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Influence of interspecific interactions on avoidance response to contamination

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HIGHLIGHTS

- The avoidance response of guppy and zebrafish to a copper gradient was evaluated.
- Contamination gradients were simulated using a non-forced exposure system.
- The effects of interspecific interactions on avoidance response was studied.
- Guppy and zebrafish avoided potentially toxic copper concentrations.
- The magnitude of avoidance by zebrafish was affected by the presence of guppy

GRAPHICAL ABSTRACT
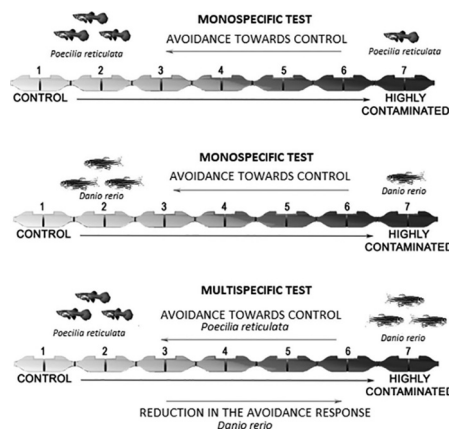
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ABSTRACT

An increasing number of studies have shown the ability of organisms to escape from toxic effects due to contamination, by moving spatially towards less contaminated habitats. However, this issue has been investigated in monospecific scenarios, without considering possible interactions between species during the contamination avoidance process. It is widely known that the spatial distribution of one species can be affected by another one, in different ways. Therefore, the main question addressed in the present study was as follows: Might interspecific interaction between the freshwater fish *Danio rerio* (zebrafish) and *Poecilia reticulata* (guppy) change their behavior patterns in terms of avoidance in the presence of a copper gradient? Zebrafish and guppies exposed to a copper gradient were tested for avoidance responses in a free-choice, non-forced, static, multi-compartmented exposure system, using two distinct approaches: (1) monospecific tests, in which only one species was exposed to the copper gradient, at two different population densities; and (2) multispecific tests, in which both species were tested simultaneously. In the control (with no copper) monospecific tests, both species were randomly distributed; however, in the control multispecific test, *P. reticulata* tended to aggregate. In the

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monospecific tests with a copper gradient, both species avoided copper in a similar way, with AC_{50} (concentration triggering avoidance in 50% of the exposed population) values between 15 and 18 $\mu g \cdot L^{-1}$, irrespective of the population density. However, in the multispecific tests, *P. reticulata* displaced *D. rerio* to previously avoided copper levels, consequently increasing the AC_{50} of *D. rerio* to 75 $\mu g \cdot L^{-1}$. This study shows the importance of understanding the interactions among species in contaminated areas, and the way that one species can prevent the avoidance behavior of another.

1. Introduction

The risks to organisms resulting from the exposure to contaminants are commonly related to damage caused to individuals, including lethal and sublethal effects (Martinez-Haro et al., 2015). Such damage is generally studied by means of ecotoxicological tests with forced exposure, involving the direct and continuous exposure of organisms to contaminants. Although less frequent, some earlier studies have indicated that organisms are able to detect and avoid contamination, thereby preventing them from suffering toxic effects (see review by Araújo et al., 2016). Evidence of avoidance to contamination was reported by Folmar (1976), who exposed the fish *Salmo gairdneri* (rainbow trout) to nine herbicides in a bi-compartmentalized system and observed how the fish preferred the clean region. Using a similar system, Gunn and Noakes (1986) evaluated the avoidance behavior of the fish *Salvelinius fontinalis* caused by low pH and addition of aluminum. In both studies, toxic effects at the individual level resulting from continuous exposure were prevented by the avoidance response to contamination.

Recently, a new approach using a free-choice, multicompartment, non-forced system was proposed for use in ecotoxicological studies to assess how the spatial distribution of organisms is driven by contaminants (Lopes et al., 2004). The aim of this nonforced exposure approach is to simulate contamination gradients or patches, so that the effects are no longer measured using individuals, but instead using their spatial distribution. This approach expands the concept of environmental disturbance beyond the traditional one based exclusively on the toxicity (and bioaccumulation) at the organism level. Evidence of contamination-driven spatial displacement (avoidance response) in non-forced exposure systems has been described for many organisms including fish (Moreira-Santos et al., 2008; Araújo et al., 2014, 2018; Silva et al., 2017, 2018), amphibians (Araújo et al., 2014; Vasconcelos et al., 2016), and invertebrate species (Lopes et al., 2004; Araújo et al., 2016) exposed to different contaminants.

Although evidence of avoidance responses to contamination has been widely reported for many fish species (see review by Araújo et al., 2016), it is recognized that habitat selection processes may be conditioned by several factors other than contamination, such as temperature (Stehfest et al., 2017), pH (Fost and Ferreri, 2015), presence of predators (Scherer and Smee, 2016) and competitors (Dunlop et al., 2006), among others. In the natural environment, organisms are simultaneously exposed to several factors, so it is necessary to understand the extent to which contamination influences the habitat selection process, and the ways that these factors affect the avoidance response. For instance, Scherer and Mcnicol (1998) observed that *Coregonus clupeaformis* avoided exposure to Cu, Zn, and Pb, but that the avoidance response was suppressed when the environment was shaded. Dunlop et al. (2006) found that the escape responses of *Carassius auratus* and *Oncorhynchus mykiss* to a harmful acute stimulus (electric shock) changed if the two species were present together. Araújo et al. (2016) observed that the habitat selection by tilapia (*Oreochromis* sp.) exposed to a contamination gradient in a non-forced exposure system was changed when food was offered, with the organisms moving to previously avoided areas in order to feed. These results indicate that while fish can avoid toxic effects by moving to less contaminated habitats, the presence of a more attractive factor (or one of immediate importance) in the potentially avoidable habitat can change the avoidance pattern.

