and CPF-m with both the CTRLs (from 0.05 TU to 0.25 TU) and $CTRL_{EtOH}$ (0.5 TU) groups.

Behavioural responses

Changes in the BSR were measured after 24 h and 48 h in juvenile *D. magna* specimens exposed to different concentrations of CPF and CPF-m (0.05 TU; 0.1 TU, 0.25 TU, and 0.5 TU). Additional information regarding the measured data after 24 h and 48 h of exposure for both pesticides and the results obtained from the statistical analyses performed is provided in the Supplementary Material (Tabs. S2 to S10). The obtained results showed the covariance of active time vs distance moved and of AVG velocity *vs* AVG acceleration for both pesticides, as confirmed by the Pearson correlation analysis results (Tab. S10 A-B). Thus, we reported the results for only the distance moved and AVG velocity. The distance moved and AVG swimming velocity of the daphnids exposed to different concentrations of CPF and CPF-m after 24 and 48 h are shown in Fig. 1.

Based on the results shown in Fig. 1, the following considerations can be made about the investigated behavioural markers and their changes during time. Distance moved was more responsive than AVG velocity. Indeed, the results of the U Mann test indicated significant differences (Tab. S5) in particular at higher concentrations. After 24 h (Fig. 1A,) any statistically significant difference was observed between the treated groups and CTRL up to 0.25 TU for both pesticides ($p>0.05$). Conversely, CPF and CPF-m caused a statistically significant a decrease in swimming activities at the highest tested concentration (0.5 TU; $p<0.0001$ for both distances moved and AVG velocity). No statistically significant difference was observed between the two compounds on both swimming parameters at all the tested concentrations ($p>0.05$). Results of statistical analysis performed are presented in the Tab.S4 of the supporting information section. After 48 h (Fig. 1 C,D), at high concentrations, CPF exhibited the opposite trend than that shown in the first 24 h of exposure. Indeed, the highest concentration determined an intensification of distance moved (e.g., 0.5 TU, the distance moved increased by 41%; $p<0.001$). Conversely, CPF-m induced two different responses. First, there was a generalised reduction of the distance moved from 0.1 TU to 0.25 TU ($p<0.0001$ at 0.1 TU and $p<0.05$ at 0.25 TU), followed by a sharp increase at the highest tested concentration, reaching a 60% increase in the distance moved ($p<0.0001$). After 48 h of exposure, statistically significant changes in AVG velocity were observed. Indeed, compared with the 24 h exposure, both pesticides caused declining trends up to 0.25 TU in 48 h of exposure (CPF: $p<0.05$ from 0.05 to 0.25 TU; CPF-m: $p<0.0001$ at 0.05 TU, $p<0.01$ at 0.1 TU, and $p<0.001$ at 0.25 TU). At the highest tested concentration, an increase in AVG velocity was observed (CPF: $p<0.05$; CPF-m: $p<0.01$). Also after 48 h, for the AVG velocity any statistical difference was observed between congeners.

Finally, the NOEC and LOEC values for the behavioural endpoints were derived after exposures of 24 h (CPF: NOECBSR_24h = 10 ng L^{-1} and LOECBSR_24h = 25 ng L^{-1}; CPF-m: NOECBSR_24h = 25 ng L^{-1} and $LOEC_{BSR\_24h}$ = 50 ng L^{-1}) and 48 h ($NOEC_{BSR\_48h}$ = 5 ng L^{-1} and $LOEC_{BSR\_48h}$ = 10 ng L^{-1} for both compounds).

