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Behavioral response of juvenile rainbow trout exposed to an herbicide mixture

Candida Shinn [a,b,*], Miguel Machado Santos [c], Sovan Lek [a], Gaël Grenouillet [a]

[a] Laboratoire Evolution et Diversité Biologique, Université Paul Sabatier/CNRS, 118 Av. de Rangueil, 31062 Toulouse, France
[b] Escuela de Ciencias Agrícolas y Ambientales, Pontificia Universidad Católica del Ecuador-Sede Ibarra, Ibarra, Ecuador, Av.Padre Aurelio Espinosa Polit, Ibarra, Ecuador
[c] CIMAR/CIIMAR, Interdisciplinary Centre for Marine and Environmental Research, University of Porto, Rua dos Bragas no. 289, 4050-123 Porto, Portugal

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ABSTRACT

Fish are capable of sensing water-borne chemicals at sub-lethal concentrations. Inadequate behavioral responses to physiological and environmental stimuli owing to adverse effects of aquatic toxicants can have serious implications for survival.

In this study we exposed juvenile rainbow trout (*Oncorhynchus mykiss*) during 5 days to a low-concentration mixture of three co-occurring herbicides: atrazine, linuron and metolachlor, at maximum concentrations of 4.5, 4.9 and 13.4 μg L^{-1}, respectively. Our hypothesis was that fish behavior – swimming activity and interactions between individuals – would be modified due to exposure to the mixture. We studied these behaviors by observing fish twice-daily throughout the exposure period at 30-s intervals for 5 min, registering the vertical distribution of fish in the water column and the number of agoniztic acts between all individuals.

Fish exposed to the mixture of herbicides were hypoactive and spent more time in the lower parts of the aquaria in comparison to non-exposed controls, reflecting inhibited swimming activity. Average swimming height of exposed fish decreased significantly with the number of agoniztic acts, whilst in control groups there was no significant relationship between the two behaviors. Overall, behavior of fish exposed for a short time to the herbicide mixture was altered in comparison to control-fish behavior. The behavioral endpoints chosen here were easily observed, simple to quantify, and of ecological relevance.

1. Introduction

A mixed-crop farmland will typically be treated with a broad range of substances with different modes of action. Thus, adjacent water bodies can carry a mixture of chemicals that are applied aiming at specific organisms but may present risks to non-target species. Herbicides, designed to inhibit germination, development and persistence of weeds, may indirectly affect aquatic fauna through disturbance of communities at the lower end of the trophic-chain, such as phytoplankton and macrophytes (Belden et al., 2007; Daam et al., 2009). However, more information is needed on the direct effect of herbicides on invertebrates, fish, and other aquatic vertebrates, and given that they are commonly applied simultaneously, studying the effects of mixtures is more environmentally relevant.

Behavior is the result of the interactions of an organism with its external environment, integrating physiological, biochemical and metabolic processes with the environmental factors that stimulate behavioral responses (Grue et al., 2002). Inadequate behavioral responses to physiological and environmental stimuli owing to adverse effects of aquatic toxicants can have serious implications for survival (Weber and Spieler, 1994): if an organism is not minimally in tune with the surrounding physical or biological conditions, basic necessities may become jeopardized. Although behavioral responses are not as contaminant-specific as other biomarkers of lower complexity (Peakall, 1994), their attractiveness relates to a higher sensitivity regarding dosage and response time, and therefore a potential as early-warning signals of effect (Hellou, 2011). Indeed, fish are capable of detecting, and sometimes responding by avoiding, water-borne chemicals at sub-lethal concentrations (Folmar, 1976; Scott and Sloman, 2004).

Fish are convenient models for behavioral ecotoxicology studies as many of their behaviors that are easily observed and

* Corresponding author. Present address: Escuela de Ciencias Agrícolas y Ambientales, Pontificia Universidad Católica del Ecuador - Sede Ibarra, Ibarra, Ecuador, Av. Padre Aurelio Espinosa Polit, Ibarra, Ecuador.
E-mail addresses: candida.shinn@gmail.com (C. Shinn), santos@ciimar.up.pt (M.M. Santos), sovannarath.lek@univ-tlse3.fr (S. Lek), gael.grenouillet@univ-tlse3.fr (G. Grenouillet).

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quantified under controlled conditions are per se more ecologically relevant than lower level biomarkers (Scott and Sloman, 2004). For example, social interactions such as schooling, courtship, and dominance hierarchies are directly linked to fitness. Disruption of those behaviors may jeopardize the chances of succeeding in fundamental processes that are crucial to individual, and eventually population, continuity (Grue et al., 2002).