Interspecific interaction might also affect the spatial distribution of organisms due to competition for food, space, or other factors intrinsic to each population (Begon et al., 2007). Studies have clearly demonstrated that the structure and functioning of communities can be greatly altered when different populations cohabit. For instance, the presence of *Salmo gairdneri* (rainbow trout) in a stream flume influenced the individual fitness of *Luxilus coccogenis* (warpaint shiner), in terms of prey capture success and feeding efficiency (Elkins and Grossman, 2014). The presence of the fish *Carassius auratus* forced the amphibian *Ichthyosaura alpestris* to remain longer in the terrestrial environment (Laurane et al., 2017). In a study performed in the North-Western Mediterranean Gulf, despite the overlap of three small pelagic fish (*Sardina pilchardus*, *Engraulis encrasicolus*, and *Sprattus sprattus*), the biomass of each species differed locally due to the influence of one over the others (Saraux et al., 2014).

In the presence of contaminants, changes in the patterns of interaction (such as competition and aggressiveness) among fish populations (Lingaraja et al., 1979; Clotfelter and Rodriguez, 2006) are expected to occur. Therefore, considering that (i) fish can avoid contamination by moving spatially towards less contaminated habitats, and (ii) contamination can affect the behavior of fish in terms of aggressiveness, competition, and spatial displacement, the main question addressed in the present study was: Might interspecific interaction between the freshwater fish *Danio rerio* (zebrafish) and *Poecilia reticulata* (guppy) change their patterns of avoidance to contamination? The goal of the present study was to assess if the avoidance responses of these two fish populations exposed to a contamination gradient might be affected by interspecific interactions. It was expected that the less aggressive species would be displaced towards previously avoided contaminated habitats. Copper (Cu^{2+}) was selected as a test contaminant as it is an avoidable pollutant for fish (Moreira-Santos et al., 2008; Araújo et al., 2015) and produces toxic effects such as chemosensory deprivation (McIntyre et al., 2008), changes in gene expression (Craig et al., 2010), feeding inhibition (Abdel-Moneim et al., 2015), effects on the swimming performance of larvae (Acosta et al., 2016), morphological and metabolic alterations (Chatterjee et al., 2016), and changes in the surfaces of the gills (Fu et al., 2016). *P. reticulata* and *D. rerio* were selected as test organisms because they (i) are easy to cultivate, (ii) are widely used as model organisms in the fields of ecology, evolution, and ethology, and (iii) have already been shown to spatially avoid chemical contamination (Moreira-Santos et al., 2008; Araújo et al., 2014, 2014; Silva et al., 2017).

2. Materials and methods

2.1. Test organisms

Individuals of the fish *P. reticulata* and *D. rerio* (2 to 3 months old and with total length between 1.0 and 1.5 cm) were obtained from the São Paulo Agency of Agribusiness and Technology (Pindamonhangaba, Brazil) and the company Power Fish (Rio de Janeiro, Brazil), respectively. The use of the fish was approved by the Ethics Committee on the Use of Animals (Institute of Biosciences, University of São Paulo; protocol #236/2015). The organisms were acclimated under laboratory conditions (at the Ecotoxicology Laboratory of the Lorena School of

Engineering (EEL-USP), Department of Biotechnology (DEBIQ), Lorena, São Paulo, Brazil) for at least one week prior to the tests, following OECD guidelines (OECD, 2000). No mortality was observed during the culture period. Fish were kept in 60 L aquaria filled with activated charcoal-filtered well water. The cultures were constantly aerated using an air diffuser and the water was renewed by 30% every week. The animals were fed daily, ad libitum, with commercial fish food flakes (Tetramin®), until 24 h prior to the experiments. The pH (7.0 ± 0.5), conductivity (120 ± 20 μS·cm^{-1}), and dissolved oxygen content (6.4 ± 0.4 mg·L^{-1}) of the culture water were monitored on a daily basis. Natural light was provided in the room, without direct exposure to the sun (natural photoperiod of around 12:12 h light:dark), and the mean temperature was 23 ± 2 °C. Before the avoidance experiments, some organisms were selected and tested for the sensitivity of the fish population by performing toxicity tests using potassium dichromate ($K_2Cr_2O_7$) as a reference substance, at concentrations ranging from 10 to 300 mg·L^{-1}, following the OECD guidelines (OECD, 2000). The LC_{50} values (mean ± standard deviation; n = 3) were 41.66 ± 14.17 mg·L^{-1} (*P. reticulata*) and 127.3 ± 19.53 mg·L^{-1} (*D. rerio*), which were considered acceptable since they were within the upper and lower limits of the control charts for these species, provided by the Ecotoxicology Laboratory of the Lorena School of Engineering (21.0 to 63.8 mg·L^{-1} and 75.8 to 167.0 mg·L^{-1}, respectively).