Cumulative responses curves

Daphnia swimming behaviour is one of the most sensitive biomarkers of toxicity (Duquesne and Küster, 2010). However, based on the literature survey herein, it is evident that BSRs are an active organism response that reflects internal changes induced by chemical contaminants. These changes are dependent on the characteristics of the compound (e.g., MoA), concentration, and time of exposure. As highlighted herein, the same chemical elicited different BSRs (e.g. decrease or increase in AVG velocity or distance moved) with different concentration levels and durations of exposure. However, a description of the behavioural swimming changes only provides qualitative information, not quantification, which prevents us from comparing the overall effects of different compounds on the swimming behaviour of *D. magna*. To overcome this limitation, we calculated the CRCs. While widely used in toxicology and pharmacology (Scholze *et al*., 2001), CRCs have never been utilised in the field of BSR studies. CRCs describe the cumulative percentage of the expected response of a population at a given concentration. The CRCs were calculated by considering the absolute values of the variation of the BSRs. Therefore, changes in swimming behaviour at different concentrations were considered as the stress response of the exposed population, independent of the direction of the BSR (decrease or increase of swimming parameters).

The CRCs of distance moved, and AVG velocity for CPF and CPF-m are shown in Fig. 2. Multiple *t*-test using the Holm-Sidak method was applied to compare the CRC

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curves of CPF and CPFm for Distance moved and AVG velocity. Results of statistical analysis are presented in Tab. S9.

DISCUSSION

Stressor-induced changes in distance moved in *D. magna,* as well as in other aquatic invertebrates, are well documented in the literature. Hansen and Roslev (2016) reported that in *D. magna* exposed to binary mixtures of glyphosate and copper [Cu(II)], distance moved decreased after 24 h depending on the tested concentrations (consisting of 1:1 molar mixtures in a range of 5.2-332 μM), and that distance moved was more responsive than AVG velocity. Ferrão-Filho *et al*. (2014) reported a decrease in distance travelled by *Daphnia* specimens exposed to saxitoxin-producer cyanobacteria (concentration range: 125–1000 $\mu g L^{-1}$). Zein *et al*. (2014) found a reduction in the distance moved in *D. magna* at the highest tested CPF concentration (concentration range: 0.016–0.25 μM), which was mainly due to the complete immobilisation of

