**EFFECTS OF AQUATIC CONTAMINANTS ON THE HABITAT
SELECTION AND SPATIAL DISTRIBUTION IN FISH: A
COMPLEMENTARY APPROACH TO TRADITIONAL
ECOTOXICOLOGICAL TESTS**

*Efeitos de poluentes aquáticos na seleção de hábitat e
distribuição espacial em peixes: Uma abordagem
complementar aos testes ecotoxicológicos tradicionais*

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São Paulo

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Universidade de São Paulo
Instituto de Biociências - Departamento de Ecologia

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Tese apresentada ao Instituto de
Biociências da Universidade de São
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Dedicatória

Dedico este trabalho aos meus pais, Ângela e Iberê, e à minha esposa e filho, Verónica e Gael, sem os quais eu não teria a inspiração e forças necessárias para chegar onde cheguei!

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Epígrafe

“Let me tell you something you already know. The world ain't all sunshine and rainbows. It's a very mean and nasty place and i don't care how tough you are it will beat you to your knees and keep you there permanently if you let it. You, me, or nobody is gonna hit as hard as life. But it ain't about how hard ya hit. It's about how hard you can get hit and keep moving forward. How much you can take and keep moving forward. That's how winning is done!”

Rocky Balboa. Directed by:
Sylvester Stallone. Hollywood
(U.S.A.): MGM Studios /
Columbia Pictures, 2007, DVD.

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Resumo Geral

Os testes ecotoxicológicos convencionais (exposição forçada) são uma ferramenta importante quando o que se busca são os possíveis efeitos agudos e crônicos dos poluentes ambientais sobre cada indivíduo que é exposto. A desvantagem dessa abordagem está no fato de que os organismos são mantidos enclausurados dentro de recipientes com uma mesma concentração por várias horas e/ou dias. O teste de exposição forçada não tem relevância ecológica quando o ambiente modelado apresenta um gradiente de contaminação e os organismos podem se mover ao longo deste gradiente. Em muitos ecossistemas aquáticos, é comum observar um gradiente de contaminação, com as concentrações diminuindo com a distância da zona de descarga, de modo que os organismos não apresentam obrigatoriamente uma exposição contínua e forçada ao contaminante. Desta forma, este trabalho teve como objetivo a análise de como os poluentes aquáticos (e.g. Triclosan, Bisfenol, Atrazina e Cobre) influenciam o padrão de dispersão / seleção de habitat por duas espécies de peixes: *Poecilia reticulata* e *Danio rerio*, utilizando um sistema estático (não forçado) com vários compartimentos, formando um gradiente de contaminação com o composto a ser analisado. Todos os poluentes testados dispararam uma resposta de fuga nos peixes em concentrações ambientalmente relevantes. As concentrações que causaram a fuga dos organismos são menores do que aquelas que causam efeitos sub-letais em organismos aquáticos, incluindo peixes. Encontramos também em uma de nossas abordagens o potencial de formação de uma barreira química (fragmentação de habitat) pela liberação de poluentes nos corpos hídricos, reduzindo o potencial de migração dos organismos aquáticos. Por fim, um dos achados mais importantes está na interação das espécies entre si quando expostas a um gradiente de poluição. Nesse caso, a presença de uma espécie interferiu na distribuição da outra (redução do potencial de migração), quando ambas se encontravam no mesmo sistema. Sendo assim, a abordagem não forçada demonstra ser uma ferramenta poderosa na avaliação de risco ambiental, complementar aos testes ecotoxicológicos tradicionais.