2.2. Contaminant solution preparation and chemical analysis

The stock copper solution used in the avoidance tests was prepared by dissolving metallic copper powder (99.99%, VETEC/Sigma) in sulfuric acid (H_2SO_4) (95–99%, VETEC/Sigma). All the concentrations employed were prepared with ultrapure water (resistivity 18.2 MΩ cm). Controls were prepared with sulfuric acid, in the same proportions as the copper treatments. The copper concentrations were checked at the end of all the tests, which were performed in triplicate (n = 3). The samples retrieved after the tests were stored at 4 °C, in the dark, for a maximum of 1 week. For transport to the analysis laboratory, the samples were cooled in thermally insulated packages. The copper concentrations were determined at the Chemical Analysis Laboratory of Federal Fluminense University (UFF), in Volta Redonda, Rio de Janeiro, using a Varian 55B flame atomic absorption spectrometer. The instrument was operated with an air/acetylene flame, at 324.8 nm, with the flame, wavelength, and sample aspiration rate adjusted according to the manufacturer's recommendations. The procedures used for the standard curve and the analyses followed the protocol of the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). A stock copper standard solution (100 mg·L^{-1}) was diluted to obtain the concentration range from 0 to 1000 μg·L^{-1}. The linear equation of the calibration curve was y = 9.5×10^{-5}x − 6.4×10^{-4} (r^2 = 0.999).

2.3. Non-forced exposure system

Non-forced avoidance tests were performed in a free-choice static exposure system composed of seven interconnected chambers (Fig. 1; Araújo et al., 2014) constructed from borosilicate glass bottles, handcrafted in a high temperature furnace at 600 °C. The chambers were connected using sections of nontoxic transparent silicone hose. The system had a total length of 105 cm and a total volume of 980 mL, with each compartment having a volume of 140 mL. For the tests, the chambers were filled with 125 mL of water containing copper at different concentrations, so that the total system volume was 875 mL.

2.4. Avoidance tests

Two distinct approaches were adopted in the avoidance tests: (1) monospecific tests, in which only one species was introduced into the system, at two different population densities (one or two individuals per chamber), and (2) multispecific tests, in which both species
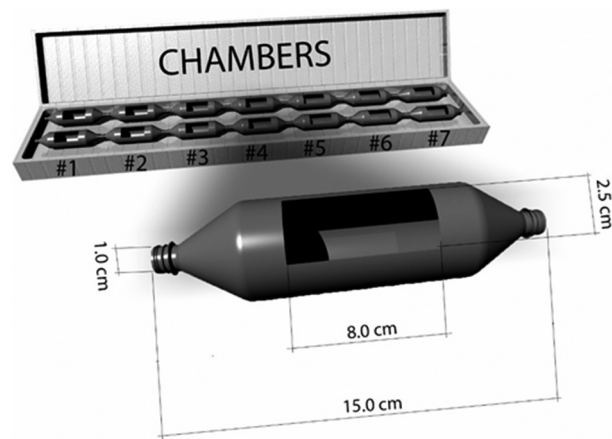

Fig. 1. Schematic diagram of the static multi-compartmented non-forced avoidance test system, highlighting one of the seven compartments.

were tested simultaneously in the system. The two different densities were employed in order to ensure that the copper avoidance response would not be affected by the population density.

Firstly, control distribution tests in which the system was filled with 875 mL (125 mL * seven chambers) of culture water (with no contamination) were conducted to confirm that the fish did not have preference for any of the seven chambers. In the monospecific approach, 14 fish were used per system (2 fish in each chamber), while in the multispecific approach, 14 fish were used in total per system, with 7 organisms of each species being introduced into the same system (one fish of each species per chamber).

After checking the spatial distribution of the fish in the absence of contamination, avoidance tests were carried out with a copper gradient. The monospecific tests were performed with 7 and 14 fish per system, while in the multispecific approach, 14 fish (7 fish of each species per system) were employed. In each system, the same copper gradient was prepared from a 100,000 μg·L^{-1} stock solution, with nominal concentrations in the chambers of 0, 10, 20, 30, 40, 55, and 70 μg·L^{-1}, hence forming a copper gradient. Prior to introducing the solutions into the chambers, plasticine plugs wrapped in PVC were inserted between adjacent chambers, using a clamp, hence blocking the connections between them and preventing mixing of the concentrations. After filling the system with the different concentration solutions, the fish were introduced and the plugs were removed.

All the tests (control with no gradient and avoidance with copper gradient) were performed in a dark room, at 23 ± 2 °C. Observations were made every 30 min during 3 h exposure (at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 h), using a red light to prevent interference due to the presence of the observer. The organisms were not fed during the tests. All the tests were performed in triplicate. At the end of the tests, plugs were inserted between adjacent chambers to close the connections, and samples were then taken for determination of the final copper concentrations.

2.5. Statistical analyses

In the non-forced approach, a mixed-design ANOVA was employed to evaluate the distributions (in %, arcsine transformed) of the fish in the control (using only culture water) and avoidance (with copper gradient) monospecific and multispecific tests. In this analysis, time was treated as a within-subjects factor (repeated measurements), while chamber was treated as a between-subjects factor. The sphericity of the repeated measurements was evaluated using Mauchly's test; in the case of noncompliance (with the variances of the differences not being equal: p < 0.05), the Greenhouse-Geisser correction for degrees of freedom was applied. When statistically significant differences (p <

0.05) were observed for time or compartment, pairwise comparisons were performed using the Bonferroni post-hoc test. In the case of the observation times, if the results of the repeated measurements were not significant (p > 0.05), the fish distribution data to be represented in the graphs were pooled, considering the means from the six times (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 h).

