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Can a mixture of agrochemicals (glyphosate, chlorpyrifos and chlorothalonil) mask the perception of an individual chemical? A hidden trap underlying ecological risk

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ABSTRACT

As aquatic environments associated with conventional agriculture are exposed to various pesticides, it is important to identify any possible interactions that modify their effects when in a mixture. We applied avoidance tests with *Danio rerio*, exposing juveniles to three relevant current use pesticides: chlorpyrifos (CPF), chlorothalonil (CTL) and glyphosate (Gly), individually and in binary mixtures (CPF-Gly and CTL-Gly). Our goal was to identify the potential of contaminants to trigger the avoidance response in fish and detect any changes to that response resulting from binary mixtures. Avoidance was assessed for three hours using an open gradient system with six levels of increasing concentrations. Fish avoided environmentally relevant concentrations of the three compounds. The avoidance of CPF [AC50 = 7.95 (3.3–36.3) μg/L] and CTL [AC50 = 3.41 (1.2–41.6) μg/L] was evident during the entire period of observation. In the case of Gly, the response changed throughout the experiment: initially (until 100 min) the fish tolerated higher concentrations of the herbicide [AC50 = 52.2 (12.1–2700) μg/L] while during the later period (after 100 min) a clearer avoidance [1.5 (0.8–4.2) μg/L] was observed. The avoidance recorded using CPF and CTL alone was attenuated by the presence of Gly. Applying an additive concentration model, Gly initially acted synergistically with the other two compounds, although this interaction was not observed during the later period. Avoidance gives us an idea of how the distribution of populations may be altered by contamination, our results suggest that in some mixtures this response may be inhibited, at least temporarily, thus masking the ecological risk of the exposure.

1. Introduction

The proper characterisation of the impact that chemical contamination has on aquatic environments has pushed the advance of ecotoxicology towards the development of more realistic, complex, and environmentally relevant approaches (Vighi and Villa, 2013). Regarding the exposure of ecosystems to such contamination, it is necessary to adapt the ecotoxicological assessments to realistic scenarios where more than one contaminant is present in the environment, and in concentrations not expected to cause acute effects in the organisms (de Souza Machado et al., 2019). In a real scenario, the community established in a contaminated habitat is exposed to more than one stress factor at a time. For example, organisms are frequently exposed to cocktails of different pesticides in agricultural landscapes (Arias-Andrés et al., 2018; Schreiner et al., 2016) and the concentrations of the substances present in such environments are frequently below the levels related with acute effects (Polidoro and Morra, 2016; Rämö et al., 2018), which may lead to an underestimation of the impact caused by anthropogenic contamination. For this reason, the studies assessing the effects of mixtures offer a better approach to reality (Altenburger et al., 2015).

The effects that contamination causes in the ecosystems have been traditionally assessed individually, using forced exposure protocols to estimate its potential toxicity to organisms (Rico et al., 2016; Villenueve and Garcia-Reyero, 2011). Early sub-lethal individual responses are relevant as they can improve the understanding of the ecological mechanisms that translate a sub-lethal exposure into changes at higher levels of biological organisation, such as populations and communities (Amiard-Triquet, 2009; Baldwin et al., 2009; Villenueve and

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Garcia-Reyero, 2011). Among such individual responses, the behaviour of organisms facing contamination has been of interest for many years, by helping explain mechanisms through which a specific behaviour is triggered or changed by a contaminant (Beitinger and Freeman, 1983; Hansen et al., 1972). The assessment of behaviour can represent an integration of many sub-individual events at cellular and physiological levels that manifest a behavioural change (Amiard-Triquet, 2009; Scott and Sloman, 2004). But also, as in the case of avoidance, it can be an early response based on the sensing of the environment, that prevents organisms from suffering negative physiological effects as they can flee from exposure to damaging concentrations of the pollutants (Moreira-Santos et al., 2019). However, this process depends on an appropriate functioning of the organisms sensorial system and the absence of interferences (Dominoni et al., 2020; Tierney, 2016).

