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Behavioral responses of juvenile *Daphnia magna* after exposure to glyphosate and glyphosate-copper complexes☆

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ABSTRACT

Glyphosate (*N*-(phosphonomethyl)glycine) is the active ingredient in a range of popular broad-spectrum herbicide formulations. Glyphosate is a chelating agent that can form stable complexes with divalent metal ions including Cu(II). Little is known about the bioavailability and ecotoxicity of glyphosate-Cu(II) complexes to aquatic organisms. In this study, we used video tracking and behavior analysis to investigate sublethal effects of binary mixtures of glyphosate and Cu(II) to juvenile *D. magna*. Behavioral responses were quantified for individual *D. magna* after 24 h and 48 h exposure to glyphosate and glyhosate-Cu(II) mixtures. Sublethal concentrations resulted in decreases in swimming velocity, acceleration speed, and distance moved whereas inactive time of *D. magna* increased. Distance moved and inactive time were the most responsive parameters to glyphosate and glyphosate-Cu(II) exposure. On a molar basis, glyphosate-Cu(II) complexes appeared more toxic to *D. magna* than glyphosate alone. The 48 h EC50 for glyphosate and glyphosate-Cu(II) determined from swimming distance were 75.2 μM and 8.4 μM, respectively. In comparison, traditional visual observation of mobility resulted in 48 h EC50 values of 52.8 μM and 25.5 μM for glyphosate and glyphosate-Cu(II), respectively. The behavioral responses indicated that exposure of *D. magna* to mixtures of glyphosate and Cu(II) attenuated acute metal toxicity but increased apparent glyphosate toxicity due to complexation with Cu(II). The study suggests that glyphosate is a likely mediator of aquatic metal toxicity, and that video tracking provides an opportunity for quantitative studies of sublethal effects of pesticide complexes.

1. Introduction

Glyphosate (*N*-(phosphonomethyl)glycine) is the active ingredient in a range of common broad spectrum systemic herbicide formulations. Glyphosate-based pesticides are currently among the most widely used agricultural chemicals, but these herbicides have also been approved for use in silviculture and for domestic weed control (Annett et al., 2014). Glyphosate affects target plants by inhibiting the enzyme EPSP synthase of the Shikimate pathway. This enzyme is not present in animals, and the environmental toxicity to non-target organisms has been considered low (Annett et al., 2014; Helander et al., 2012). However, adverse effects have been observed in a range of non-target organisms including bacteria, algae, crustaceans, amphibians, and fish (Annett et al., 2014; Cuhra et al., 2013; Folmar et al., 1979; Sihtmäe et al., 2013). Glyphosate may inhibit specific enzymes in invertebrates (Tu et al., 2001), including alkaline phosphatase (Ørsted and Roslev, 2015), but may also alter expression of stress-responsive genes (Le et al., 2010). Glyphosate exposure can also cause oxidative stress, inhibition of acetylcholinesterase, and increase susceptibility to infection by parasites (Annett et al., 2014). These findings regarding the ecotoxicity of glyphosate suggest multiple modes of action, and have led authors to conclude that the adverse effect to non-target organisms is underestimated (Cuhra et al., 2013; Helander et al., 2012).

Glyphosate contains three functional groups: one carboxylic, one amino, and one phosphonate group. The double bonds of the carboxylic and phosphonic groups can react and form covalent bonds with divalent cations (Franz et al., 1997; Morillo et al., 2002). Hence, glyphosate is a chelating agent and before its herbicidal properties were discovered, glyphosate was patented as a metal chelator. In the environment, glyphosate may act as a ligand and

☆ This work is valuable, as it shows, how complex formation between glyphosate, a commonly used herbicide, and copper affects the apparent toxicity of the herbicide to a non-target organism. In addition, it reports a good methodology for determining behavioural endpoints useful in ecotoxicology.

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form complexes with divalent metal ions including zinc, iron, manganese, and copper (Morillo et al., 1997, 2002; Subramaniam and Hoggard, 1988; Undabeytia et al., 2002). Metals often occur naturally in the environment but they can also be found as pollutants in many surface waters were glyphosate is also present. Complexation of glyphosate with metals may results in altered physical-chemical characteristics relative to the parent compounds (Subramaniam and Hoggard, 1988), but the environmental role of such complexes is poorly understood. It has been suggested that glyphosate-metal complexes will have decreased environmental mobility due to precipitation (Subramaniam and Hoggard, 1988), whereas other studies suggest that complexation with metals will decrease sorption and increase environmental mobility (Barrett and McBride, 2006). Unfortunately, little is known about the bioavailability and ecotoxicity of glyphosate-metal complexes. Complexation of glyphosate with metals may increase environmental persistence due to slower biodegradation and delay biological detoxification. Some studies indicate that glyphosate-metal complexes may increase metal bioaccumulation, and result in altered toxicity compared to the parent compounds (Duke and Powles, 2008; Tsui et al., 2005). Environmental glyphosate concentrations are often relatively low, and toxic effects may not be evident using traditional testing protocols and endpoints (Annett et al., 2014). Hence, it has been suggested to use sub-lethal endpoints for investigating adverse effects (Bahrndorff et al., 2016; Hellou 2011; Untersteiner et al., 2003). Behavioral endpoints can provide increased sensitivity, and reveal early effects at the organism level that may subsequently be linked to effects at the population level (Hellou, 2011).