Dominance hierarchies are a key factor in ensuring enough resources (food and shelter) for individual fish optimal growth, and are established via intraspecific competition (Chapman, 1966). Alterations in agoniztic acts due to the presence of toxicants may lead to either failure to maintain a territory or metabolic fatigue (Triebskorn et al., 1997). The action of toxic substances can also directly interfere with feeding and predator recognition-and-escape behaviors through physiological interaction with sensory organs/cells (Saglio and Trijasse, 1998; Tierney et al., 2007b). Fish swimming activity might also be altered in the presence of contaminants (Little et al., 1990; Zhou and Weis, 1999; Steinberg et al., 1995).

The three herbicides studied here-atrazine, linuron and metolachlor-are representatives of three chemical groups and two modes of action. Due to their application to the same crops (e.g., corn, sorghum, soybeans), they have been reported to co-occur in environmental water samples collected from different watersheds (Gilliom, 2007; Faggiano et al., 2010).

Atrazine is part of the s-triazine chemical group and inhibits photosynthesis by blockage of electron transport in the photosystem II (van Rensen, 1989). It is included in the EU priority substance list due to its high mobility and persistence in the environment (Directive 2008/105/EC of the European Commission, 2008) and has been banned from use on most crop types in the EU since 2004 (Directive 2004/248/EC of the European Commission, 2004). The Canadian Water Quality Guideline (WQG) for the protection of freshwater life against atrazine has set a maximum allowed limit of 1.8 $\mu g\,L^{-1}$ (CCME, 1999). A lowest observed effect concentration of $\leq 5\ \mu g\,L^{-1}$ has been reported for swimming behavior of zebrafish (Steinberg et al., 1995). Saglio and Trijasse (1998) reported increased surfacing activity, decreased grouping behavior, and decreased sheltering in response to an alarm signal in goldfish exposed to 5 $\mu g\,L^{-1}$ for 24 h. A 30-min exposure to 1 $\mu g\,L^{-1}$ atrazine eliminated preference behavior for a natural odorant in rainbow trout, and 10 $\mu g\,L^{-1}$ atrazine significantly reduced L-histidine-evoked olfactory sensory responses (Tierney et al., 2007a). Atrazine at 1 $\mu g\,L^{-1}$ significantly reduced male Atlantic salmon olfactory response to a female pheromone (Moore and Waring, 1998; Moore and Lower, 2001).

Linuron is a phenylurea that acts upon photosynthesis in a similar way as atrazine (van Rensen, 1989). The Canadian WQG for the protection of freshwater life against linuron has set the maximum allowed limit to 7 $\mu g\,L^{-1}$ (Caux et al., 1998). Linuron is suspected to alter olfactory-mediated behaviors in fish (Tierney et al., 2007b). The structurally similar herbicide diuron was reported to alter olfactory-based behaviors in goldfish, such as the decrease of grouping behavior in the presence of an alarm signal, after a 24-h exposure to 5 $\mu g\,L^{-1}$ (Saglio and Trijasse, 1998). Tierney et al. (2007b) detected reduction of L-serine-evoked olfactory sensory responses in rainbow trout exposed to linuron at 10 $\mu g\,L^{-1}$ for 15 min, suggesting that linuron has the potential to disturb predator avoidance and food location in salmonid fish.

Metolachlor is a chloroacetanilide that promotes the inhibition of cell division in seedling shoots and roots (Takacs et al., 2002). The Canadian Council of Ministers of the Environment has established the WQG limit at 7.8 $\mu g\,L^{-1}$ for the protection of aquatic life (CCME, 1999). This herbicide has been reported to affect the perception of chemical stimuli by the crayfish *Orconectes rusticus*, leading to inappropriate decisions regarding detection of food and response to an alarm signal (Wolf and Moore, 2002), as well as interfere with the ability of crayfish to respond to social signals involved in agoniztic behaviors when exposed to 80 $\mu g\,L^{-1}$ for 96 h (Cook and Moore, 2008).

In the present study we investigated the effect of a mixture of three herbicides on the behavior of juvenile trout (*Oncorhynchus mykiss*). Occupation of the water column, number of movements, and number of agoniztic acts, observed at regular intervals throughout the experiment, were compared between exposed and control organisms.