Chemical cocktails, characteristic of agricultural landscapes, can be related to different levels of their effect on ecosystems. Peaks of contaminants carried by heavy runoff or occasional discharges can cause events and massive acute mortalities (Gormley et al., 2005). However, the presence of compounds with different biocide actions at sub-lethal concentrations that affect the physiology of organisms and lead to a gradual system degradation (Rossi et al., 2020; Teixeira-Marins et al., 2020) is more common. For motile animals, capable of recognising the presence of a contaminant, there is still the chance of escaping from the toxic effects that could affect them (Araújo et al., 2016). This ability, that can spare the organisms from suffering damage, can still have an impact on the ecosystem, as the displacement of a population alters the community and can lead to local extinctions (Araújo et al., 2016; Moreira-Santos et al., 2019). The relevance of this approach should increase if the avoidance response is observed with environmentally relevant concentrations of contaminants. This would mean that any repercussions concerning the structure of communities might be expected to occur earlier than any abrupt changes as the outcomes of classic toxicological processes (acute). But there is still another point to consider: the avoidance response is generally expected as the outcome of the contaminant's inherent repellency (Araújo et al., 2020); however, some contaminants may actually be attractive to the organisms. In the specific case of agriculture-associated contamination, some herbicides have been proven to attract fish (Tierney et al., 2011). So, what happens if the environment contains a mixture of several pollutants, of which some are capable of affecting the organism's physiology or even killing them when certain concentrations are reached, while other compounds are attractive to those same organisms?

Taking Costa Rican agriculture as a reference for intensive pesticide use (Food and Agriculture Organization of the United Nations (FAO) and FAOSTAT, 2019), chlorpyrifos (CPL), chlorothalonil (CTL) and glyphosate (Gly) are examples of substances with different biocide actions that are widely used for several local crops (Bravo et al., 2013). The first is an organophosphate insecticide, known to cause neurotoxicity, and highly toxic to fish and aquatic biota; chlorothalonil is a fungicide, which is also highly toxic to fish and other aquatic organisms; and glyphosate is a herbicide with moderate toxicity to fish (Lewis et al., 2016). However, glyphosate has been proven to be attractive to fish (*Danio rerio*) at environmentally realistic concentrations (Tierney et al., 2011). Therefore, the presence of glyphosate could prevent the expected flight from the contaminated areas, leading the organisms into a trap with serious ecological consequences. Hence, this work was carried out with the aim of, firstly, characterising the avoidance response of a model fish to three currently used pesticides (chlorpyrifos, chlorothalonil and glyphosate) and, secondly, to elucidate whether a binary mixture of chlorpyrifos and chlorothalonil with glyphosate could modify the avoidance response of the fish. *D. rerio* was used as the test organism due to its known ability to detect and avoid contamination (Moreira-Santos et al., 2008; Islam et al., 2019) and a multi-compartmented exposure approach was employed to create a gradient of contamination, allowing organisms to move among different concentrations of chemicals and avoid the most repellent ones.

2. Materials and methods

2.1. Test organism

Juveniles of zebrafish (*D. rerio*; Ethics Committee; *Junta de Andalucía*, Spain; #2020999001515058 and #202199901083791), 1.5 cm average standard length, were obtained from the Andalusian Center for Development Biology (CABD), Spain (license: # ES 410910008004), placed in a tank with dechlorinated freshwater and constant aeration under the following laboratory conditions: dissolved oxygen: 6.5 mg/L, temperature: 25 °C, photoperiod: 12:12 light/dark. Food (TetraMin Flakes) was offered daily ad libitum until 24 h before the assays.

2.2. Pesticides

Solutions of chlorpyrifos (Sigma-Aldrich, batch number: BCCC1983; purity: ≥ 98%), chlorothalonil (Sigma-Aldrich, batch no.: BCBZ8713; purity: ≥ 98%) and glyphosate (Sigma-Aldrich, batch no.: BCBZ6585; purity: ≥ 98%) were prepared from standard grade stocks in ultrapure (Milli-Q) water (Millipore Q-POD™) in the ranges of concentrations detailed in Table 1. Samples of the exposure solutions were collected at the beginning and at the end of the experiments and the content of the substances was measured by liquid chromatography tandem mass spectrometry (LC–MS/MS) in the case of Gly and CPF, and by gas chromatography mass spectrometry, in the case of CTL. Chemical analyses demonstrated that the gradients of each pesticide were maintained during the experiments (Table 1).

2.3. Avoidance tests

The avoidance tests were carried out using a multi-compartment system (Fig. 1) that enables the simulation of a gradient of pesticide concentrations where the fish can swim freely among the different concentrations. Control tests were run previously to verify whether the fish's distribution in the system was random. In this test, the compartments were filled (1000 mL in each compartment) with pesticide-free water (the same water used in the fish tank) then, five fish were put in each compartment. In the test with pesticides, the connections between the compartments were closed before the addition of each concentration of the pesticide, using plasticine plugs. Afterwards, each compartment was filled with the corresponding exposure solution in order to establish the gradients indicated in Table 1. Five fish were immediately introduced in each compartment, then, the plugs were removed. In both control and avoidance tests, three replicates were performed. The period of exposure was of 3 h and the distribution of the fish was recorded every 30 min. The exposure was carried out in the dark to avoid any visual interference on the behaviour of the animals. A red light was used during the observations of the organisms to reduce any possible interference caused by the observer.