In the avoidance tests with a copper gradient, calculation of the avoidance response (in %) was based on the method described by Moreira-Santos et al. (2008). Firstly, the number of avoiders was determined using the following equation: $Avoiders = N_E - N_O$, where N_E is the number of expected organisms and N_O is the number of observed organisms. For the compartment with the highest copper concentration (the seventh chamber), N_E was equal to the number of fish introduced into the chamber at the beginning of the test, since it was not expected that the organisms would move from the lower concentrations to that chamber. For the remaining chambers, N_E included the organisms initially introduced into the chamber, plus the organisms introduced into the adjacent chamber with higher concentration. For instance, if two fish were introduced at the beginning of the experiment, N_E for the sixth chamber was equal to 4, because it considered the two organisms introduced into the sixth chamber and the two organisms introduced into the seventh chamber (these two fish were expected to move to lower concentrations). The calculation of N_O considered the organisms recorded in a given concentration and those recorded in higher concentrations. For instance, for the seventh chamber, N_O represented the organisms found in that chamber, while for the sixth chamber, N_O represented the organisms recorded in both the sixth and the seventh chambers. This reasoning was based on the fact that organisms able to inhabit concentrations higher (e.g., the seventh chamber) than that to be studied (e.g., the sixth chamber) were not considered avoiders. As the control chamber contained only culture water, avoidance was not expected ($N_E - N_O = 0$). Finally, the avoidance percentage for each chamber was calculated as follows: $(Avoiders/N_E) * 100$. From the avoidance percentages, the AC_{20}, AC_{50}, and AC_{80} values (the copper concentrations causing avoidance in 20, 50, and 80% of the exposed population, respectively) and their corresponding confidence intervals (CI) were calculated using PriProbit software (Sakuma, 1998).

3. Results

3.1. Control of fish population distributions

The distributions of fish in the control tests (using culture water) were analyzed for the monospecific (14 fish per system) and multispecific (7 fish of each species, totaling 14 fish per system) experiments, in order to determine whether the distributions occurred in a random fashion (Fig. 2). In the monospecific tests with *P. reticulata*,

the fish distribution was random (see details in Tables S1, S2a, S2b, and S2c of the Supplementary material), with no statistically significant differences in the percentages of organisms among the chambers ($F_{6, 14} = 0.355$, p = 0.895). The repeated measurements indicated that the movement of the organisms in the system did not vary with time ($F_{2.857, 40.005} = 0.062$ after Greenhouse-Geisser correction, p = 0.976). The interaction between the two factors (time and chamber) was not statistically significant ($F_{17.145, 40.005} = 1.069$ after Greenhouse-Geisser correction, p = 0.404). The same random distribution pattern was observed for *D. rerio* in the control test (time: $F_{5, 70} = 0.129$, p = 0.985; time and chambers: $F_{30, 70} = 1.018$, p = 0.461; chambers: $F_{6, 14} = 0.312$, p = 0.920; see details in Tables S3, S4a, S4b, and S4c of the Supplementary material).

In the multispecific tests (see details in Table S5 of the Supplementary material), the data were treated considering the species individually (7 fish) and in combination as a community with two populations (14 fish). In the case of *P. reticulata*, the fish distribution was not totally random, with statistically significant differences in the percentages of organisms among the chambers ($F_{6, 14} = 3.320$, p = 0.030), particularly between chamber 1 and chamber 7 (Bonferroni test). The repeated measurements indicated that the movement of organisms in the system did not vary with time ($F_{5, 70} = 0.038$, p = 0.999). The interaction between the two factors (time and chamber) was not statistically significant ($F_{30, 70} = 1.091$, p = 0.373) (see details in Tables S6a, S6b, and S6c of the Supplementary material). For *D. rerio*, the fish distribution was random and the repeated measurements indicated that the movement of the organisms in the system did not vary with time or according to the two factors (time and chamber) (chambers: $F_{6, 14} = 0.667$, p = 0.678; time: $F_{3.220, 45.077} = 0.055$ after Greenhouse-Geisser correction, p = 0.986; time and chambers: $F_{19.319, 45.077} = 1.401$ after Greenhouse-Geisser correction, p = 0.174; see details in Tables S7a, S7b, and S7c of the Supplementary material).

Considering the distribution of both species together (14 fish per system), there was no statistically significant difference among the chambers ($F_{6, 14} = 1.731$, p = 0.186) and the repeated measurements indicated that the movement of the organisms in the system did not vary with time or considering the interactions of both factors (time and chamber) (time: $F_{5, 70} = 0.050$, p = 0.998; time and chambers: $F_{30, 70} = 1.126$, p = 0.335; see details in Tables S8a, S8b, and S8c of the Supplementary material).