2. Material and methods

2.1. Herbicide mixture selection

Three herbicides, each belonging to 3 different chemical groups (s-triazines, acetanilides and phenylureas) were selected to be assessed in the mixture toxicity tests. The selection was based on the pesticide concentrations measured by the AEAG throughout the Adour-Garonne river basin during the year 2007 (140 sampling sites $\times$ 5 or 6 samplings per site). Hierarchical clustering analysis was performed in R using complete linkage and Euclidean distances between the maximum concentrations of contaminants that were detected in more than 5% of the samples. The contaminants were thus grouped according to their co-occurrence and concentration, i.e. the mixture occurs in the natural environment with all 3 herbicides co-occurring in 22% of sampling sites. The selected herbicides were atrazine, linuron and metolachlor.

2.2. Herbicide concentrations

Test concentrations of each compound were established according to the highest concentration found in the year 2007 (Cmax) in the Adour-Garonne river basin, multiplied by 50 for atrazine and linuron and by 5 for metolachlor. The difference in the multiplication factor aimed to avoid metolachlor (Cmax = 9 $\mu g\,L^{-1}$) dominating the mixture – Metolachlor contributed to 70% of the total contamination of 2009-spring flood water in a medium-sized river of the Adour-Garonne river basin (Polard et al., 2011) –, and thus possibly determining fish response regardless of the presence of atrazine (Cmax = 0.2 $\mu g\,L^{-1}$) and linuron (Cmax = 0.3 $\mu g\,L^{-1}$). A more representative mixture of year-round pesticide concentrations would be one in which metolachlor was not dominating, but to nevertheless maintain the weight of metolachlor contamination in the environment, increasing the concentrations of the other two pesticides was chosen over decreasing that of metolachlor. The final nominal concentrations of each chemical in the mixture was thus 10, 15 and 45 $\mu g\,L^{-1}$ for atrazine, linuron and metolachlor, respectively. Although the tested concentrations do not copy the Cmax found in the environment, they closely reflect levels that have been studied previously and that induced sub-lethal effects in fish.

2.3. Exposure setup

A flow-through system (Fig. 1) was constructed in a closed room with air at a constant temperature of 16 °C and light:dark regime of 16:8 h. Four control (only water) and four treated 40.4 L ($28 \times 38 \times 38\ cm^3$) glass aquariums received a continuous flow of filtered (25 µm, DomSource-COMAP, Lyon, France), aerated and refrigerated tap water. A multichannel peristaltic pump (Watson-Marlow 205U, La Queue Lez Yvelines, France), equipped with silicone tubes (63 µm internal diameter) delivered the mixture of herbicides to each mixing vessel at the desired rate. Water inflow, regulated by flow meters (Cole Parmer, Chicago, USA), was

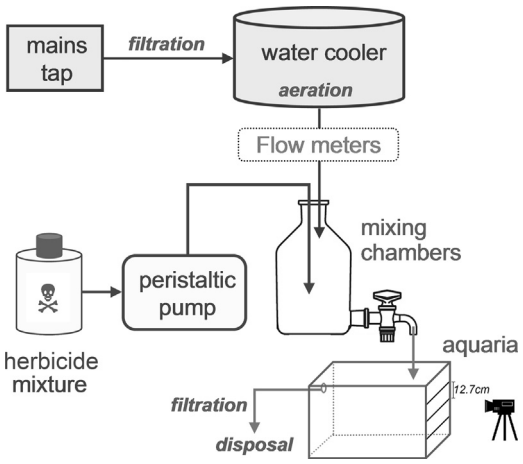

Fig. 1. Schematic diagram of flow-through system built for exposure testing with continuous renewal of test water. Black lines on the aquarium facing the video camera represent guidelines for division of the water column into 3 sections.

established according to the required test concentration and renewal rate (see details in Section 2.4.). Mixing vessels, containing a magnet and placed above a magnetic stirrer, received water and contaminants and provided test water to two aquaria each via 15 mm diameter tubing. Passive outflow from aquaria was attained by constant overflow through an opening in the upper part of the aquaria, connected to a drain and a self-assembled carbon filter. The structure of the flow-through system used here was adapted from and similar to that of Brian et al. (2005).

2.4. Herbicide mixture administration

Stock solutions of each pure compound (Sigma-Aldrich, Lyon, France) were individually prepared in a carrier solvent (acetone; Carlo Erba, Val-de-Reuil, France). From each stock solution aliquots were taken to prepare concentrated aqueous mixture solutions containing all three herbicides. The aqueous mixture of herbicides was delivered to each mixing vessel at a rate of 0.15 mL min^{-1}. Mixing vessels received fresh water via the flow meters at a rate of 276 mL min^{-1}. Each mixing vessel provided water for two replicate aquaria at a rate of 7.80 L h^{-1}, thus resulting in one complete water change of each aquarium every 5 h 8 min. Control treatments equally received water from mixing vessels but without the addition of herbicides via the peristaltic pump. The amount of solvent in the final test water of the 4 spiked treatments did not exceed 0.1 mL L^{-1}, as recommended by the OECD guidelines (e.g. OECD guideline 215; OECD 2000).