In this study, we used behavior analysis to investigate sublethal effects in juvenile *D. magna* after exposure to glyphosate and mixtures of glyphosate and Cu(II). *D. magna* were incubated in defined arenas, and behavioral effects were quantified by image analysis followed by estimation of swimming velocity, acceleration speed, distance moved, and active-inactive time. Inhibition and EC50 values estimated from video tracking experiments were compared with traditional visual observation of mobility.

2. Materials and methods

2.1. D. magna *cultivation*

D. magna Straus was cultivated from a laboratory-reared clone originating from pure culture ephippia (MicroBioTests Inc., Mariakerke, Belgium). Females were grown at 20 ± 1 °C in 24 L tanks with 16 h light and 8 h dark cycles under cool white light. The tanks contained 20 L of modified ISO 6341 synthetic freshwater medium (ISO 6341, 2012). $NaHCO_3$ addition was omitted from the medium because the source water contained abundant natural $NaHCO_3$ and displayed a stable pH of pH 7.8 ± 0.5. The source water consisted of high-quality non-disinfected groundwater abstracted directly from pristine aquifers (16°dH). The culture tanks were constantly aerated using an air pump. *D. magna* cultures were fed a mixture of dry yeast (Malteserkors, Denmark), and dried organically grown *Chlorella pyrenoidosa* and *Arthrospira maxima* (Naturland, Denmark). Feeding corresponded to approximately 0.05 mg C/animal/day, and a mixed diet was used to avoid nutritional deficiencies (ISO 10706, 2000). Crowding and density-dependent male production was avoided by weekly culling of the population. All experiments were conducted using juvenile *D. magna* of the same age (<24 h). Hatching was carried out in 2 L breeding stations containing 1 L aerated freshwater medium and approximately 50 pregnant females. Neonates were sorted, and then randomly assigned to different treatments as described below.

2.2. Chemicals

Stock solutions of the reference toxicant $K_2Cr_2O_7$ (CAS 7778-50-9; Sigma-Aldrich, Denmark), and $CuSO_4{\cdot}5H_2O$ (CAS 7758-99-8; Merck, Germany) were prepared in distilled autoclaved water. Stock solutions of glyphosate was prepared from a 40% wt/vol *N*-(Phosphonomethyl)glycine, monoisopropylamine salt solution (CAS 38641-94-0, Sigma-Aldrich, Denmark). Binary mixtures of $CuSO_4{\cdot}5H_2O$ and glyphosate (hereafter referred to as "glyphosate-Cu(II)") were prepared by mixing stock solutions of $CuSO_4{\cdot}5H_2O$ and glyphosate, and then placing the mixture on a shaker for 18 h at 20 °C to allow complexation (Tsui et al., 2005; Undabeytia et al., 2002). Glyphosate-Cu(II) mixtures were prepared 1:1 (mole:mole) or with a fixed glyphosate concentration and varying $CuSO_4{\cdot}5H_2O$ concentrations (see below). All stock solutions were stored in the dark at 10 °C. Test solutions of metals and glyphosate were prepared in autoclaved freshwater medium (ISO 6341, 2012). Nominal concentrations of glyphosate were verified by quantifying concentrations in stock solutions using fluorescence spectroscopy (Hasson and Schechter, 2000). Initial scanning of glyphosate and glyphosate-Cu(II) stocks were carried out using a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent, Santa Clara, CA, USA). An excitation wavelength of 294 nm was used, and emission peaks were recorded at a scan rate of 600 nm/min. Fluorescence emission maxima were observed at 594 nm and a minor peak at 618 nm.

2.3. Video tracking and behavior analysis

Video recording of neonate *D. magna* was conducted using an USB 3.0 color video camera with a e2v CMOS sensor (UI-3240CP, IDS Imaging Development Systems GmbH, Obersulm, Germany). The camera was equipped with a 25 mm monofocal lens without IR filter (Spacecom, Tokyo, Japan), and uEye PC software was used for video recordings (IDS Imaging Development Systems GmbH). 24 juvenile *D. magna* were tracked individually for each toxicant concentration in arenas consisting of Falcon 24-well transparent flat bottom non TC-Treated polystyrene multiwell cell culture plates (Corning, New York, USA). The incubation was initiated when neonates where transferred to freshly prepared ISO 6341 medium containing the different toxicant concentrations. Each well contained 1 neonate and 2 mL medium. The 24-well plates were incubated at 20 °C in a temperature controlled chamber. Video recordings were carried out by placing the 24-well plate with 24 animals on a LED Light Panel (Dörr Professionel), and then tracked for 10 min using camera settings with 30 frames per seconds. Videos of each animal were recorded after 0 h, 24 h and 48 h of incubation. Video recordings were analyzed using the software LoliTrack v. 4 (Loligo Systems, Tjele, Denmark). This software is designed for animal behavior analysis and can be calibrated to include analyses of swimming velocity (mm/s), acceleration speed (mm/s^2), distance moved (mm), and active/inactive time (%). Tracking was based on differences in contrast between objects (animals) and background (water) without use of markers. When the object appeared against a contrasting background, the software assigned a coordinate pair (x, y) to the centroid of the contrasting object. Each well in the 24-well plates was defined as one arena, and each animal was treated as a single object. Each animal tracking consisted of approximately 18000 frames.