Palavras-chave: ecotoxicologia, análise de risco ambiental, poluentes aquáticos

General Abstract

Conventional ecotoxicological tests (forced exposure) are an important tool when what is sought are the possible acute and chronic effects of environmental pollutants on each individual that is exposed. The disadvantage of this approach lies in the fact that the organisms are kept enclosed within containers of the same concentration for several hours and / or days. The forced exposure test has no ecological relevance when the modeled environment exhibits a contamination gradient and organisms can move along this gradient. In many aquatic ecosystems, it is common to observe a contamination gradient, with concentrations decreasing with distance from the discharge zone, so that organisms do not necessarily exhibit continuous and forced exposure to the contaminant. The objective of this work was to analyze how aquatic pollutants (e.g. Triclosan, Bisphenol, Atrazine and Copper) influence the dispersion / habitat selection pattern of two species of fish: *Poecilia reticulata* and *Danio rerio*, using a static system (non-forced exposure) with several compartments, forming a gradient of contamination with the compound to be analyzed. All pollutants tested triggered an avoidance response in fish at environmentally relevant concentrations. The concentrations that caused avoidance on the organisms are lower than those that cause sub-lethal effects on aquatic organisms, including fish. We also find in one of our approaches the potential for the formation of a chemical barrier (habitat fragmentation) by the release of pollutants into the water bodies, reducing the migration potential of aquatic organisms. Finally, one of the most important findings is the interaction of the species with each other when exposed to a pollution gradient. In this case, the presence of one species interfered in the distribution of the other (reduction of the migration potential), when both were in the same system. Thus, the non-forced approach demonstrates to be a powerful tool in the evaluation of environmental risk, complementary to the traditional ecotoxicological tests.

Keywords: ecotoxicology, environmental risk analysis, aquatic pollutants

1. General Introduction

Water pollution is a problem related to urban and industrial development. Land use in watersheds is one of the most important factors in the degradation of aquatic ecosystems, due to point (discharge of effluents) and non-point (surface runoff) source pollution. A large number of contaminants is discharged indiscriminately in water bodies, and there is not always the treatment for the removal of pollutant loads (López-Doval et al., 2016). In the last decades, environmental protection agencies have paid attention to the question of the influence of these elements on the quality of life of aquatic organisms. To this end, there are legal tools, such as the Resolution of the National Council for the Environment (CONAMA) n° 357/2005 (CONAMA, 2005) and n° 430/2011 (CONAMA, 2011), which indicate the permitted values of several quality parameters in the aquatic environments and treated effluent in Brazil. The legislation indicates the ecotoxicological tests to complement the results of the physical-chemical analyzes, with the use of bioindicators, showing how much the pollutants interfere in the development of the individuals exposed to them.

According to Zagatto & Bertolotti (2008), for the ecotoxicological evaluation of a given environment, it is essential to be aware of the emission sources of the pollutants, as well as their transformations, diffusions and destinations in the environment. It is also important to know the potential risks of these pollutants to biota, including humans. The diversity of studies with a focus on acute and chronic effects is great, and to get an idea of the intensity of research involving the keyword "ecotoxicology", 4,246 records were found in the Web of Science database, of which 1,382 are related to aquatic pollution, and finally 342 involving fish. The responses surveyed range from the molecular level to the more complex level (the organism). Despite the importance of bioassays, there are still some outstanding issues regarding the relevance of measured responses, the type of exposure and the behavior of organisms when in contact with contaminants.

The major problem from the point of view of the environmental relevance of standard ecotoxicological tests (acute and chronic) is the matter of the confinement of organisms and the homogeneous environment to which they are exposed. It should be

noted that it is important to know the relationship between dose and response between pollutants and bioindicators, as this provides important data on the level of individual, knowing how much an aquatic pollutant can interfere in the vital processes of each organism. The question is: In natural environments, are organisms really confined and exposed to the same dose of a pollutant for several hours, if not for several days? Does this dose tend to remain constant in such environment, without the influence of the mass of water on its dilution?

Another important approach that deserves to be highlighted is how environmentally relevant are the concentrations applied in ecotoxicological tests, that is, the doses produced for ex-situ tests correspond to those occurring in natural environments? And if so, how often? Several studies focus only on effects on organisms, even if the doses have to be high, and when the exposure of organisms has no effect, it is often understood that the compound does not pose a risk to the bioindicators who are exposed to it. International environmental agencies, such as the United States Environmental Protection Agency (USEPA), establish different safe concentrations for various compounds studied. Below these concentrations toxicity to aquatic organisms is not expected. As an example we have a report from the same agency showing the safe concentration of Bisphenol A that must be present in surface water: $\leq 1\mu\text{g}\cdot\text{L}^{-1}$ (USEPA, 2010). From a comparison of these concentration values with those that cause sub-lethal effects on fish, it can be seen that forced testing uses concentrations higher than levels considered safe, so that toxicity is normally expected to occur.