2.4. Analysis of the effects of the mixtures

The effect of the mixtures, and analysis of the interaction between the substances in the binary mixtures, on the avoidance response of fish was estimated according to the additive concentration model used by DeLorenzo and Serrano (2003). Briefly, a sum of biological activity (S) was calculated:

$$S = (Am/Ai) + (Bm/Bi)$$

In the equation, $Am = AC_{50}$ of the compound A in mixture; $Ai = AC_{50}$ of the compound A individually; $Bm = AC_{50}$ of the compound B in mixture; $Bi = AC_{50}$ of the compound B individually. An additive index was then calculated as following:

If $S \leq 1.0$, additive index $= (1/S) - 1$.

If $S > 1.0$, additive index $= S(-1) + 1$.

Table 1
Ranges of nominal and quantified concentrations in μg/L for the three pesticides used in the avoidance assays. For each substance, the values reported for the assays with the compound alone and in mixture are the quantified initial and final concentrations for each compartment.

	Chlorpyrifos (CPF)				Chlorothalonil (CTL)				Glyphosate (Gly)					
	Single		Mix-Gly		Single		Mix-Gly		Single		Mix-CPF		Mix-CTL	
Nominal	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
10	9.05	7.09	9.63	7.09	8.93	6.33	8.28	3.40	7.03	7.83	10.45	8.83	10.20	6.02
1	0.95	0.77	0.98	0.75	1.07	0.62	0.95	0.49	0.83	0.80	1.03	0.84	1.25	0.78
0.5	0.43	0.40	0.51	0.39	0.47	0.27	0.49	0.28	0.33	0.28	0.54	0.43	0.51	0.31
0.1	0.08	0.08	0.10	0.07	0.10	0.07	0.10	0.07	0.12	0.10	0.11	0.09	0.11	< LOQ
0	< LOQ	< LOQ	0.012	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

LOQ (limit of quantification): < 10 ng/L for CPF, < 10 ng/L for CTL and 100 ng/L for Gly.

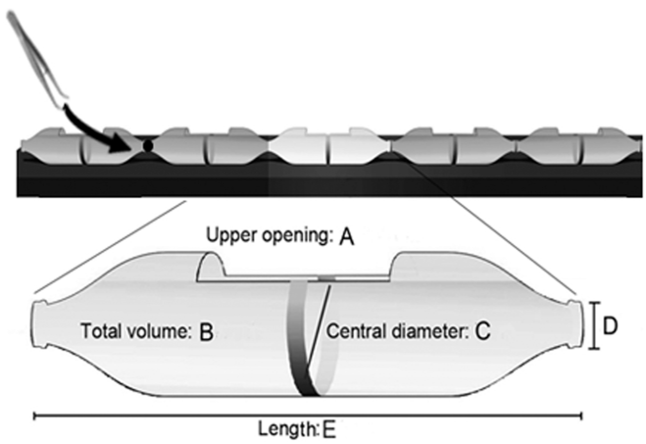

Fig. 1. Multi-compartmented exposure system used in the non-forced avoidance tests (total length: 245 cm; total volume: 5 L); A: 5 × 28 cm, B: 1 L, C: 16 cm, D: 2.2 cm, and E: 49 cm. Tweezers were used to introduce the plasticine plugs that initially blocked the connections between the adjacent compartments.

An antagonist interaction was inferred with an additive index less than zero, synergism was indicated by an additive index greater than zero and an additive index with confidence limits overlapping zero was an indication of an additive effect.

Particularly in the case where the AC50 value estimated was higher than the highest concentration used (e.g., X), although it is more correct to represent it as > X, we applied the extrapolated value to calculate the interactions, because it is closer to the real AC50 value.

2.5. Statistical analysis

A mixed-design ANOVA was used to check for differences in the distribution of fish throughout the compartments of the system in the avoidance tests. The data concerning the percentage of fish were separated as a function of time (within-subjects factor; repeated measures) and compartments (between-subjects factors). The sphericity of the repeated measures was evaluated using Mauchly's test and if the sphericity was violated (the variances of the differences were not equal) the Greenhouse-Geisser correction for degrees of freedom was applied. When statistically significant differences ($p < 0.05$) were observed for time or compartment, the Bonferroni test was used.