2.4. Inhibition of mobility

Video tracking results were compared to the conventional acute toxicity test for *D. magna* in which the measured toxicological endpoint is inhibition of mobility determined by visual inspection of the animals (ISO 6341, 2012). Initial screening was carried out

using 20 juvenile *D. magna* (≤24 h old) distributed among 4 glass vials with 5 animals and 10 mL freshwater medium in each vial (ISO 6341, 2012). In subsequent experiments, inhibition of mobility was determined for 24 juvenile *D. magna* incubated individually using the 24 well microplate set-up described for the video tracking experiments (see above). Mobility was determined visually as the ability of *D. magna* to swim during 15 s following gentle agitation of the incubation medium in each well using a small glass rod. Mobility was observed directly in 24-well plates after 0 h, 24 h and 48 h of incubation using the juvenile *D. magna* that had been video recorded at similar time points.

2.5. Toxicity experiments

Concentration-dependent effects of glyphosate and Cu(II) to *D. magna* were tested for individual compounds, and for binary mixtures with different molar ratios between Cu(II) and glyphosate. Juvenile *D. magna* were exposed to individual chemicals in concentrations of 0, 5.2, 10.4, 20.8, 41.6, 83.2, 166, and 332 μM glyphosate (=0, 0.875, 1.75, 3.5, 7, 14, 28, and 56 mg/L); or 0, 0.2, 0.5, 1.4, 4.3, 12.8, and 38.3 μM Cu (II) (=0.0, 0.01, 0.03, 0.09, 0.27, 0.81 and 2.43 mg/L). Binary mixtures of glyphosate and Cu (II) consisted of 1:1 molar mixtures with 0, 5.2, 10.4, 20.8, 41.6, 83.2, 166, and 332 μM of glyphosate-Cu(II). In addition, an experiment with a fixed glyphosate concentration of 15.9 μM was combined with varying Cu(II) concentrations of 0.8, 1.6, 3.2, 6.4, 12.8, 25.6 and 52 μM resulting in Cu(II):glyphosate ratios between 0.05 and 3.3.

2.6. Data analysis and statistics

The response measured as changes in behavioral parameters were expressed as inhibition (I) relative to control samples: $I = 1-(R_i/R_c)$ where R_i and R_c are responses measured for inhibited and control samples, respectively. Concentration-response curves were fitted to a logistic model using iterative non-linear regression:

$$I = \frac{1}{1 + exp^{(-(\alpha+\beta\times logC))}} \quad (1)$$

where C is the toxicant concentration, and α and β are model parameters. From these parameters EC50 values were calculated using the following model:

$$EC50 = 10^{\left[\frac{\alpha}{-\beta}\right]} \quad (2)$$

Iterative non-linear regressions were performed using Microsoft Excel 2013 (Microsoft Corporation, USA), and KaleidaGraph 4.5.1 software (Synergy Software, USA). 95% confidence limits were calculated as described in Annex 2B in ISO 6341 (ISO 6341, 2012). Comparisons of results were carried out using the nonparametric Kruskal-Wallis H test for evaluating differences among multiple treatments, and the Mann-Whitney *U* test (Wilcoxon rank sum test) for evaluating differences between two defined treatments. Statistical analyses were carried out using KaleidaGraph 4.5.1 (Synergy Software, USA) with a significance level of $p < 0.05$. Behavioral profiles were analyzed by Principal Component Analysis using Pearson correlation coefficients and XLSTAT 2007 software (Addinsoft, France).

3. Results

3.1. Effect of glyphosate and glyphosate-Cu(II) on D. magna behavior

Four behavioral parameters in *D. magna* were compared for their response to glyphosate and glyphosate-Cu(II) exposure. Initial experiments with glyphosate exposure of neonate *D. magna* showed a significant concentration dependent effect on "mean swimming velocity", "mean acceleration speed", "active time" and "distance moved" (Kruskal-Wallis, $p < 0.0001$) (Fig. 1). The behavioral parameters were significantly different from controls at glyphosate exposure at 83.2 μM (14.1 mg/L) and above (Mann-Whitney, $p < 0.0001$). In particular, "active time" and "distance moved" was sensitive to glyphosate at concentrations ≥83.2 μM (Fig. 1C and D).

Exposure of *D. magna* to a mixture of glyphosate and Cu (II) for 24 h and 48 h also resulted in clear concentration dependent effects (Kruskal-Wallis, $p < 0.0001$) (Fig. 2). In general, activity measured after 48 h was lower compared to activity measured after 24 h, and changes relative to control samples were more distinct after 48 h especially for active time and distance moved (Fig. 2C and D). After 24 h, responses for active time and distance moved were significantly lower relative to controls for glyphosate-Cu(II) concentrations of 41.6 μM and 20.4 μM, respectively (Mann-Whitney, $p < 0.002$ and $p < 0.048$). In contrast, responses for active time and distance moved after 48 h were significantly lower than controls for glyphosate-Cu(II) concentrations of 5.2 μM and above (Mann-Whitney, $p < 0.002$).