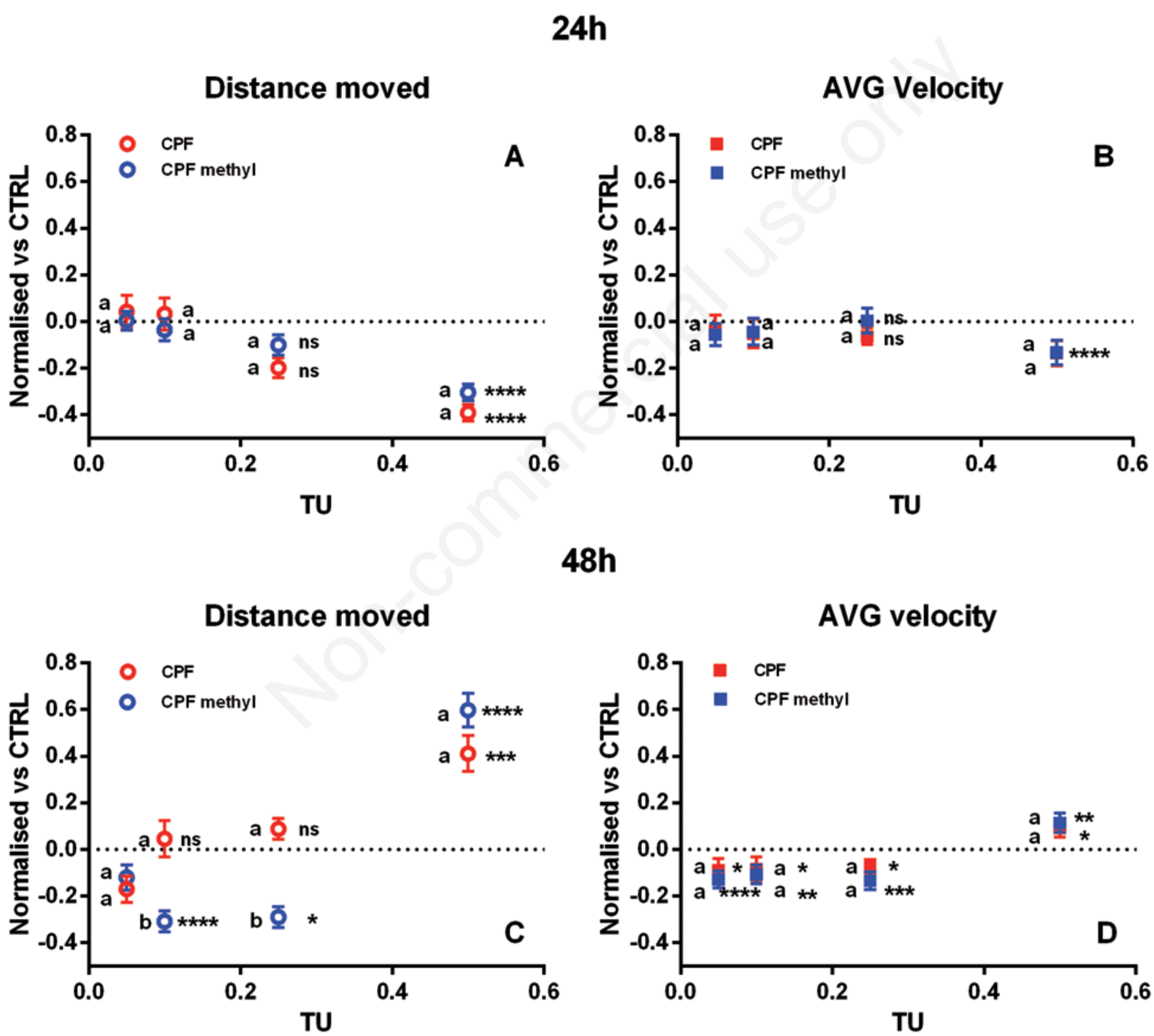

Fig. 1. BSRs of juvenile *D. magna* after 24–48 h of exposure to CPF (in red) and CPF-m (in blue) Data are expressed as values normalised to CTRL or CTRLco. Statistical significance of each treatment group respect to the respective CTRL are indicated with asterisks (****p<0.0001; ***p<0.001; **p<0.01; *p<0.05; ns, non-significant). Letters indicate the statistical significance between treated groups of correspondent level of TU of CPF and CPF-m. Groups treated with 0.05 TU, 0.1 TU and 0.25 TU were compared with the CTRL. Groups treated with 0.5 TU of pesticide were compared with the ethanol treated control group ($CTRL_{EtOH}$). Variability is represented as the standard error of the mean.

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organisms. They also found a concentration-dependent stimulatory effect on distance moved in *D. magna* treated with imidacloprid (concentration range: 4–1024 μM), nicotine (concentration range: 1–256 μM), and physostigmine (concentrations range: 0.25–4 μM). Moreover, daphnids exposed to the OP insecticide diazinon (concentration range: 0.125–2 μM) showed a concentration-dependent effect (Zein *et al*., 2015), in which there was an increase and decrease in the distance moved at lower and higher concentrations, respectively.

In addition, several studies reported variations in AVG velocity. Wolf *et al*. (1998) and Christensen *et al*. (2005) found that sub-lethal concentrations of cadmium (3.5–5 μg L^{-1}) and cypermethrin (0.05–1 μg L^{-1}) significantly inhibited the swimming ability of *D. magna*. A different result was obtained by Duquesne and Küster (2010), who investigated the effects of paraoxon-CH3 (a metabolite of the parathion-CH3 insecticide) on the swimming capability of *D. magna*. They found that after a 24 h exposure (concentration range: 0.3–1.5 μg L^{-1}), the mean velocity was significantly higher at concentrations ≥0.7 μg L^{-1}), Ferrario *et al*. (2018) found the oppo-