The formulas proposed by Moreira-Santos et al. (2008) were applied to calculate the avoidance percentage, more details of this calculation have been described previously by Silva et al. (2018). The concentrations of each pesticide that elicited avoidance in 50% [AC_{50}, ± confidence interval (CI)] of the exposed population was calculated using a Probit regression in R (R core team, 2020) with the Ecotox package (Hlina et al., 2021).

3. Results

3.1. Avoidance of individual substances

The three substances evaluated repelled *D. rerio* at environmentally relevant concentrations. However, the avoidance response differed among them and, in the case of glyphosate, the avoidance varied during the course of the experiment. For this reason, the results were analysed for the whole period of observation (3 h) as well as for the later period of exposure, after 100 min, so they could be compared more easily.

Fish exposed to chlorpyrifos showed a drastic displacement towards the uncontaminated compartment of the system, with a significant difference in the number of fish between this compartment and the lowest concentration (0.1 μg/L) of the insecticide. This significant avoidance of even the lowest concentration of chlorpyrifos tested was clear for the whole period of observation (Fig. 2, A). In fact, the confidence intervals of the AC_{50} calculated for the whole experiment overlapped with those from the period after 100 min (Fig. 2, B).

When the fish were exposed to a gradient of chlorothalonil, their distribution was affected by the fungicide and a significantly lower number of individuals were counted in concentrations higher than 1 μg/L (Fig. 2, C). As observed with chlorpyrifos, the avoidance was consistent during the whole period of the experiment, but the lowest AC_{50} values indicate that chlorothalonil was the most repellent compound (Fig. 2, D).

The fish exposed to the gradient of glyphosate showed two phases of response during the 3 h of observation: considering the whole period of the assessment, the fish tended (not significantly) to stay in the least contaminated extreme of the system; however, if the distribution of the organisms in the system is considered after 100 min of exposure, the number of fish present in the compartments with the two higher concentrations of the herbicide was significantly lower when compared to the cleaner compartments (Fig. 2, E). A graphical representation of the movement of fish throughout the three-hour exposure period in the open system is given in Supplementary Fig. 1 (Supplementary material). There, it is possible to see the early avoidance to CPF and CTL, and the delayed avoidance to Gly. Further, the avoidance was only evident during that second part (after 100 min) of the exposure period, while during the first 100 min, the fish tolerated higher concentrations of the herbicide (Fig. 2, F).

3.2. Avoidance of mixtures

Taking into consideration the references that state glyphosate is attractive for some fish species (Tierney et al., 2011) and the outcome of our previous experiment, we tested binary mixtures of glyphosate-chlorothalonil and glyphosate-chlorpyrifos to see if the avoidance responses would change. Interestingly, the distribution of the fish throughout the system was altered in the presence of glyphosate. The most evident change was observed in the mixture of glyphosate-chlorpyrifos. In this case, the fish were distributed more evenly along the compartment system, only showing a significant