D. magna in control samples from experiments with glyphosate-Cu(II) was more active after 48 h (Fig. 2C and D) than in the initial experiments with glyphosate (Fig. 1C and D), which was due to differences in initial handling time in the two experiments (Mann-Whitney, $p < 0.0001$). However, both experiments suggested that active time and distance moved were the more responsive parameters compared to mean velocity and acceleration. We also performed additional control experiments were effects of $CuSO_4$ were tested without glyphosate, and these experiments also suggested that active time and distance moved were the more responsive parameters (data not shown). Hence, active time (%) and distance moved (mm/min) were chosen as focus parameters for subsequent experiments with glyphosate-Cu(II).

3.2. Comparison of visual mobility and video tracking results

Video tracking results were compared with traditional visual observation of mobile/immobile individuals as described in standard protocols (ISO 6341: 2012). Concentration-effect curves for *D. magna* exposed to glyphosate-Cu(II) showed somewhat comparable responses for active time and distance moved (Fig. 3). Apparent inhibition was observed at relatively low concentrations (5–10 μM) of glyphosate-Cu(II) for these parameters (Fig. 3, note the log scale). In contrast, use of visual mobility as an endpoint resulted in little response at these concentrations (Fig. 3).

Concentration-effect curves were used to calculate EC50 values for inhibition of mobility, active time and distance moved (Table 1). The 48 h EC50 values for glyphosate was estimated to 52.8 μM using visual mobility as endpoint, which corresponded to 8.9 mg/L (Table 1). The 48 h EC50 values did not vary much for the 3 endpoints as seen from the overlapping confidence limits (Table 1). However, the 48 h EC50 values for glyphosate were somewhat higher than those of glyphosate-Cu(II) which was estimated to only 8.38–25.6 μM corresponding to 1.4–4.3 mg/L glyphosate complexed with Cu(II). Interestingly, 48 h EC50 values based on video tracking were somewhat lower (8.38–11.1 μM) than those estimated from visual observation of mobility (Table 1). A similar trend was observed for EC10 values where glyphosate-Cu(II) resulted in apparent 48 h EC10 values that were <1 μM for video tracking whereas apparent 48 h EC10 values based on visual observation were more than 10 fold higher (data not shown).