3.2. Avoidance response to copper using the monospecific approach

Two distinct scenarios (with 7 and 14 fish per system) were analyzed in the monospecific tests with a copper contamination gradient, in order to evaluate the effect of the population density on the distribution of the organisms. For *P. reticulata* (Tables S9, S10a, S10b, and S10c of
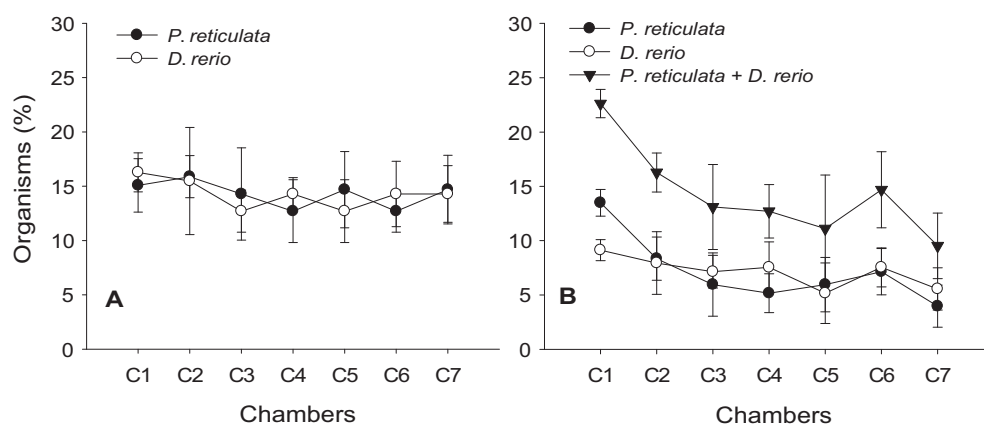

Fig. 2. (A) Distributions (in %) of *Poecilia reticulata* and *Danio rerio* in the control monospecific tests (without contamination). (B) Distributions (in %) of *P. reticulata* and *D. rerio* in the control multispecific tests (without contamination). The lines show the mean distributions of the fish species individually and in combination.
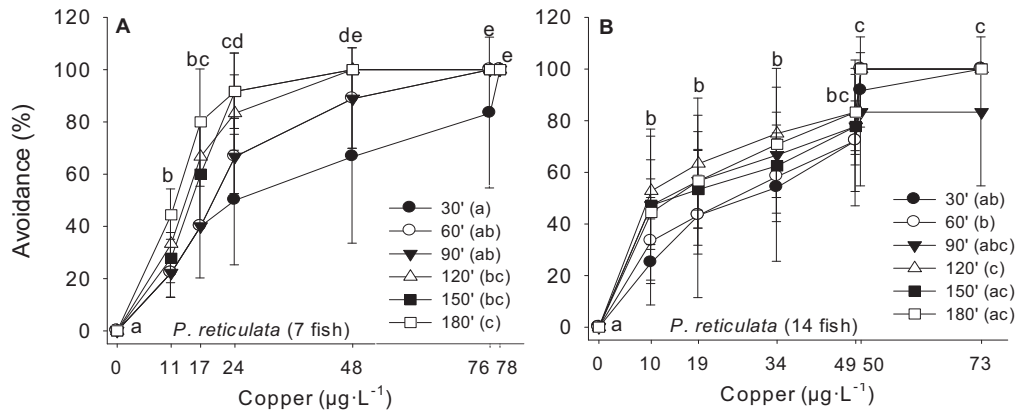
Fig. 3. Avoidance (in %) of *P. reticulata* in the monospecific test with a copper gradient, using (A) 7 fish and (B) 14 fish. The lines show the mean distributions of the fish at different times. Different letters associated with the copper concentrations indicate statistically significant differences, considering the mean from the six observation times. Statistically significant differences among the times are shown by different letters in the legends.

the Supplementary material), in the first scenario (7 fish), the distribution of the organisms was influenced by the different copper concentrations ($F_{6, 14} = 92.129$, $p < 0.001$) and time ($F_{2.537, 35.520} = 10.313$ after Greenhouse-Geisser correction, $p < 0.001$) (Fig. 3-A). However, for the interaction of the two fixed factors (time and chamber), there was no statistically significant effect ($F_{15.223, 35.520} = 1.572$ after Greenhouse-Geisser correction, $p = 0.131$). The avoidance means and standard deviations (in %, considering the six observation times) for each concentration (0, 11, 17, 24, 48, 76, and 78 µg·L^{-1}) were 0.0, 29.0, 54.0, 75.0, 91.0, 97.0, and 100.0, respectively. In the second scenario (14 fish), only the concentrations influenced the distribution ($F_{6, 14} = 30.046$, $p < 0.001$). For time and the interaction of the two factors (time and chamber) there were no statistically significant effects (time: $F_{2.160, 30.241} = 3.230$ after Greenhouse-Geisser correction, $p = 0.050$; time and chamber: $F_{12.961, 30.241} = 0.839$ after Greenhouse-Geisser correction, $p = 0.618$) (Fig. 3-B; Tables S11, S12a, S12b, and S12c of the Supplementary material). Irrespective of the population density, the organisms tended to avoid the contaminated chambers, moving towards the control chamber. The avoidance means and standard deviations (in %, considering the six observation times) for each effective copper concentration (0, 10, 19, 34, 49, 50, and 73 µg·L^{-1}) were 0.0, 41.67, 52.78, 64.58, 77.78, 95.83, and 97.22, respectively.