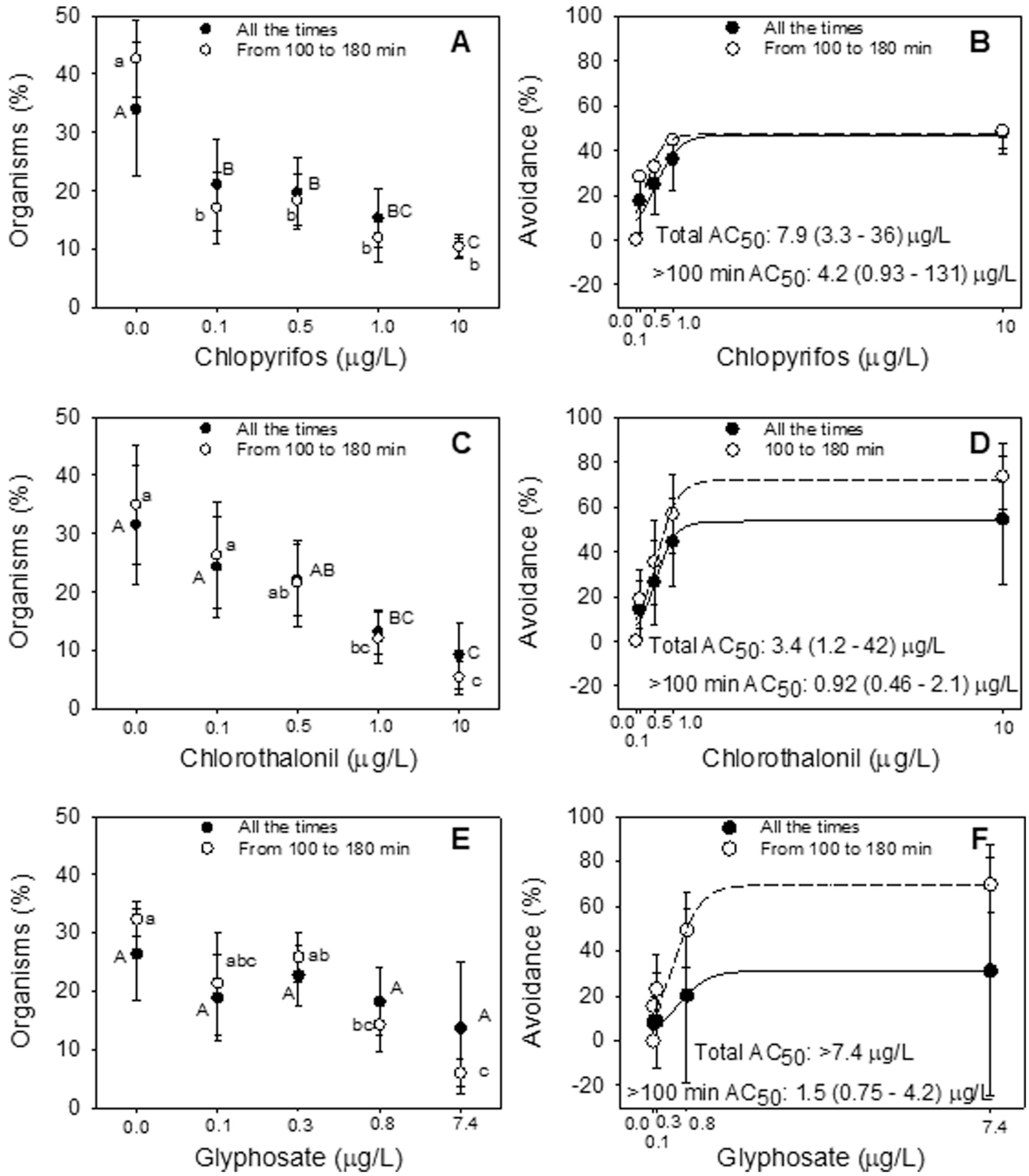

Fig. 2. Distribution of *D. rerio* along the open gradient system (left), and avoidance response (right) during the exposure to chlorpyrifos (A–B), chlorothalonil (C–D) and glyphosate (E–F). The experiment was analysed considering all the observations recorded in a period of 3 h (solid lines) and for the later part, after 100 min (dashed lines). Different letters indicate significant differences (Bonferroni test; $p < 0.05$) in the proportion of organisms in the compartments. Avoidance concentrations of 50% (AC_{50}) with 95% confidence intervals are included for the total time (30–180 min) of the experiment and for the period after 100 min.

reduction in the number of organisms in the compartment with the higher concentrations of the substances and resembling the behaviour observed when exposed only to glyphosate (Fig. 3, A).

With the exposure to the glyphosate-chlorothalonil mixture, the distribution of the organisms in the system, again, resembled the behaviour of the fish exposed to glyphosate alone more than the behaviour observed with chlorothalonil alone. In this case, if the whole period of observation is considered, the number of animals was only significantly lower in the compartment with the higher concentrations of the pesticides. If the experiment is observed past 100 min, a significantly higher number of fish preferred the cleanest compartment and a significantly lower number were present in the most contaminated (Fig. 3, D), in agreement with the behaviour observed in the fish exposed only to glyphosate.

The exposure to these mixtures suggests that the immediate repellence observed was due to chlorpyrifos and chlorothalonil, as the AC_{50} values calculated for these substances in the mixture experiments (Fig. 3, B and D) were similar to the ones observed for the individual exposure to them. On the other hand, the AC_{50} calculated for glyphosate during the whole experiment with the other substance present in the system was similar to the avoidance observed during the later phase of the exposure with glyphosate alone, when the avoidance was clearer. No change was observed in the AC_{50}s estimated for the substances in the experiments with the mixtures regarding the period of observation. For that reason, only the AC_{50}s for the whole experiment are presented.