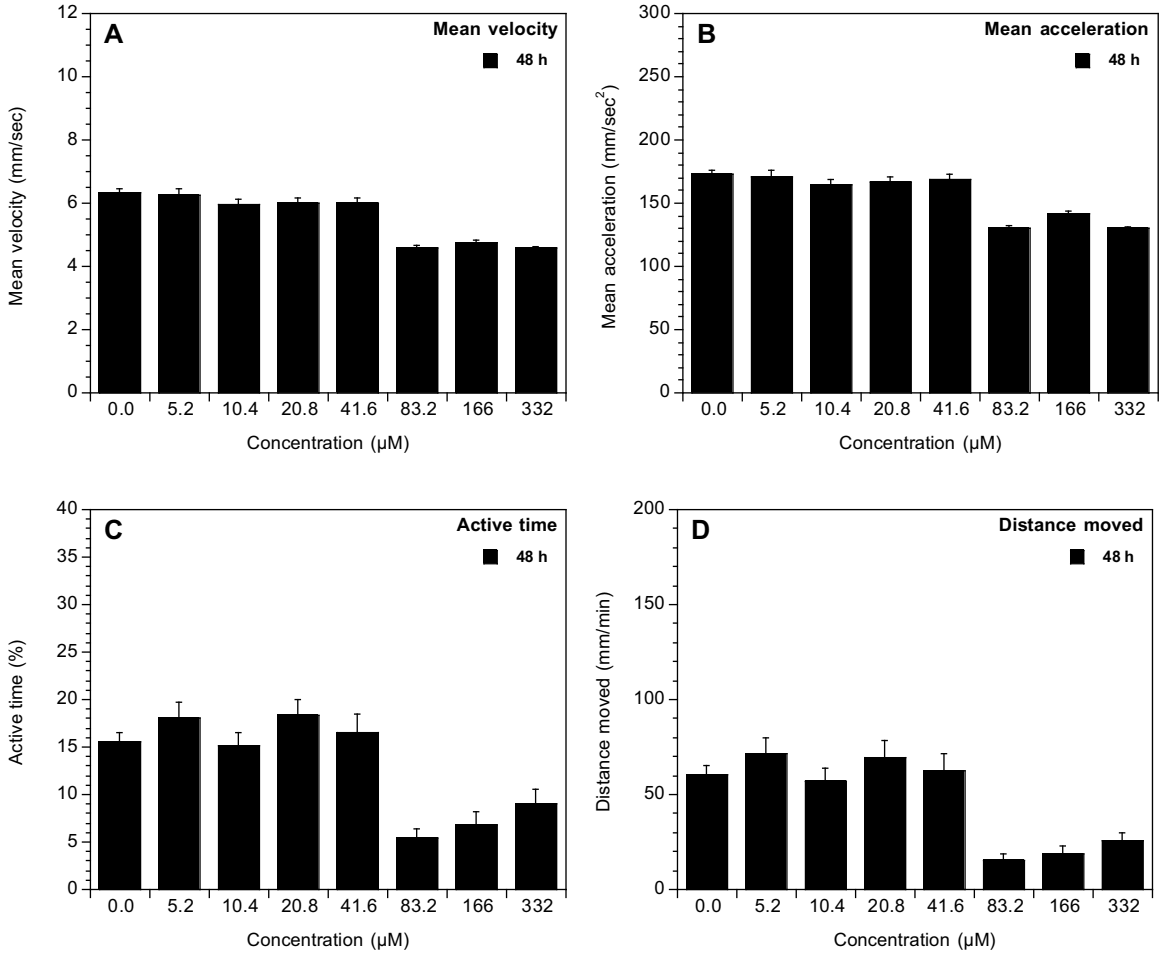

Fig. 1. Effect of 48 h glyphosate exposure on swimming activity of *D. magna* determined as mean velocity per sec (A), mean acceleration per sec^2 (B), percent active time (C), and distance moved per minute (D). Error bars indicate standard error (n = 24).

Table 1
Median effective concentrations (EC50) for *D. magna* exposed to glyphosate or glyphosate-Cu(II). EC50 values were calculated from visual observations of mobility, and by video tracking followed by estimation of active time and distance moved. Values in brackets indicate the 95% confidence limits (n = 24).

Compound	Incubation time	EC50 (μM)		
		Mobility (visual observation)	Active time (video tracking)	Distance moved (video tracking)
Glyphosate	24 h	81.9 [75.4–89.2]	60.7 [25.9–142.4]	35.3 [13.3–94.1]
Glyphosate	48 h	52.8 [49.3–56.6]	99.4 [62.1–159.1]	75.2 [68.7–82.2]
Glyphosate-Cu(II)	24 h	47.4 [41.1–54.7]	57.9 [27.8–120.5]	34.3 [17.4–67.8]
Glyphosate-Cu(II)	48 h	25.6 [22.1–29.6]	11.1 [2.78–44.5]	8.4 [3.33–21.1]

3.3. *Effect of varying ratios between Cu(II) and glyphosate*

Neonate *D. magna* were exposed to mixtures with different molar ratios between Cu(II) and glyphosate to examine fitness responses of low glyphosate concentrations in combination with variable copper concentrations (Fig. 4). The Cu(II):glyphosate molar ratio varied between 0.05 and 3.33 in binary mixtures with a fixed glyphosate concentration of 15.9 μM and variable Cu(II) concentrations. A glyphosate concentration of 15.9 μM corresponded to the 48 h EC10 value for free glyphosate determined in parallel experiments (2.69 mg/L). Exposure to mixtures with a Cu(II):glyphosate molar ratio <1 resulted in responses that were somewhat comparable (Fig. 4). In contrast, significant negative effects on active time and distance moved were observed after 24 h (Mann-Whitney, $p < 0.001$) and 48 h (Mann-Whitney, $p < 0.002$) for Cu(II):glyphosate ratios with an excess of Cu(II) (Fig. 4A and B), when each was compared to controls without Cu(II) or glyphosate. Behavioral profiles based on 4 parameters (swimming velocity, acceleration speed, active time and distance moved) were compared after 24 h and 48 h exposure to different molar ratios between Cu(II) and glyphosate (Fig. 4C). A PCA analysis suggested three distinctive groupings of behavioral profiles corresponding to three different exposure regimes (Fig. 4C). Behavioral profiles grouped similarly after 24 h and 48 h for mixtures dominated by Cu(II) in excess whereas mixtures with low Cu(II) and glyphosate in excess resulted in profiles that grouped separately after 24 h and 48 h exposure (Fig. 4C).