2.5. Fish acclimation and exposure

Juvenile rainbow trout (*O. mykiss*) aged 5 months old were obtained from a commercial fish farm in the Eastern Pyrenees (Gaec des Chutes D'Aston, Tarascon sur Ariège) in July 2009. The fish were acclimated to laboratory conditions in 160 L holding tanks for 7 days. They were then weighed and measured (controls: 3.23 ± 0.55 g, 59.6 ± 3.7 mm; exposed: 3.21 ± 0.63 g, 59.4 ± 4.4 mm) and 8 fish were randomly allocated to each of the 8 testing aquaria, already running on flow-through mode with non-spiked water. Care was taken to only include fish that did not differ more than 25% in size between and within treatments. All weight measurements during this experiment were performed after a 12-h fasting period. Fish were fed fish pellets (Neo start, Le Gouessant, France) twice daily with a total daily food-body weight ratio of 3%.

After a 10-day acclimation period all pre-selected fish were again weighed and measured for feeding rate recalculation. The fish were returned to their original aquarium to maintain groups identical to those of 10-day pre-exposure period. A resettling period of 24 h was allowed before the 5-day exposure period was initiated. Euthanasia was conducted by immersing fish in a solution of 250 mg L^{-1} Tricaine methanesulfonate (Sigma-Aldrich, Lyon, France) following animal welfare measures established in the European Union (Directive 2010/63/EU of the European Commission, 2010).

Feces were removed daily by siphoning and during the acclimation period aquaria walls were brushed twice throughout the whole experiment. Dissolved oxygen, pH, conductivity (WTW Multi 340i/ SET), and nitrite (VISOCOLOR®, Macherey-Nagel GmbH, Düren, Germany) were checked daily, and temperature was registered at 30-min intervals using submerged dataloggers (Tinytag Plus 2). The average test water temperature did not deviate more than 0.7 °C in each individual aquarium throughout the experiment, ranging from 11.9 to 13.4 °C over all aquaria. Dissolved oxygen, pH, conductivity and nitrite concentrations maintained within recommended limits (84–95%; 7.70–8.05; 252–275 µ S cm^{-1}; < 0.01 mg NO_2^- L^{-1}, respectively).

2.6. Analysis of herbicide concentrations

Using glass bottles, samples of 1 L test water were collected from 2 of the 4 treated aquaria at 24 and 72 h after start of herbicide exposure. The water samples were treated with 10 mL of analytical grade dichloromethane (Carlo Erba, Val-de-Reuil, France) and stored at 4 °C until chemical analysis.

Atrazine, linuron and metolachlor concentrations were measured following a protocol developed at Ecolab campus ENSAT (F-31326 Castanet Tolosan, France; Devault et al., 2007). Liquid–liquid extraction was performed with 850 mL of non-filtered samples in a 3-step procedure: 70 mL dichloromethane (DCM; Pestipur, SDS-Carlo-Erba, Val-de-Reuil, France) in the 1st step, and 60 ml DCM in the 2nd and 3rd steps. After settling, each DCM phase was collected and all three were pooled. Water residues were then removed from each sample using fiber glass filters and anhydrous sodium sulfate (SDS-Carlo-Erba, Val-de-Reuil, France). The samples were evaporated at 40 °C, resuspended with hexane, placed in dark vials, and reduced to a known volume under a nitrogen stream. Before analysis, an internal standard (Fenitrithion-D6, Ehrenstorffer provided by Clouzeau Info Labo F-33220 Ste Foy la Grande, France) was added to each sample extract at a ratio of 1:50 (internal standard:sample).

Final quantification of the tested herbicides was performed using a gas chromatograph coupled to a mass spectrometer under conditions as stated in Devault et al. (2007). Two replicate aliquots of 1 µL of each sample extract were individually injected. The detection limit established was 0.001 µg g^{-1}. Recovery after sample preparation, extraction and purification obtained for each herbicide varied from 82.4% to 104.6%, leading to a mean recovery of 95.4 ± 6.5%, with an acceptable repeatability of < 14%. The efficiency of this method is confirmed by the test on organo-chlorine derivatives which gave a mean recovery yield of 98.5%, in accordance with methods used in other studies.