Additionally, an interesting trend was observed in all the experiments where glyphosate was present. Consistently, a higher number of fish seemed to gather in the centre of the system, in the middle

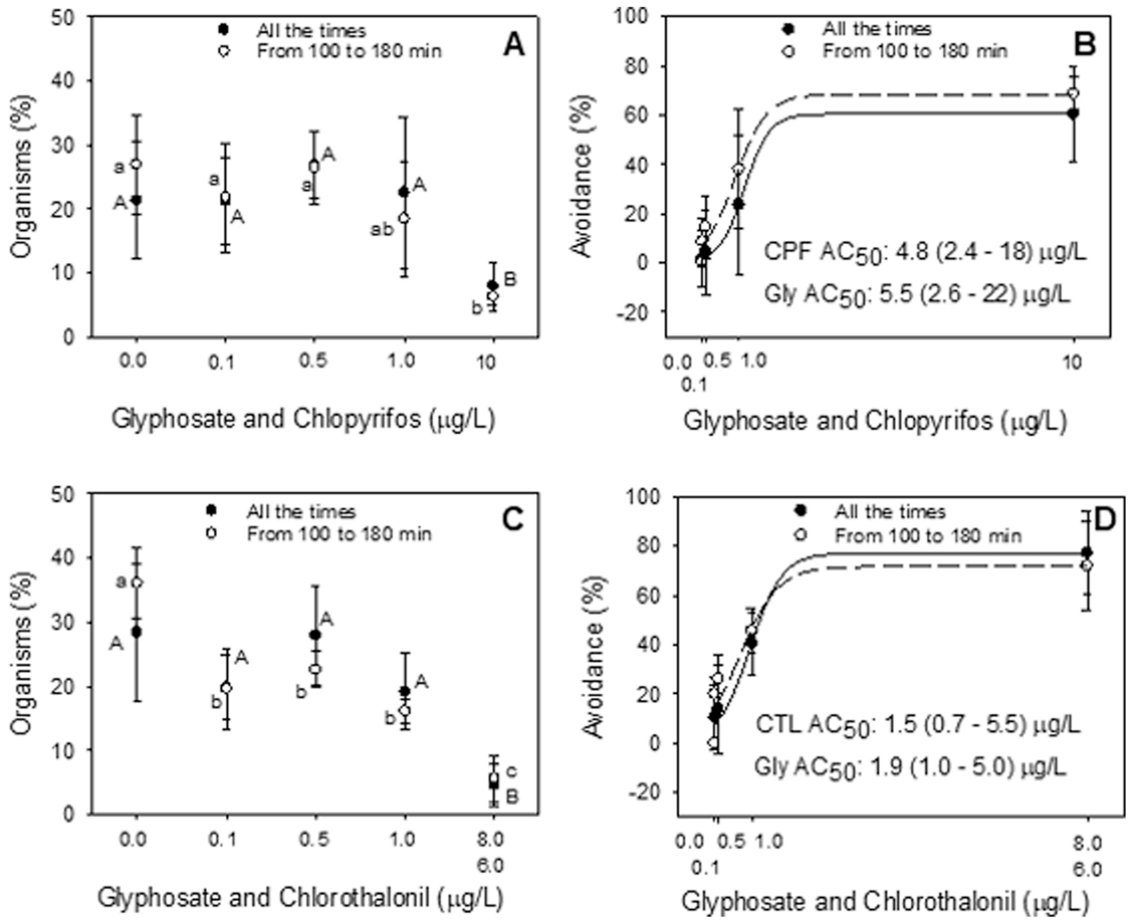

Fig. 3. Distribution of *D. rerio* throughout the open gradient system (left) and avoidance response (right) during the exposure to mixtures of glyphosate-chlorpyrifos (A–B) and glyphosate-chlorothalonil (C–D). The experiment was analysed considering all the observations recorded in a period of 3 h (solid lines) and for the later part, after 100 min (dashed lines). Different letters indicate significant differences in the number of organisms in the compartments. Avoidance concentrations of 50% (AC_{50}) with 95% confidence intervals are included for the total time of the experiment, for each substance in the mixture.

concentration of the contaminants, perhaps attracted to some concentrations. Such increased permanence in the middle concentration can be observed in Supplementary Fig. 2 (Supplementary material).

3.3. The effect of the mixtures on avoidance

The interactions in the mixtures of chlorpyrifos-glyphosate and chlorothalonil-glyphosate were evaluated (Table 2). According to the additive indexes calculated, if the whole period of the experiment is considered (3 h in total), the interactions were synergistic in both mixtures. If only the late period of the exposure (> 100 min until 180 min) is assessed, synergism was not observed in any cases. We should be cautious with the interpretation of these results as they are based on the estimated AC_{50}s and, at least in the later period of the experiment, the AC_{50}s for each substance, individually or in mixture, were very similar (with overlapping confidence intervals). So, we highlight the observation that no synergistic (instead of antagonistic; Table 2) interaction was evident for the mixtures during that later period, which corresponds to a period when the fish were clearly repelled by all the substances individually.