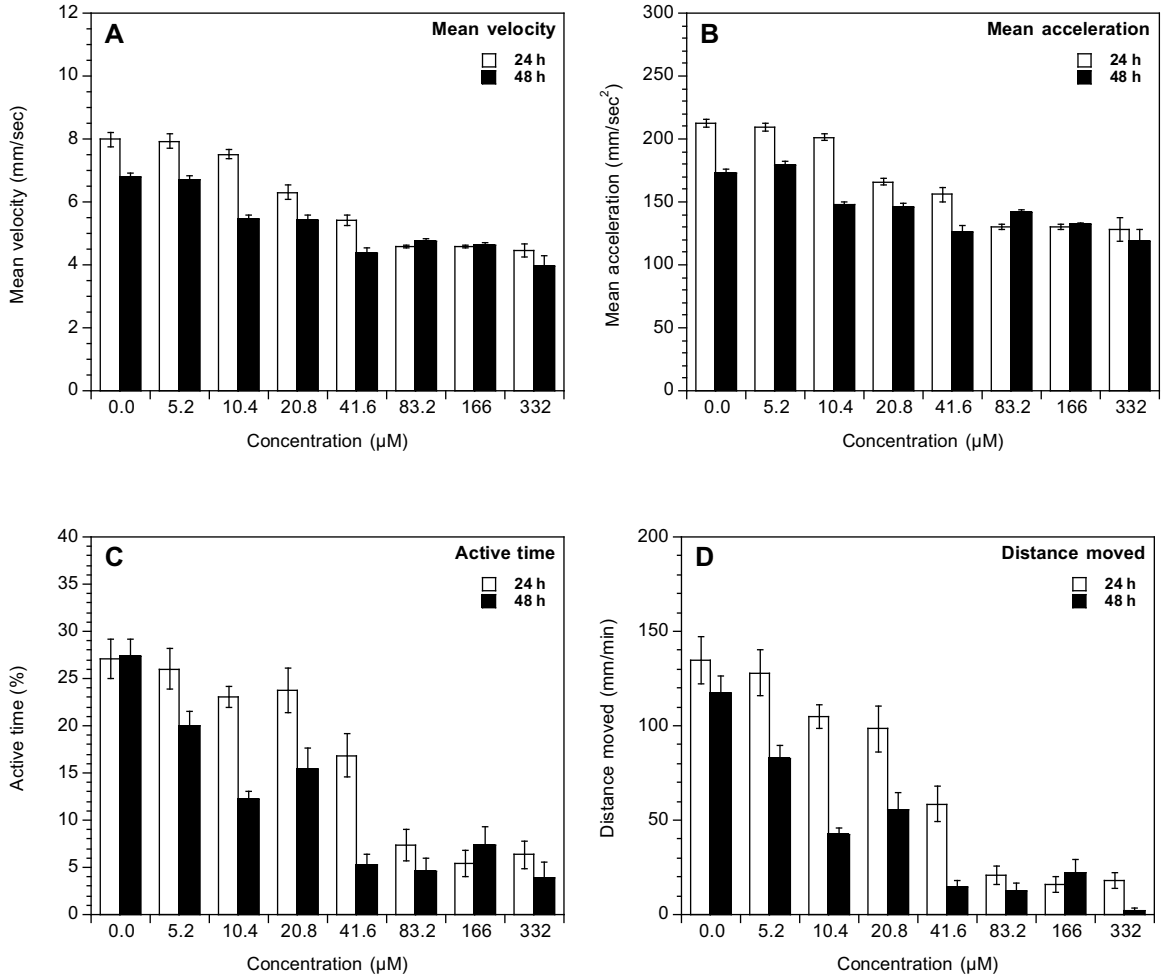

Fig. 2. Effect of 24 h and 48 h glyphosate-Cu(II) exposure on swimming activity of *D. magna* determined as mean velocity per sec (A), mean acceleration per sec^2 (B), percent active time (C), and distance moved per minute (D). Error bars indicate standard error (n = 24).

4. Discussion

The carboxylic and phosphonic groups of glyphosate can react and form covalent bonds with divalent ions (Franz et al., 1997; Morillo et al., 2002; Subramaniam and Hoggard, 1988). As a result, glyphosate can form complexes with environmental metals including copper. Divalent metals often coexist in soils and surface waters together with glyphosate pollution. Glyphosate complexed with environmental metals has recently been implicated in occurrence of human kidney disease in areas with intensive herbicide use (Jayasumana et al., 2014). In the present study, measurements of absorbance and fluorescence spectra in mixtures of glyphosate and Cu(II) suggested complexation of glyphosate and Cu(II). Such complexes are considered very stable in water, and can result in altered toxicity profiles compared to glyphosate alone (Morillo et al., 1997; Tsui et al., 2005).

Exposure of juvenile *D. magna* to glyphosate alone resulted in 48-h EC50 values of 52.8-99.4 µM (8.9–16.8. mg/L) using behavioral endpoints. These EC50 values correspond to the toxicity to *D. magna* of glyphosate and glyphosate-based herbicides determined in other studies using endpoint such as mobility, mortality, reproduction, and enzyme activity (Alberdi et al., 1996; Cuhra et al., 2013; Folmar et al., 1979; Melnichuk et al., 2007; Pérez et al., 2011; Ørsted and Roslev, 2015). In the past, relatively large toxic differences were sometimes observed between pure glyphosate compared to tests with commercial glyphosate-based herbicides such as Roundup® (Brausch et al., 2007; Folmar et al., 1979; Pérez et al., 2011; Tsui and Chu, 2003). These differences were often attributed to the presence of additives such as surfactants in the latter. However, recent studies suggest only minor differences in the apparent toxicity of some Roundup® formulations compared to the glyphosate-IPA salt (Cuhra et al., 2013; Demetrio et al., 2012; Ørsted and Roslev, 2015). This suggests that recent glyphosate formulation may contain adjuvants or additives at concentrations that only contribute marginally to toxicity compared to the active ingredient glyphosate. Glyphosate-IPA salt was used in the present study and we suggest that the findings are also relevant for many glyphosate-based herbicides formulations.

It is often stated that behavioral responses are sensitive stress indicators because these measurements integrates both biochemical and physiological processes (Chevalier et al., 2014, 2015; Fonga and Ford, 2014; Gerhardt, 2007; Hellou, 2011; Untersteiner et al., 2003; Wolf et al., 1998). In our study, the behavioral responses "active/inactive time" and "distance moved" did not appear to be more sensitive to glyphosate exposure than more traditional endpoints such as visual mobility which is a simple dichotomous endpoint. However, exposure of *D. magna* to glyphosate-Cu(II) resulted in lower EC values than determined for glyphosate alone, and especially "distance moved" appeared to be a rather sensitive endpoint for glyphosate-Cu(II) exposure after 48 h. In addition, a more than 4-fold increase in apparent toxicity was observed between 24 h and 48 h glyphosate-Cu(II) exposure for "distance moved" and "active/inactive time" (Table 1). If one considers all experiments in the present study, a good linear correlation was