Is the exposure time used in ecotoxicological tests really ecologically relevant? Are the doses of the pollutants so continuous and stable that organisms remain in the same region for several hours or days in the same concentration conditions as they were primarily exposed? In this sense, we must take into account the characteristics of the habitat in which the organism is inserted, whether it is a lotic or lentic environment, deducing then if they are environments with more homogeneous or heterogeneous characteristics, and if this factor is intrinsically related to the dilution potential of the pollutants present (Esteves, 1998).

The study of avoidance behavior of aquatic organisms exposed to pollutants is a complementary approach to ecotoxicological tests, focusing no longer on the individual, limiting the environmental risk assessment, but on the population and its

spatial distribution. Several methodologies were employed to simulate more realistically the environmental conditions in which individuals were exposed. Among these approaches, the first experiment with a focus on avoidance of pollutants was developed by Jones (1947, 1948), simulating a clean and contaminated area with the stressor, injecting clean contaminated water on one side and contaminant on the other (Figure 1).

Figure 1. General scheme of the apparatus using a central glass tube with 30 mm of diameter and 58 cm of length with a capacity of 400 ml (Source: Jones, 1947).

Another system used (Figure 2) consisted of the formation of two tanks with a flow rate of $400 \text{ mL} \cdot \text{min}^{-1}$ per side (one control side and the other treatment) (Gun and Noakes, 1986).

Figure 2. Bi-compartmentalized system. Dimensions: 23x5x10cm (Source: Gun and Noakes, 1986).

In both systems there are two distinct areas with a marked difference in contaminant concentration (control and treatment), allowing fish to discriminate between the two environments. This approach is applicable to verify the ability to detect the contaminants and chose the zone with the least stress, but does not allow to simulate a real field situation where the formation of gradients is expected (Araújo et al., 2016a).

A new approach has recently been applied, taking into consideration the concept of avoidance and the question of the formation of contamination gradients, simulating with more precision what would occur in environments where dilution may occur, such as rivers and large lakes (lotic regions). The two linear systems (Moreira-Santos et al, 2008 [Figura 3-A]; Araújo et al, 2014 [Figura 3-B]) presented below, are formed by interconnected chambers, which allow the free passage of the organisms, allowing them to move in any direction (left or right). In this approach, a contamination gradient between the first and last chamber is produced, with free access by the organisms to any chamber. At the same time as it allows the passage of the fish, the reduced opening avoids diffusion between the compartments. The difference between both examples is that in the first one (figure 3-A) there is a renewal of the solution in each chamber through peristaltic pumps, while the second is static (Figure 3-B).

Figure 3. Illustration of non-forced systems for conducting small fish tests. (A): Avoidance system with renewal of solutions by peristaltic pumps. (B) Static avoidance system (Source: Moreira-Santos et al, 2008 [A] and Araújo et al, 2014 [B]).

Generally, in aquatic ecosystems, there is dispersion / dilution of the pollutants released (López-Doval et al., 2017), forming a gradient of contamination. That is,

avoidance can be estimated in an environment simulating real environmental conditions (Moreira-Santos et al., 2008; Rosa et al., 2008; 2012). Thus, toxicity assays whose organisms are exposed in confined conditions with no possibility of escape or even in bicompartimental systems are often a non-realistic simulation of exposure (Araújo et al., 2016a).