Table 2
Additive indexes and interactions calculated for the mixtures of chlorpyrifos-glyphosate and chlorothalonil-glyphosate on the avoidance response by *D. rerio*.

Mixture	Period	Additive Index	Synergism
CPF-Gly	Total	0.4 (0.08–1.0)	Yes
CPF-Gly	> 100 min	-1.6 (− 2.1 to − 1.6)	No
CTL-Gly	Total	1.1 (0.5–6.4)	Yes
CTL-Gly	> 100 min	-1.4 (− 1.7 to − 1.4)	No

4. Discussion

The assessment of the effects caused by pesticide mixtures on aquatic organisms is a field of much interest in ecotoxicology (Hernández et al., 2017). However, much of the effort in this respect has been dedicated to the interaction of substances and related toxicological outcomes derived from their modes of action, implying at least physiological alterations (Altenburger et al., 2015). Meanwhile, little has been done regarding the effects of mixtures, especially at environmentally relevant concentrations, on the behaviour of organisms (Rodney et al., 2013).

Behavioural alterations in fish have been assessed after exposure, individually, to the three pesticides that we tested. Several studies have been conducted with chlorpyrifos and, probably because this cholinesterase-inhibiting insecticide targets the nervous system, behavioural effects have been observed. In most cases, the studies have assessed behavioural responses after days of forced exposure to sublethal concentrations of the substance. In such conditions, altered locomotion and swimming patterns have been common for *Gambusia affinis* (Venkateswara Rao et al., 2005), *Cyprinus carpio* (Halappa and David, 2009) and *Channa Punctatus* (Stalin et al., 2019). The fish *Oryzias latipes* showed a contrasting behaviour as higher concentrations of chlorpyrifos caused hypoactivity, while a lower concentration (10% of LC_{50}) caused hyperactivity (Khalil et al., 2013). Regarding studies with a shorter duration or lower concentration of exposure, a short (2-h) exposure of *D. rerio* larvae to chlorpyrifos, at concentrations above 100 μg/L, caused an increase in locomotion (Keinle et al., 2009), while 96-h exposure of *Oncorhynchus kisutch* to environmentally relevant concentrations (below 2.5 μg/L) caused a reduction in swimming and feeding that correlated with significant cholinesterase inhibition (Sandahl et al., 2005). All these studies have contributed to the characterisation of the impact of chlorpyrifos on fish behaviour. Our results suggest that *D. rerio* is capable of an early recognition of the insecticide in the water that could spare it from suffering further physiological effects. However, some neurotoxic effects would be expected to affect the avoidance response as well, and this needs to be further assessed.

In the case of chlorothalonil, fewer behavioural studies have been carried out. Thus far, Teather et al. (2005) found that a concentration of 0.06 ug/L of the fungicide did not significantly affect the swimming of *O. latipes*. However, this compound is known to be an irritant and is highly toxic for fish (Lewis et al., 2016). Our results demonstrate that it is highly repellent as well.

Similar studies with glyphosate have demonstrated that concentrations in the range of mg/L can affect: the swimming and ventilation in surubim (Sinhorin et al., 2014), *D. rerio*'s position in the water column, a behaviour related to anxiety (da Costa Chaulet et al., 2019), or inhibit feeding in *Piaractus mesopotamicus* (Cardoso Giaquinto et al., 2017). Meanwhile, more environmentally relevant concentrations, in the range of μg/L, can reduce the locomotion of zebrafish, also affecting the natural aversive behaviour of larvae and the memory of adults (Bridi et al., 2017). Prolonged exposure of zebrafish to relevant concentrations of the herbicide affected social and exploratory behaviours with increased anxiety; accompanied by evidence of antioxidant activity in the brain and induction of the dopaminergic response (Faria et al., 2021). Although the toxicity of glyphosate to fish is considered moderate (Lewis et al., 2016), these behavioural responses indicate that significant changes can occur at sub-lethal concentrations. Regarding our results, it is interesting that a clear avoidance was only observed during the later period of exposure. This suggests that *D. rerio* could tolerate, at least for a short period, concentrations of the herbicide that have been related to behavioural and even physiological impairments after prolonged exposure. Nonetheless, it is not possible to entirely eliminate the hypothesis that this tolerance is caused by an attractive effect produced by glyphosate, as it has been observed in other vertebrates, like Japanese quails (*Coturnix japonica*) that preferentially fed on glyphosate-based herbicide-contaminated food (Ruuskanen et al., 2020).