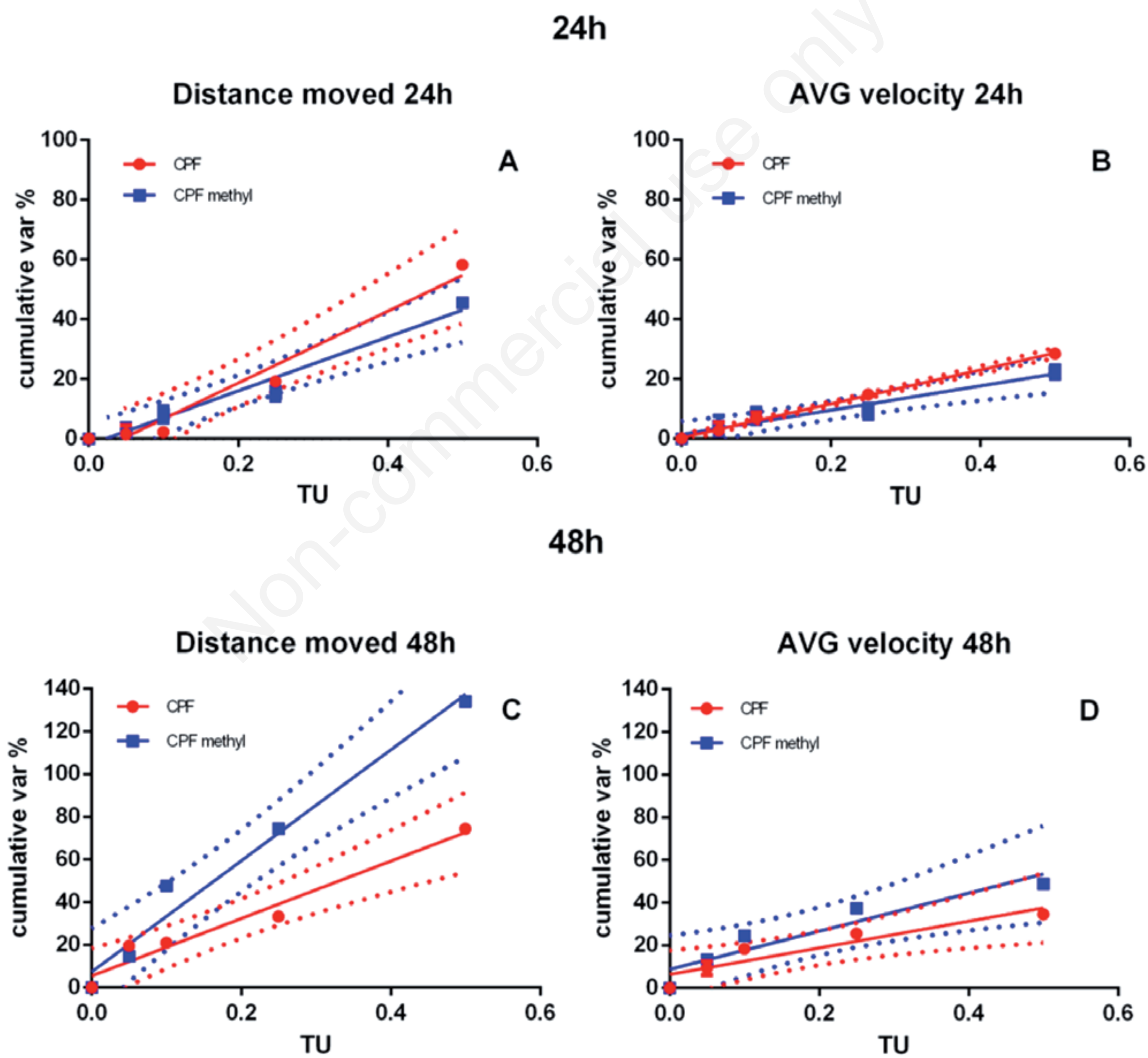

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Fig. 2. Cumulative variations (% to the control group (CTRL)) in distance moved and average (AVG) velocity for chlorpyrifos (CPF) and chlorpyrifos-methyl (CPF-m) (24 h and 48 h). Dashed lines indicate the stress variation tendency.