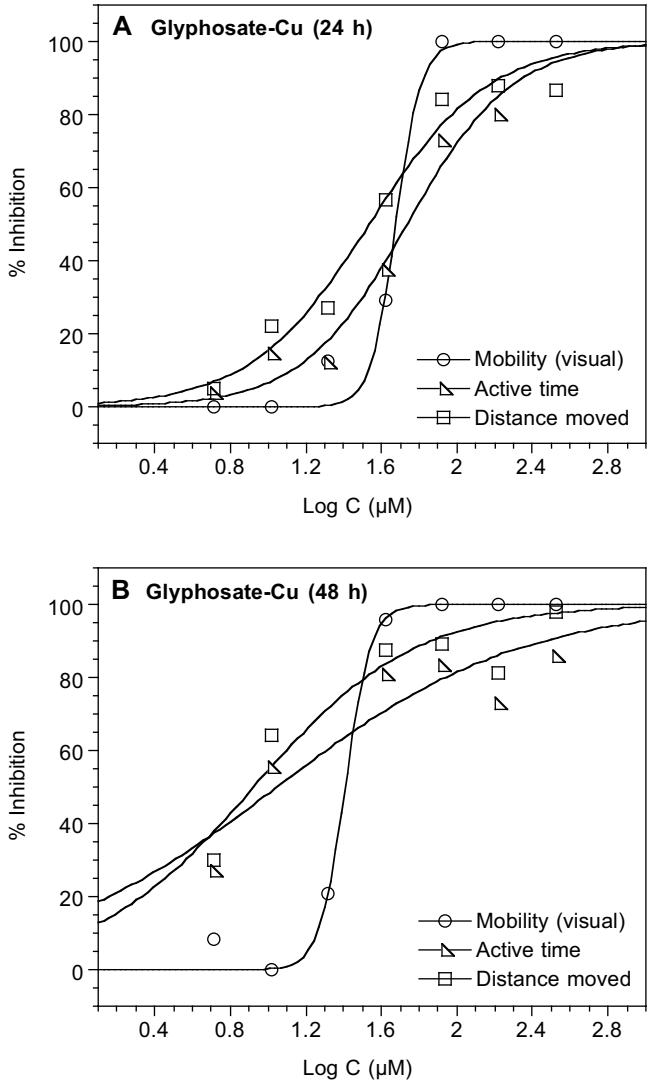

Fig. 3. Inhibition of swimming activity in *D. magna* determined with different endpoints after 24 h and 48 h exposure to glyphosate-Cu (II) (n = 24).

observed between swimming distance and percent active time ($R^2 = 0.98$).

Exposure of *D. magna* to binary mixtures with different molar ratios between Cu(II) and glyphosate resulted in significantly lower fitness for Cu(II):Glyphosate ratios of 1.67 and 3.33. In contrast, a Cu(II):Glyphosate ratio between 0 and 0.77 resulted in somewhat comparable values for active time and distance moved suggesting that glyphosate attenuated acute Cu(II) toxicity in mixtures with an excess of glyphosate. These findings also suggested that complexation between Cu(II) and glyphosate likely occurred at a ratio around 1:1 in these experiments. This corresponds to theoretical ratios that have been estimated in a study of biosorption of glyphosate and copper (Trinelli et al., 2013). Complexation between Cu and glyphosate (GPS) was found to be pH-dependent, and at circumneutral pH three complexes were proposed to dominate including Cu(HGPS)(GPS), $Cu(HGPS)_2$, and Cu(GPS) (Trinelli et al., 2013). These complexes have a Cu (II):glyphosate ratio of either 1:2 or 1:1.

It has been suggested that complexation of glyphosate with Cu(II) will result in compounds with decreased sorption and increased mobility in mineral and organic soils (Barrett and McBride, 2006). Along this line, we have observed decreased sorption of glyphosate-Cu(II) complexes to organic polymers in lab-