Juveniles of *D. rerio* avoided concentrations of chlorpyrifos, chlorothalonil and glyphosate that are environmentally realistic for the surface water in tropical agricultural landscapes (de Souza et al., 2020; Sangchan et al., 2014; Ruiz-Toledo et al., 2014). This implies that the individual presence of any of these substances would be enough to repel fish, with the consequent effect on the distribution of populations and the possible loss of biodiversity in the contaminated areas (Araújo et al., 2020). Among the three pesticides, glyphosate was the substance that fish presented lower repellence to at higher concentrations. We observed that *D. rerio* stayed in the highest concentrations of the herbicide for a period of about one hour, showing an attraction towards an intermediate concentration (i.e. 1 μg/L) but, after this first period, the fish finally avoided the substance. This observation agrees with Rosa et al. (2016), who reported avoidance of glyphosate (5 mg/L) by adult *D. rerio* after 30 min of exposure. This preference of fish for some environmental contaminants has been observed. Tierney et al. (2011) reported the preference of zebrafish for three herbicides, including glyphosate. However, contrasting responses have been reported for different species. For example, Folmar et al. (1979) observed that rainbow trout did not avoid glyphosate, while Rosa et al. (2016), as mentioned above, observed that glyphosate was aversive to *D. rerio* and concluded that such repellence would benefit organisms as they would avoid a polluted environment. Regarding the attraction of fish to other compounds, Saglio et al. (2001) reported that goldfish were attracted by three pesticides (prochloraz, bentazone, and nicosulfuron) at concentrations in the range of mg/L. Recently, Jacob et al. (2021) observed that the pharmaceutical product diazepam exerted an attractive effect over the fish *C. carpio,* regardless of its lethal effect. Together, these studies suggest that factors like concentration and time of the exposure might modulate the response that different species display when exposed to different pesticides.

Regarding the avoidance response of fish exposed to mixtures, our aim was to assess whether the presence of glyphosate could change the usual avoidance response to another chemical. Our results indicate that the presence of glyphosate could, during a time, attenuate the avoidance response of *D. rerio* regarding the other two compounds, as shown by the change in the distribution of fish throughout the open system. This behaviour agrees with the experiment carried out by da Costa Chaulet et al. (2019), where the response of *D. rerio* to a mixture of glyphosate-fipronil resembled its response to the exposure to glyphosate alone. The mixture with glyphosate produced a curious response from 100 to 180 min: the additive index indicated that the interaction with CPF and CTL tended to be antagonist. This result should be assessed with extreme caution, because the differences in the AC_{50} values of individual chemicals in relation to the chemicals in the mixture are minimal. In fact, the confidence intervals overlap. Therefore, although the statistical calculation highlights an antagonist relationship, we prefer simply to indicate an absence of additivity or synergism. As avoidance of chemical contaminants in water is a response based on sensorial (mainly olfactory) information (Olsen, 2011), the interpretation of interactions must be made with great care. In our experiment, avoidance varied throughout the period of the experiment and this demonstrates the complexity of this response and that many physiological processes might condition the behaviour of avoiding or not a toxicant.

Chemical pollution is recognised as one of the relevant anthropogenic factors influencing the fragmentation of aquatic habitats (Fuller et al., 2015), with its consequent effect on the distribution of organisms (Araújo et al., 2019). As documented by Dominoni et al. (2020), chemicals can act as sensory pollutants, interfering with organisms' correct processing of environmental information. In our case, the cue for avoidance, represented by one pesticide, seems to have been masked by the presence of a less repellent or even attractive second pesticide. In this context, the interference of one substance with the avoidance response to another could create an ecological trap that leads the population into a maladaptive selection of habitat, with the potential cost to the fitness of the individuals and the resulting impact at the population level.

5. Conclusions

Juveniles of *D. rerio* are sensitive to three current use pesticides, showing a clear avoidance of environmentally relevant concentrations of them. The avoidance of glyphosate presented a particular behaviour with an early period during which fish showed some tolerance to the herbicide. In the context of a mixture of pollution, a component in the mixture may interfere with the behavioural response of avoidance and thereby favour exposure to other components.

CRediT authorship contribution statement

Freylan Mena: Conceptualization, Methodology, Investigation, Writing – original draft. **Adarli Romero**: Conceptualization, Validation, Supervision, Writing – original draft. **Julián Blasco**: Conceptualization, Validation, Resources, Supervision, Funding acquisition, Writing – original draft. **Cristiano V.M. Araújo**: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113172.

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