2.7. Behavior data collection and analysis

Three horizontal black lines were drawn on the visible side of each aquarium such that the water column was visually divided into three equal parts: top, middle and bottom (Fig. 1). Five-minute video recordings of each aquarium were performed twice-daily throughout the test period (i.e. from the start of test water spiking): one between 10 and 12 a.m. and one between 3 and 5 p.m.

The camera (JVC HD camcorder) was positioned behind a dark, opaque curtain with openings for aquarium observation. Care was taken not to disturb the fish at least 2 h prior to video recordings. Over the 5-day exposure, 20 measurements were performed daily per aquarium (2 times per day × 10 measurements per observation), considered sufficient given the easily viewed and clearly distinct behaviors recorded, and similar to other behavioral studies with fish (e.g. Sloman et al., 2001: 3 times per day × 5 measurements per observation = 15 measurements).

Videos were observed on a PC using standard media viewing software. Starting at 0 s, the number of fish in each section of the aquarium (presence data) was recorded at 30-s intervals, throughout the 5-min video recording. As rainbow trout are territorial organisms (Cole and Noakes, 1980) and alteration of the rate of agoniztic acts has been reported when organisms are exposed to contaminants (trout and heavy metals: Sloman et al., 2003; crayfish and metolachlor: Cook and Moore, 2008), we selected an easily detectable behavior that would represent and quantify this characteristic. The number of agoniztic behaviors were registered during the whole video recording. A single agoniztic behavior here was counted when fish A approached fish B, resulting in the escape of fish B, i.e. fish B changed swimming direction and/or speed after the encounter. All observations were performed by the same person without knowledge of the treatment of each aquarium.

Regarding the evaluation of fish vertical swimming activity, the swimming intensity and the spatial distribution in the water column of exposed fish has been shown to be a responsive endpoint in a number of previous behavioral ecotoxicology studies: e.g. swimming velocity (Triebskorn et al., 1997), swimming activity (Zhou and Weiss, 1999), swimming depth (Eissa et al., 2006b). Therefore, using the presence data, the total numbers of movements between top and middle, and middle and bottom compartments of the aquaria (not accounting for possible fish movements between top and bottom compartments) were calculated for each aquarium/day/observation event and divided by the total number of fish present. This statistic was named M_F (movements per fish). A weighted water column height per number of fish was also calculated using presence data per aquaria section, referred to as *Height* (cm): $\sum(n^\circ\ fish\ in\ compartment_i \times average\ height\ of\ compartment_i)/\sum n^\circ\ fish$. The average height of the top, middle and bottom compartments were, respectively, 31.7, 19 and 6.3 cm (total water column height: 38 cm; height of each division: 12.7 cm).

The total number of aggressions per aquarium/day/observation was divided by the number of fish present and hereafter referred to as *Aggress* (aggressions per fish). Pearsons' correlation tests were performed between pairs of variables M_F, *Height*, and *Aggress*, for controls and exposed treatments separately.

2.8. Statistical analysis

To test the effects of the herbicide mixture on behavioral parameters, Generalized Linear Mixed Models (GLMMs) were used. Treatment (exposed and control) and observation hour (time since start of observations on day 1 of exposure; continuous) were set as fixed factors. Aquarium was a random factor and observation hour was scaled (transformed values are centered around zero and have a unit variance). In order to evaluate all behavioral parameters GLMM with Gaussian error distributions were used (model fit by residual maximum likelihood approximation). Statistical significance of model outputs were given by calculating the 95% confidence intervals (1000 runs of Markov chain Monte Carlo generations) and checking for absence of overlap of the interval with zero that indicates a significant effect; e.g. Table 1. The confidence interval of the parameter "Movements fish^{-1}" for the effect of "Treatment" is between −1.31 and −0.19, thus does not include 0, therefore Treatment had a significant effect on the number of movements per fish.

Table 1
Results of Generalized Linear Mixed Models performed on behavior parameters measured during treatment period. In bold are significant effects (*p-value* < 0.05). See Section 2.8 for details on model construction.

	t-Value	95% CI	
		Lower	Upper
Movements fish^{-1}			
Treatment	−2.2700	**−1.3066**	**−0.1910**
Observation hour	1.3250	−0.0597	0.3229
Water column height			
Treatment	−1.2170	**−7.9552**	**−1.6319**
Observation hour	−1.8140	−0.9477	0.2973
Aggressions fish^{-1}			
Treatment	−0.7180	−2.4859	1.1695
Observation hour	2.2760	−0.0009	0.8581

CI, confidence interval.

All statistical analyzes were performed in R 2.11.1 (R-project, 2010) using the lme4 package for GLMM runs, and level of significance set at $p < 0.05$.