site result in daphnids exposed to CPF (96 h), observing a significant decrease in AVG velocity at the lowest tested concentration (50 ng L^{-1}) and an increase at the highest concentration (250 ng L^{-1}). Bownik *et al*. (2020) reported that daphnids exposed (24 h) to ketoprofen manifested a time- and concentration-dependent decrease in swimming speed at concentrations of 0.005–50 mg L^{-1}. Conversely, benzoylecgonine exposure (concentration range: 0.5–1 µg L^{-1}) induced a significant increase in the swimming velocity of treated daphnids (Parolini *et al*., 2018). Evidence of behavioural changes determined by chemicals on other aquatic invertebrates is also present in the literature. Xuereb *et al*. (2009) found that after *Gammarus fossarum* was exposed to methomyl (carbamate) and CPF for 96 h, there was a significant inhibition of the swimming capability at the highest tested concentrations (986.4 nM and 2.86 nM, respectively). Villa *et al*. (2018) reported contradictory results for the BSRs (distance moved and AVG velocity) of *Diamesa zernyi* larvae (Chironomidae) exposed to different chemical contaminants tested at their respective LOECs. After 24 h, they found a significant reduction in larvae exposed to the herbicide metolachlor (27.4 mg L^{-1}) and the fungicide captan (3.14 mg L^{-1}), whereas CPF and boscalid did not significantly differ from the controls (0.001 mg L^{-1} and 0.5 mg L^{-1}, respectively). Furosemide and ibuprofen stimulated the distance moved (500 mg L^{-1} and 100 mg L^{-1}, respectively). After 48 h, a completely different reaction was observed, in which CPF, boscalid, and furosemide experienced an increase in distance moved.

Based on the literature, it is difficult to reach a definitive conclusion regarding the BSRs of aquatic invertebrates exposed to sub-lethal chemical concentrations. In general, the BSRs appear to be species-specific and depend on the stressor properties (e.g. MoA). Moreover, according to the stepwise stress model (Gerhardt, 1999, 2007; Gerhardt *et al*., 2005), which is related to the time of exposure and/or increasing of toxicant concentration.

Overall, our results are consistent with those reported in the literature, wherein almost all the results showed that, for both compounds, there were relationships between the tested concentrations and BSRs in *D. magna*, in which distance moved is more responsive than AVG velocity. In addition, the results show that when exposed to equipotent concentrations of the two OPs, the investigated behavioural parameters changed analogously (with a partial exception of the distance moved at 0.1 TU and 0.25 TU after 48 h of exposure). For instance, after 24 h, both OPs induced a gradual decrease in distance moved and AVG velocity with increasing concentrations. In particular, the weakening of both parameters became significant at 0.25 TU (only for the CPF in the case of AVG velocity). Similarly, the changes in BSRs were time dependent. By comparing the results obtained after 24 h and 48 h of exposure, some differences became evident for each of the investigated parameters. After 24 h of exposure, the decrease in AVG velocity was significant only at the higher tested concentrations. Conversely, after 48 h of exposure, both chemicals induced statistically significant drops in the AVG velocity even at lower tested concentrations (0.05 TU), whereas the highest concentration (0.5 TU) provoked a statistically significant increase. In addition, after 48 h, both compounds induced changes in the distance moved that were completely different from those of the 24 h exposure. Here, CPF reversed the trend with an increase in distance moved with increasing concentration. Meanwhile, CPF-m induced two opposing responses by eliciting a higher reduction and increase of distance moved at the lower and highest TUs, respectively. The overall similar BSRs provided by organisms exposed to two OPs suggest a possible implication of the MoA of the compounds in the BSR trend. The selected insecticides are congener compounds that differ only in the presence of methyl or ethyl substituents linked to the thiophosphate group, and exert their toxicity mainly by inhibiting acetylcholinesterase enzyme activity (Fukuto, 1990).