For the tests with *D. rerio*, in both scenarios (with 7 and 14 fish per system) the distributions were determined by the concentration gradient, with the organisms tending to migrate to less contaminated areas (Figs. 4-A and B; Supplementary material Tables S13, S14a, S14b, and

S14c (7 fish), and S15, S16a, S16b, and S16c (14 fish)). *D. rerio* showed similar gradient-dependent distribution patterns in both scenarios, with the concentration and time (for 7 fish only) being statistically significant factors (for 7 fish: chambers: $F_{6, 14} = 510.238$, $p < 0.001$; time: $F_{2.041, 28.574} = 5.494$ after Greenhouse-Geisser correction, $p < 0.05$; time and chambers: $F_{12.246, 28.574} = 1.073$ after Greenhouse-Geisser correction, $p = 0.417$; for 14 fish: chambers: $F_{6, 14} = 1957.549$, $p < 0.001$; time: $F_{2.082, 29.150} = 3.034$ after Greenhouse-Geisser correction, $p = 0.06$; time and chambers: $F_{12.493, 29.150} = 0.456$ after Greenhouse-Geisser correction, $p = 0.928$).

The avoidance mean and standard deviation values (in %, considering the six observation times) for each effective copper concentration (0, 10, 18, 21, 27, 50, and 67 µg·L^{-1}) were 0.0, 22.22, 43.33, 65.28, 87.04, 97.22, and 100.0, respectively (7 fish), and (for effective copper concentrations of 0, 11, 14, 21, 31, 43, and 64 µg·L^{-1}) 0.0, 22.69, 36.11, 50.69, 67.59, 98.61, and 100.0, respectively (14 fish).

3.3. Avoidance response to copper using the multispecific approach

The data from the multispecific tests were treated according to species and for the community, merging the data for both species (Tables S17 to S20, Supplementary material). In all the scenarios and for both species, only the concentrations/chambers presented statistically significant effects in terms of the fish distributions (*P. reticulata* (7 fish): chambers: $F_{6, 14} = 19.129$, $p < 0.0001$; time: $F_{1.819, 25.463} = 1.533$ after Greenhouse-Geisser correction, $p = 0.235$; time and
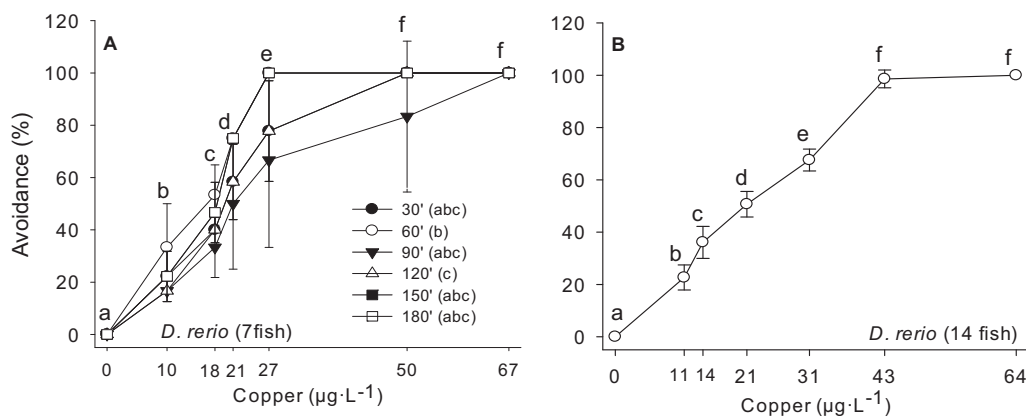

Fig. 4. Avoidance (in %) of *Danio rerio* in the monospecific tests with (A) 7 (with values for the six observation times) and (B) 14 (with mean values for the six observation times) fish exposed to a copper gradient. Different letters above the lines indicate statistically significant differences, considering the means from the six observation times. Statistically significant differences among the times are shown by different letters in the legend.
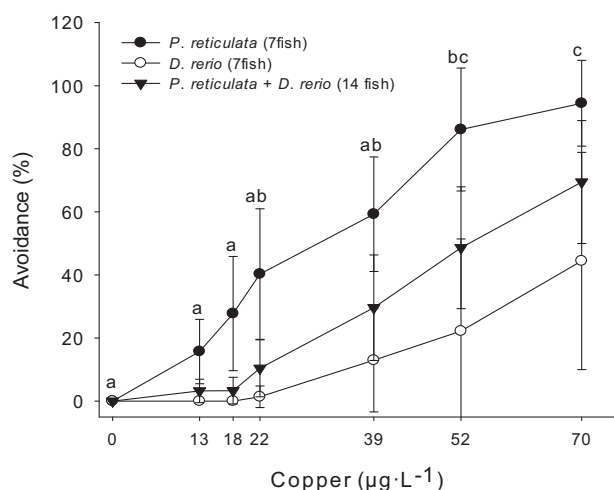
Fig. 5. Mean avoidance values (in %) from the six observation times for *Poecilia reticulata* and *Danio rerio*, individually and in combination, in the multispecific test with 14 fish exposed to a copper gradient. Different letters indicate statistically significant differences (*P. reticulata* + *D. rerio*).

chambers: $F_{10.913, 25.463} = 0.718$ after Greenhouse-Geisser correction, p = 0.710. *D. rerio* (7 fish): chambers: $F_{6, 14} = 3.533$, p < 0.05 (in this case, although ANOVA detected differences, they were not discriminated by the post-hoc test); time: $F_{2.680, 37.522} = 1.965$ after Greenhouse-Geisser correction, p = 0.142; time and chambers: $F_{16.081, 37.552} = 1.023$ after Greenhouse-Geisser correction, p = 0.456. *P. reticulata* and *D. rerio* (14 fish): chambers: $F_{6, 14} = 11.435$, p < 0.0001; time: $F_{5, 70} = 2.084$, p = 0.078; time and chambers: $F_{30, 70} = 1.048$, p = 0.423).