3. Results

Out of the 64 fish tested (8 per aquarium), 1 fish died in 3 different aquaria during the acclimation period, and none died during the exposure period. Mortality was thus negligible and not related to a specific treatment, also indicating that stress levels were insignificant.

Measured concentrations of atrazine, linuron and metolachlor in each of the two analyzed samples were, respectively, 0.01–3.83, 0.03–3.84, and 0.02–6.52 μg L^{-1} at the first sampling time (24 h after start of exposure), and 4.51–3.54, 4.95–4.69, and 13.4–12.71 μg L^{-1} at the second sampling time (72 h after start of exposure). The nominal/maximum measured ratio was of 45%, 33% and 30% for atrazine, linuron and metolachlor, respectively. The deviation between nominal and actual pesticide concentrations in the treated aquaria may have been due to: biological degradation; adsorption of compounds on parts of the flow-through system (silicone tubing, glass mixing vessel, aquarium walls) despite care to use material with lower adsorptive tendency; additionally, flow-through systems like the one used in the present work usually require several days to reach the nominal concentrations which will depend on several variables such as the chemical's physico-chemical properties. Comparable findings have been reported in previous studies (e.g. Mensink et al., 2002; Brian et al., 2005). However, the measured concentrations in samples taken towards the end of the exposure time (72 h) were close to maximum levels allowed by Canadian regulation in surface waters; the regulated limit/average final concentration (± standard deviation) measured for atrazine, linuron and metolachlor, were, respectively: 1.8/4.0 ± 0.6, 7/4.8 ± 0.2 and 7.8/13.1 ± 0.4 μg L^{-1} (Regulated values: Caux et al., 1998; CCME, 1999). The concentrations that the fish were ultimately exposed to were thus within ecologically relevant levels.

Inter-replicate variance of measured concentrations after 24 h of exposure was high. Although the use of a flow-through system has many advantages, one disadvantage is that the aimed concentrations are not obtained immediately, only after some hours. However, given that the replicates presented very similar concentrations at 72 h, the maximum concentration was reached between 24 and 72 h. Statistically this does not pose a problem as the