The observed variability in the BSRs could be an active response of *D. magna* to different stresses elicited by the OPs during a certain time. Presumably, in the first 24 h of exposure, both chemicals act as narcotic compounds with hypo-activity and reduced responsiveness (Dell'Omo, 2002). Narcotic-type toxicity is dependent on hydrophobicity (Verhaar *et al*., 1992; Tremolada *et al*., 2004). Thus, the higher octanol/water partition coefficient (log Kow) value detected by the ethyl form of the OPs with respect to the methyl one (log Kow CPF = 4.7; log Kow CPF-m = 4.0 from PPDB) could explain a smaller effect recorded for CPF-m at the end of the 24 h exposure (Figs. 1 A and B). Further, the OPs activate other regulatory mechanisms (e.g., antioxidant and detoxifying enzymes) (Ferrario *et al*., 2018), which allow the recovery of specimens and the activation of other BSRs, such as feeding searching behaviour or avoidance. Thus, faster swimming may be related to feeding behaviour (Larsson and Kleiven, 1996) and an energy requirement as a result of compensatory mechanisms, or avoidance (the attempt of the organism to "escape" from a polluted aquatic environment), which is one of the first behaviour modulations in *D. magna* (Ren *et al*., 2007).

As reported in the results section, determining the CRCs allows us the determination of the overall magnitude of the behavioural effects for each of the investigated chemicals, and their comparison. Particularly, from Fig. 2 and the statistical analysis (Tab. S9), it is evident that, compared with the AVG velocity, both pesticides induced higher statistically significant cumulative response in distance moved at both exposure periods. Moreover, after 24 h, CPF seems to produce higher levels of stress on swim-

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ming behaviour in *D. magna* than that of CPF-m. In particular, the distance moved and AVG velocity were cumulatively affected by 58% and 28%, and 45% and 22% for CPF and CPF-m, respectively. As shown in Fig. 1 A,B, the cumulative curves both have decreased in distance moved and AVG velocity with increasing concentrations, which are probably caused by the narcotic effects of both compounds. A completely different result was obtained after 48 h, wherein CPF-m induced the highest hypo activity in daphnids in the range of 0.1-0.25 TU (Fig. 1C). On the contrary, at these concentrations, CPF had already induced a switch in the swimming behaviour of *D. magna*. This could be because CPF being more toxic to *D. magna* than CPF-m, could have achieved a body burden capable of activating other regulatory mechanisms, resulting in hyperactivity in swimming behaviour.

It should be recall that the observed differences in behavioural markers could be clone-dependent. In fact, it is known that interclonal variability may be relevant in the Daphnia toxicity tests (Baird and Barata, 1998; Picado *et al*., 2007).

CONCLUSIONS

Behaviours represent an active animal's response to physiological (internal) and environmental (external) factors, including exposure to chemical contaminants or other stressors. Toxicant-induced behavioural changes may indicate an early warning sign of toxicity as well as the adaptive responses of animals to mitigate the potentially detrimental effects of exposure. Therefore, this study evaluated the BSRs of *D. magna* exposed to sublethal concentrations of two neurotoxic insecticide congeners (CPF and CPF-m), in which the aim of the study was to verify whether substances that share a MoA induce similar BSR profiles. In the first 24 h of exposure, both compounds elicited the same decreasing trend in swimming behaviour. These behavioural changes may be caused by the narcotic toxicity induced by both insecticides. After 48 h of exposure, CPF provoked an increase in the distance moved with increasing concentrations, whereas CPF-m elicited an increase in the distance moved (compared with the CPF) only at the highest tested concentration. These reversed trends in BSR can be attributed to the feeding, searching, and/or avoidance behaviours of the animals once the chemical achieved sufficient internal concentrations to stimulate these adaptive responses. Finally, for the first time, we propose the use of cumulative curves of the absolute values of the variation of swimming parameters in *D. magna*, which could be useful in environmental risk assessment procedures as they allow researchers to obtain concentration-response relationships in order to understand the magnitude of the behavioural effects of different chemicals.

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