For *P. reticulata*, the avoidance mean and standard deviation values (in %, considering the six observation times) for each effective copper concentration (0, 13, 18, 22, 39, 52, and 70 $\mu g \cdot L^{-1}$) were 0.0, 15.74, 27.78, 40.28, 59.26, 86.11, and 94.44, respectively. For *D. rerio*, the values were 0.0, 0.0, 0.0, 1.39, 12.96, 22.22, and 44.44, respectively. For the combined population (*P. reticulata* + *D. rerio*), the values were 0.0, 3.24, 3.33, 10.42, 29.63, 48.61, and 69.44, respectively (Fig. 5).

3.4. Effective concentrations: AC_{20}, AC_{50}, and AC_{80}

Table 1 shows the copper concentrations that caused avoidance in 20, 50, and 80% of the fish populations (AC_{20}, AC_{50}, and AC_{80}) in the monospecific and multispecific tests, together with the corresponding confidence intervals. For both species, the avoidance responses (AC_x values) for the different population densities (7 or 14 fish) were very similar. In the case of the multispecific tests, the most evident feature was the much lower avoidance of *D. rerio* exposed to copper, with decreases in avoidance percentages of 4.27 and 4.06 times, comparing the AC_{20} (14 fish) and AC_{50} (14 fish) values of the monospecific test with the AC_{20} and AC_{50} values of the multispecific test, respectively.

4. Discussion

4.1. Control distribution in the absence of a contamination gradient

In the monospecific tests in the absence of contamination, the distribution of the organisms within the system was random, with no preference for any compartment. This confirmed that there were no external interferences (such as light or noise) or interactions among the organisms affecting the displacement of the organisms in the system. In other studies using the non-forced exposure system, similar behavior was observed for the fish species *D. rerio* (Moreira-Santos et al., 2008), *Rachycentron canadum* (Araújo et al., 2015), *Oreochromis* sp. (Araújo et al., 2016), and *P. reticulata* (Silva et al., 2017, 2018).

In the multispecific tests without a contamination gradient, *P. reticulata* showed a tendency to aggregate, which was not observed for *D. rerio*. Since this tendency was not observed in the monospecific tests, the response of *P. reticulata* was probably the result of interspecific interaction. This type of interaction has been reported previously. For example, in a study conducted along a stretch of the Au Sable River (Michigan, USA) with two species of trout, brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*), the brown trout were forced to move to other resting areas when the brook trout were present, due to competition for space (Fausch and White, 1981). According to Chapman (1966), fish have minimum spatial requirements that are related to the size of the individual fish, visual isolation, and maintenance of distance, which are mediated by psychological and physiological factors associated with food. Stream fish seem to exhibit reduced aggression and greater tolerance of contemporaries at closer range, if food is temporarily superabundant. It is therefore likely that the gregarious behavior of *P. reticulata* could be explained by competition for space.

Several studies have identified aggregation/exploitation behavioral patterns and shoal formation in populations of *P. reticulata* and *D. rerio*. Seghers and Magurran (1995) showed that changes in behavior can be attributed to variation in the predation regime or the presence of another species. For example, guppies that co-occur with the pike cichlid *Crenicichla alta* spend more time shoaling and form larger shoals than their counterparts from low-risk habitats (without other species). Chapman et al. (2008) found that guppy populations vary markedly in their tendency to form shoals, with an important factor being individual experience during early ontogeny, which can lead to the formation of larger or smaller shoals in the adult phase. The scientific literature recognizes two main aggregation patterns: shoals (aggregations of individuals that remain close to each other) and schools (aggregations of aligned, or polarized, individuals). *D. rerio* tends to form small shoals (Gimeno et al., 2016). Buske and Gerlai (2011) analyzed the age-dependent changes of shoaling in zebrafish and found a significant increase of shoaling with age (with decreased distance between shoal members). Under some conditions, both species studied here can form shoals. This was observed for *P. reticulata* in the multispecific control assay, but not in the tests using the fish species separately, when the individuals were randomly distributed.

Table 1

AC_{20}, AC_{50}, and AC_{80} values (in $\mu g \cdot L^{-1}$, concentrations triggering avoidance in 20, 50, and 80% of the exposed population) for *Poecilia reticulata* and *Danio rerio* in the monospecific (Mono.) and multispecific (Multi.) tests.

Test	Species	Density[a]	AC_{20}	AC_{50}	AC_{80}
Mono.	*P. reticulata*	7	8.5 (6.6–10.3)	15.9 (13.7–18.0)	29.6 (26.1–34.3)
		14	6.1 (0.70–11.6)	15.5 (5.8–23.1)	39.1 (26.6–81.9)
	D. rerio	7	10.6 (7.7–12.7)	16.7 (14.2–19.1)	26.6 (23.0–32.6)
		14	11.2 (7.0–14.4)	18.7 (14.5–22.9)	31.0 (25.0–44.8)
Multi.	*P. reticulata*	7	15.2 (11.9–17.9)	27.1 (23.7–30.9)	48.4 (41.3–60.4)
	D. rerio	7	48.0 (45.4–50.6)	75.9 (70.6–83.2)	119.9 (105.9–141.4)
	Both populations	14	30.2 (27.2–32.9)	52.6 (48.1–58.3)	91.6 (79.6–110.2)

[a] Number of individuals of a species in the system.