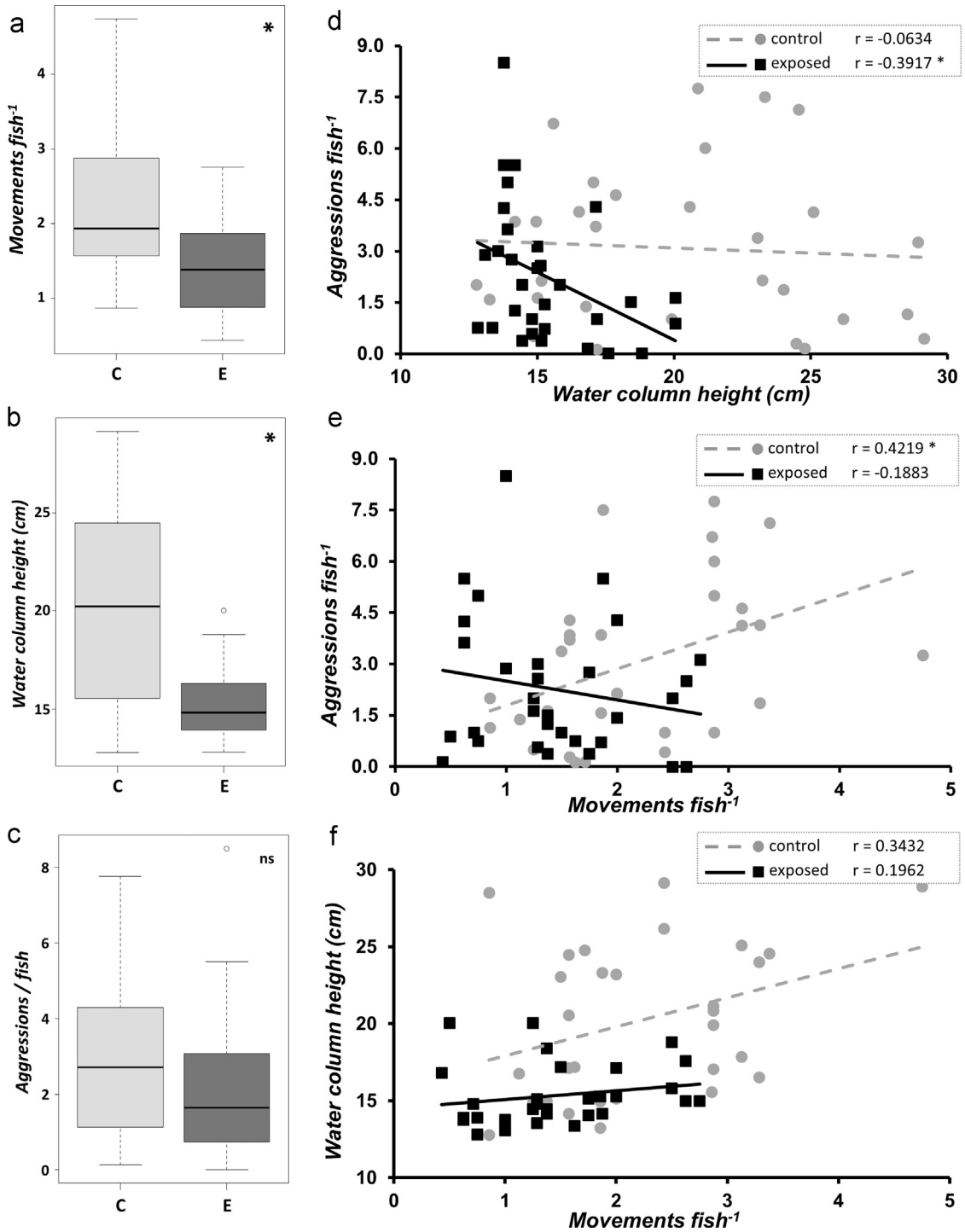

Fig. 2. (a–c) Box-plots of quantitative behavioral observations: (a) movements per fish, (b) weighted average water column height, and (c) aggressions per fish; treatment significance as in Table 3 (GLMM analysis) is indicated in the bottom-right corner of each plot (*$p < 0.05$; ns, not significant). (d–f): Linear regression plots between (d) weighted average water column height and aggressions per fish, (e) movements per fish and aggressions per fish, and (f) movements per fish and weighted average water column height.

factor time (named "Observation hour"; Table 1) did not have a significant effect on the measured behavioral parameters.

Results of GLMMs revealed a significant ($p < 0.05$) effect of exposure to the herbicide mixture on M_F and *Height*, but not on *Aggress* (Table 1). The average number of movements per exposed fish was fewer than that of control fish (1.45 ± 0.7 and 2.21 ± 0.9 respectively; Fig. 2a). Exposed fish explored preferably the bottom compartment of the aquaria whilst control fish were generally in the upper sections of the water column (respective average water column heights: 15.3 and 20.2 cm; Fig. 2b). The average number of agoniztic acts was, although less in exposed aquaria, not different from control conditions (Fig. 2c). The time point at which observations took place throughout the exposure (observation hour; Table 1) did not have an effect on any of the behavioral endpoints studied.

Pearson's correlation tests detected significant relationships between *Aggress* and *Height* in exposed fish and between *Aggress* and M_F in controls (Fig. 2d–e). In exposed fish but not in controls,

an increase in aggressiveness co-occurred with a decrease in the average water column height that the fish explored (Fig. 2d; $p=0.029$); points closest to the exposed fish regression line: 13.1 cm with 2.9 aggressions fish^{-1} and 20.1 cm with 0.9 aggressions fish^{-1}. An increase in aggressiveness co-occurred with an increase in the mobility of control fish but not of exposed fish (Fig. 2e; $p=0.020$). A positive correlation between M_F and *Height* was found to be almost statistically significant among control fish and not significant among exposed individuals (respectively: $p=0.063$, $r=0.3432$; $p=0.290$, $r=0.1962$; Fig. 2 f).

4. Discussion

In this study we sought to identify whether the behavior of juvenile rainbow trout is affected by exposure to a low-concentration mixture of atrazine, linuron and metolachlor, three co-occurring herbicides (Faggiano et al., 2010). Fish exposed to the mixture spent more time in the lower parts of the aquaria and moved less in general, whilst control fish tended to explore at higher levels in the aquaria with increased mobility, reflecting better swimming activity. Eissa et al. (2006b) reported changes in spatial distribution of juvenile carp exposed to Cd^{2+}, namely reflected in alteration in the preferred swimming depth.