The environmental risk analysis is a methodological tool that allows estimating the risk of producing certain consequences in the environment, becoming an artifice for the making of environmentally justified decisions (GEOdata, 2017). These studies try to answer such questions as: What is the danger presented? Who is affected and what are the most appropriate actions to control risks? For example, in the case of pollution by pharmaceuticals, on the basis of ecotoxicological data, approaches can occur mainly through the estimation of hazard quotients (HQ), which are defined as the ratio of predicted environmental concentrations or measures and its chronic toxicity (Ginebreda et al., 2010). In this sense, avoidance tests aim to analyze how much any substance negatively affects the pattern of distribution / selection of habitat by aquatic organisms, being an approach to environmental risk analysis.

Therefore, the traditional use of forced exposure in bioassays can be justified based on many reasons, among which the main three are cited: (i) they are easy to use, (ii) they allow the establishment of an unambiguous concentration relation on an individual level and (iii) provide an easy interpretation of the results. However, if the main ecological effects of stressors to be measured are those occurring at community and ecosystem levels, individual effects measured under forced exposure may limit the assessment of environmental risks (Amiard-Triquet, 2009).

Several studies have been using the non-forced exposure system, and fish responses are similar to those described in this paper, such as *D. rerio* (Moreira-Santos et al., 2008), *Rachycentron canadum* (Araújo et al., 2015) and *Oreochromis* sp. (Araújo et al., 2016). This system has also been applied in other researches, with distinct organisms, showing the ability of bionicators to detect stressors and move to less contaminated areas: amphibians (Vasconcelos et al., 2016) and invertebrates (Lopes, et al., 2004; Araújo et al., 2016).

This methodology, as already mentioned, allows a more realistic approach, since it simulates the environmental conditions in which organisms are exposed. It is important to highlight that the avoidance response can be influenced by different

environmental factors, and not only the pollution. Among these factors (biotic and abiotic) are: Escape due to the presence of predators (Wolf and Phelps, 2017), differences in the natural concentration of CO₂ (Cupp et al., 2017), reduction in dissolved oxygen concentration / increase in temperature (Stehfest et al., 2017), turbidity (Robertson et al., 2007), among others.

Thus, the thesis demonstrates the avoidance response in fish exposed to different contaminants, such as:

- Triclosan: is added as a preservative or antiseptic agent to medical products such as hand disinfecting soaps, medical skin creams, and dental products (Jones et al., 2000).
- Bisphenol A: is used in the manufacture of many products including food and beverage packaging, flame retardants, adhesives, building materials, electronic components, and paper coatings (Fromme et al., 2002; Liu et al., 2017).
- Atrazine: its one of the most common herbicides used to control weeds and grasses in several crops (Graymore et al., 2001; Kannan et al., 2006; Jablonowski et al., 2011; Botelho et al., 2015)
- Copper: It is used in reservoirs as algicide (Cardoso-silva et al., 2016), besides its strong presence in high concentrations in regions with metal mining (Abraham and Susan, 2017).

Therefore, the approach given in this work was the behavior of fish when exposed to such contaminants, being chosen these substances for their adverse effects on aquatic biota (lethal and sub-lethal), as will be presented in the course of this document.

This work is structured in 4 chapters in the format of articles structured according to the periodicals that have been or will be submitted. Below are some questions that will be answered within each chapter:

- Chapter (i): How can different contamination scenarios (wider and shorter gradients) influence the avoidance response in fish? Can the results (mortality) obtained in forced tests be considered overestimated, since fish in real conditions can move to less contaminated areas? What is the expected population immediate decline in environmentally relevant concentrations?

- Chapter (ii): How environmentally relevant is the avoidance response to fish and how safe are the limits set by international agencies for the concentrations of pollutants found in surface waters when studying the avoidance response?
- Chapter (iii): Can contaminants cause habitat fragmentation, producing a chemical barrier to the flow of individuals?
- Chapter (iv): How can species density and interaction influence the avoidance response in environments where there are gradients of contamination by aquatic pollutants?

The central hypothesis of this work is that fish have the ability to detect pollutants and move to less polluted areas, and that due to pollution loads in rivers and lakes (where gradients occur), there will be a local decline in population, taking into account organisms that scaped and those who for some reason stayed and died on the spot. Thus, we can say that the pollutants can interfere on the spatial distribution and therefore in the selection of habitat.