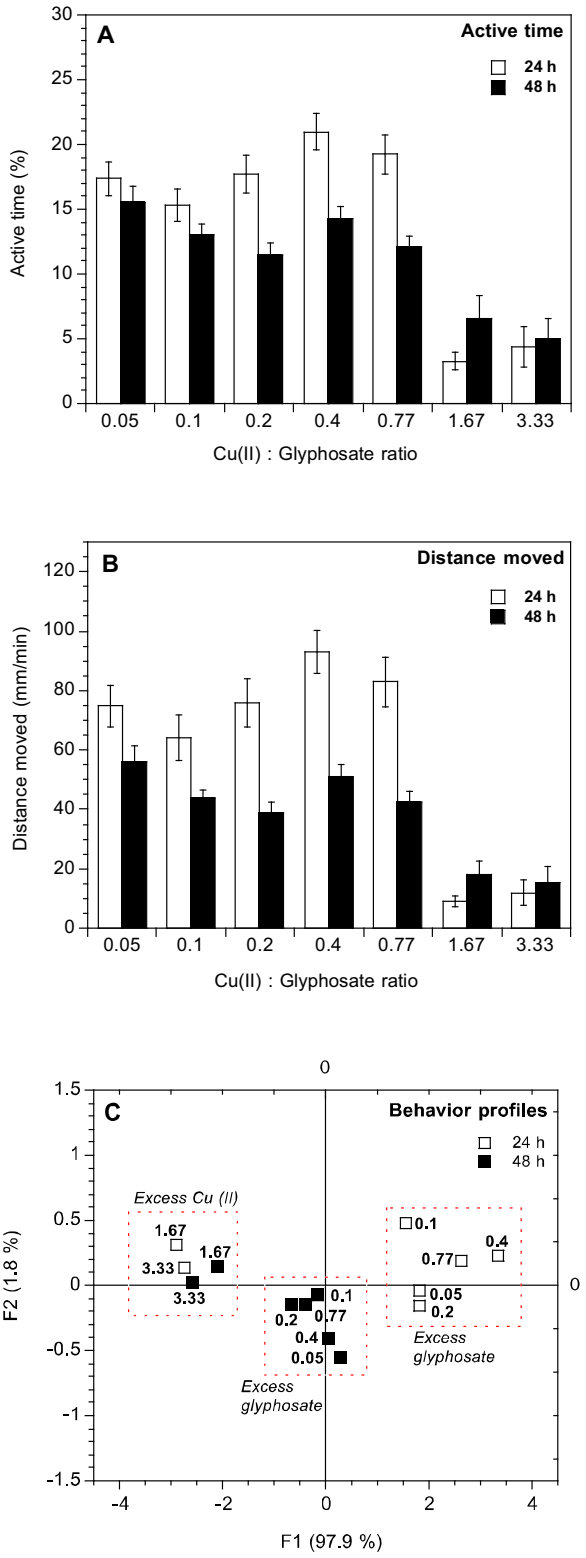

Fig. 4. Exposure of *D. magna* to mixtures with different molar ratios between Cu(II) and glyphosate. Effects were determined as percent active time (A) and distance moved per minute (B) after 24 and 48 h exposure. The Cu(II) concentration varied between 0.3125 μM and 20.0 μM whereas glyphosate was fixed at 15.9 μM. The glyphosate concentrations corresponded to the 48-h EC10 value for free glyphosate. Error bars indicate standard error (n = 24). (C) PCA analysis of behavioral profiles after 24 and 48 h exposure to different molar ratios between Cu(II) and glyphosate. Molar ratios are shown in bold in the PCA plot (C) and correspond to ratios shown in (A) and (B). The behavioral profile included swimming velocity, acceleration speed, active time and distance moved.

oratory experiments with *D. magna* (unpublished). Glyphosate may also mobilize metals that are otherwise adsorbed on surfaces in soils and sediments, and free metals may desorb bound glyphosate (Morillo et al., 1997; Morillo et al., 2002). Hence, glyphosate-metal interactions may affect bioavailability of glyphosate and/or metals to parts of the aquatic biota. In a study by Tsui et al. (2005), it was observed that glyphosate or a commercial Roundup® formulation decreased acute metal toxicity to the freshwater cladoceran *Ceriodaphnia dubia.* In some combinations, the presence of glyphosate increased metal bioaccumulation (Tsui et al., 2005). In the present study, we observed that although glyphosate reduced acute copper toxicity, the inhibitory effects of the glyphosate-Cu complexes were stronger on a molar basis than those of glyphosate. This excess toxicity may be explained by effects of free Cu(II) ions in the mixtures and/or increased toxicity of glyphosate-Cu(II) complexes. It is noteworthy that somewhat comparable mixture toxicity was observed in solutions with variable concentrations of Cu(II) between 0.8 and 12.8 μM as long as glyphosate was present in excess concentrations (Fig. 4). This result does not suggest an increasing role of free Cu(II) in these mixtures since Cu(II) is far more toxic on a molar basis than glyphosate (Okamoto et al., 2015).

Glyphosate concentrations that resulted in behavioral effects in this study were generally above levels found in most surface waters that are often in the μg/L range (Annett et al., 2014; Ruiz-Toledo et al., 2014). However, elevated environmental concentrations of glyphosate have been observed associated with spills or immediately after pesticide application (Annett et al., 2014; Ronco et al., 2008). Increased leaching to aquatic systems is often associated with the intensity and timing of rain events relative to the pesticide application (Borggaard and Gimsing, 2008; Norgaard et al., 2014). Worst-case scenarios for glyphosate concentrations in surface waters has been reported as 1.7–5.2 mg/L (Annett et al., 2014), and pesticide spraying can result in stream water concentrations of 1.8–10.9 mg/L (Ronco et al., 2008). This is within the range of toxic concentrations observed in the present study for both glyphosate and glyphosate-Cu(II). The lowest 48 h EC50 concentration for glyphosate-Cu(II) determined by behavior analysis corresponded to 1.4 mg/L glyphosate complexed with Cu(II). It should also be noted that this result is related to acute behavioral effects recorded within 48 h, whereas chronic thresholds are often lower and closer to environmental concentrations (Annett et al., 2014; Cuhra et al., 2013). Hence, the adverse effects of glyphosate-metal complexes to aquatic biota including chronic effects and long term metal accumulation may warrant further attention.

5. Conclusion

The study suggested that behavioral responses are relatively sensitive endpoint for adverse effects of glyphosate-Cu(II) to *D. magna.* The results also indicated that glyphosate is a mediator of metal toxicity to aquatic invertebrates, and that environmental metals can play a role in apparent glyphosate toxicity.

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