4.2. Avoidance response to a copper gradient: monospecific tests

The avoidance responses for the same species at different densities (7 or 14 fish) were very similar. Therefore, the densities that were tested did not seem to influence the avoidance behavior. Comparison of the responses of the two fish species in the monospecific tests showed that *P. reticulata* was more sensitive than *D. rerio*. The greatest difference between the two species occurred using 10 μg·L^{-1} (*P. reticulata*) and 11 μg·L^{-1} (*D. rerio*) of copper and 14 organisms, where the mean guppy avoidance percentage during the test (41.67%) was almost 2-fold higher than the value obtained for the zebrafish (22.69%). This difference became less pronounced as the copper concentrations increased. Similar avoidance patterns have been reported in other studies with guppy and zebrafish exposed to different contaminants. Araújo et al. (2014) exposed zebrafish to a fungicide (pyrimethanil) gradient, while Silva et al. (2017, 2018) analyzed the escape response of *P. reticulata* exposed to the compounds triclosan and bisphenol. In those cases, the organisms were able to detect the compounds present at environmentally relevant concentrations and moved to less contaminated areas. The zebrafish were more susceptible to copper and were capable of identifying the contamination risk, moving to less contaminated regions in order to reduce the exposure to high concentrations and minimize potential toxic effects. Moreira-Santos et al. (2008) exposed *D. rerio* to copper in monospecific tests and found dose/avoidance responses very similar to those observed here, with AC$_{50}$ values ranging from 16 to 22 μg·L^{-1}, compared to AC$_{50}$ of ~16 μg·L^{-1} obtained in the present work.

From comparison of the avoidance responses to copper observed in the present work and the sublethal effects described elsewhere, it could be concluded that avoidance tends to occur in a short time and at environmentally relevant concentrations. Drummond et al. (1973) found that changes in the locomotor activity and feeding behavior of *Salvelinus fontinalis* appeared after 24 h exposure to copper at 15 μg·L^{-1}. Vieira et al. (2009) reported that *Pomatoschistus microps* exposed to copper at 50 μg·L^{-1} for 96 h suffered disruption of cholinergic function (due to AChE inhibition), decreased detoxification capability (due to EROD inhibition), additional energetic demands to face chemical stress, and oxidative stress and damage, which decreased the swimming performance of the fish. In another study, exposure of *Pimephales promelas* to copper at 45 μg·L^{-1} for 21 days caused reductions in the number and size of eggs (Driessnack et al., 2017).

4.3. Avoidance response to a copper gradient: interspecific interactions

The interaction of the species caused different patterns of avoidance response in the multispecific copper gradient test. In the absence of *P. reticulata*, the avoidance response of *D. rerio* was more intense than when both species were present together in the system. The guppies clearly influenced the dispersion pattern of the zebrafish, which moved towards previously avoided copper concentrations. This could have been related to an effect of copper on the aggression/contention behavior of *D. rerio*, which decreased in the presence of *P. reticulata*. It is also possible that *P. reticulata* displaced *D. rerio* to a more contaminated zone because the former is naturally more aggressive. Other studies have highlighted the effects of contaminants on the behavior of fish. When the fish *Gasterosteus aculeatus* was exposed to the hormone estradiol, control males increased their aggressive response to a live male conspecific over time, while males exposed to ethinyl estradiol decreased their aggressive response (Bell, 2001). Alyan (2013) exposed males of *Betta splendens* to the pesticide methyl parathion for up to 24 h. After exposure, the males became less aggressive towards the conspecific, according to analyses of the frequency and duration of opercular erection. McCormick et al. (2013) evaluated the effect of alteration of the pH of water by an increase of CO$_2$ concentration on the relation between the two fish species *Pomacentrus amboinensis* and *P. moluccensis*. Field experiments showed that elevated CO$_2$ changes the pattern of aggression between the two species, hence altering the processes that

shape communities and ecosystems. Due either to a reduction in the territorial fighting ability of *D. rerio* when exposed to copper, or to increased aggressiveness of *P. reticulata*, the present results showed that under a contamination scenario with a copper gradient, the guppies occupied the less contaminated zones and displaced the zebrafish to harsher environments. Although the avoidance response is important, the presence of other species with which there is some degree of competitive interaction may cause a species to be displaced (hypothesis based on the effect of *P. reticulata* on the displacement of *D. rerio*) or to lose the ability to compete (hypothesis based on the effect of copper on the displacement of *D. rerio*).

5. Conclusions

In the monospecific tests, both species (*P. reticulata* and *D. rerio*) were able to detect copper and move to a less contaminated area. In the multispecific tests, even in the presence of previously avoidable copper concentrations, the avoidance response of *D. rerio* was affected by the presence of *P. reticulata*. This study shows the importance of understanding the interactions among different fish species in contaminated areas and the way that one species can affect another species, in order to be able to predict the avoidance behavior likely to occur in polluted environments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.06.127.

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