Alteration of fish behavior in exposed groups was also detected via the decrease of average aquarium height explored with increase of agoniztic acts, whilst in control groups the number of agoniztic acts did not correlate strongly with swimming height. Although more agoniztic acts were occurring at lower levels of the aquarium in exposed groups, the number of movements per fish was not significantly correlated to swimming height in those groups. This ambiguousness in exposed fish could be the result of an effect of the herbicide mixture on the perception and integration of external stimuli such as the approach and aggressiveness of a conspecific. The disruption of external stimuli perception has previously been reported in studies exposing fish to contaminants (Fuiman and Magurran, 1994; Moore and Waring, 1998; Moore and Lower, 2001; Tierney et al., 2007a, 2007b). For example, Tierney et al. (2007a, 2007b) showed that linuron led to impairment of amino-acid detection by salmonids, important in processes such as predator evasion and conspecific recognition, while exposure to atrazine eliminated preference behavior for a natural odorant. On the other hand, it is logical that the more confined the fish, the more aggressive they tend to be; thus the aggressiveness may not (only) be a direct effect of the contaminants, but indirect, as a consequence of another behavioral response such as a denser grouping at the bottom of the aquarium.

A certain degree of hypoactivity was detected in exposed juvenile rainbow trout, as animals from those aquaria presented a significantly smaller number of movements. Hypoactivity of contaminant exposed fish has been reported in other studies, such as for goldfish exposed to parathion (Rand, 1977), rainbow trout exposed to carbaryl (Little et al., 1990), and mummichog exposed to lead (Weis and Weis, 1998). Hypoactivity may increase vulnerability to predation indirectly through reduced feeding and thus reduction of energy levels available for escape, or directly via reduction of active escape response (Kramer, 1987). In an effort to classify chemicals according to general mode of action, Drummond and Russom (1990) used behavioral and morphological signs of stress in fathead minnow subjected to acute exposure, to then group chemicals into classes of biological toxic effects. The triazine group was classified within the hypoactivity syndrome class. Although we tested a mixture of pesticides, in contrast to Drummond and Russom's single-substance tests (1990), there is evidence of a similarity in the behavioral trends in both studies. Given that the herbicide concentrations we studied here are similar to those found in aquatic ecosystems, the behavioral alterations observed – although they are very subtle and thus may go unnoticed in the wild – may occur in feral organisms, possibly contributing to population decline.

The herbicide mixture tested in our study is comprised of compounds with different modes of action which are well studied in their target organisms – plants. However, for non-target organisms, such as freshwater fish, the relation between herbicide exposure and effect on a particular biomarker is not yet as clear-cut, especially regarding behavioral biomarkers. Metolachlor has been reported to reduce acetylcholinesterase activity and protein production in midge larvae, leading to reductions in the activity of major detoxification enzymes (Jin-Clark et al., 2008), while atrazine changes antioxidant parameters in Spotted snakehead (Nwani et al., 2010). Such interferences in the baseline physiological capacity of exposed organisms may impede the metabolic detoxification of other compounds, increasing their susceptibility (Jin-Clark et al., 2008). Relating the behavioral changes observed in the exposed rainbow trout of our study to specific herbicidal modes of action is complex, despite the literature containing some relationships between toxicant mode of action and behavioral outcomes. It is important to keep in mind that although the ecological relevance of the marker studied increases with increasing biological complexity, it inevitably becomes also less sensitive and contaminant-specific (Hellou, 2011). Furthermore, behavior, similarly to many other individual or population-level markers, is seldom able to diagnose the effects of a chemical or class of chemicals in particular and is generally regarded as a biomarker of effect than of exposure (Peakall, 1994).

After 72 h of exposure, the measured atrazine and linuron concentrations were slightly below albeit within close range of concentrations at which exposed zebrafish (Steinberg et al., 1995) and goldfish (Saglio and Trijasse, 1998) presented altered swimming capacity, increased surfacing and decreased grouping and sheltering (ca. 5 μg L^{-1}). This comparison feeds the hypothesis that the combination of atrazine and linuron were driving the behavioral alterations seen in the exposed juvenile trout, although the lack of information on the effects of metolachlor on fish behavior does not allow for a definite conclusion.

4.1. Concluding remarks

Being several times more sensitive than high-concentration toxicity tests that provide information on lethality thresholds and other acute effect derived indices, behavioral endpoints can help determine adequate no- and lowest-observed-effect-levels, important for water quality standard definition. However, although very sensitive, these endpoints need to be object of research regarding their relevance to survival in the wild, including field investigations. Much work is still to be done to further support the inclusion of behavioral endpoints in the evaluation process of sublethal contaminant toxicity in organisms. The behavioral endpoints chosen here were easily observed and simple to quantify, and within a short timeframe, overcoming some of the behavioral toxicology challenges outlined by Little et al. (1993a). Using simple and cost-effective test material and ready-available observation devices (glass aquaria with horizontal guidelines and a video camera) we were able to study a range of easily detected, albeit complex, behavioral endpoints that were affected by the presence of the contaminants.

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