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2. Justification

Traditionally, internationally standardized ecotoxicological studies assessing the effects of contaminants are carried out using forced exposure systems, simulating a confined environment. In such systems, the organisms are exposed to a certain concentration of the substance and measurements of different biological responses are made after a specified time. The forced exposure test lacks ecological relevance when the environment modeled presents a gradient of contamination and organisms can move along this gradient. In many aquatic ecosystems, it is common to observe a gradient of contamination, with concentrations decreasing with distance from the discharge zone, so the organisms do not mandatorily experience a continuous and forced exposure to the contaminant. Therefore, non-forced exposure is a more environmentally realistic method to assess the effects of contaminants on the spatial distribution of organisms since, instead of exposing organisms to a single concentration, it offers them the possibility of migrating to other regions. Another important advantage of this approach is that it improves the environmental risk assessment by integrating the effects of contamination across a wider spatial scenario, because the avoidance response and the spatial displacement is not focused exclusively on the organisms and the contaminated area, but also considers the ecosystem at a wider spatial scale, including the adjacent uncontaminated areas.

Since various pollutants are capable of causing disturbances in fish behavior, the present study provides as the main objective an evaluation of the potential effects of different chemical compounds on the spatial distribution of freshwater fish (*Poecilia reticulata* and *Danio rerio*), analyzing the following factors: (#1) the population immediate decline (PID) caused by the avoidance response, (#2) formation of chemical barriers isolating fish populations, (#3) comparison of the ecotoxicological responses (sub-lethal effects) of some contaminants with avoidance response and how environmentally realistic are both approaches regarding the safe concentrations pointed by international agencies and (#4) analysis of the effects of interespecific interaction between two fish species on the individual patterns of avoidance to contamination. The results demonstrated the importance of using non-forced exposure in order to increase the ecological relevance of environmental quality studies. This

approach (i) helps to understand how contaminants interferes in the spatial distribution of organisms by triggering an avoidance response, and (ii) integrates effects at the ecosystem level (such as local population immediate decline, loss of biodiversity, and imbalance in the food web, among others), considering the contaminated area and the adjacent environments.

3. Chapter I

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Potential effects of triclosan on spatial displacement and local population decline of the fish *Poecilia reticulata* using a non-forced system

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ABSTRACT

Triclosan (TCS) is an emerging contaminant of concern in environmental studies due to its potential adverse effects on fish behavior. Since avoidance has been shown to be a relevant behavioral endpoint, our aims were: (i) to determine if TCS is able to trigger an avoidance response in *Poecilia reticulata*; (ii) to predict the population immediate decline (PID) caused by TCS exposure, by integrating lethality and avoidance responses; and (iii) to verify the overestimation of risk when mortality is assessed under forced exposure. Fish were exposed to TCS in a forced exposure system, to assess mortality, and to a TCS gradient in a non-forced exposure (NFE)

system. Two NFE scenarios were simulated: (#1) a spatially permanent gradient, including low and high concentrations; and (#2) a scenario with high concentrations, simulating a local discharge. The fish avoided TCS concentrations as low as $0.2 \mu\text{g}\cdot\text{L}^{-1}$ (avoidance of 22%). The AC50 obtained from scenario #1 ($8.04 \mu\text{g}\cdot\text{L}^{-1}$) was about 15 times more sensitive than that from scenario #2 ($118.4 \mu\text{g}\cdot\text{L}^{-1}$). In general, up to the highest concentration tested ($2000 \mu\text{g}\cdot\text{L}^{-1}$), the PID was determined by the avoidance. Mortality from the forced exposure was overestimated (48 h-LC50 of $1650 \text{mg}\cdot\text{L}^{-1}$), relative to the NFE. The reduced mortality in a non-forced environment does not imply a lower effect, because part of the population is expected to disappear by moving towards favorable environments. TCS is a potential environmental disturber, since at environmentally relevant concentrations ($<2 \mu\text{g}\cdot\text{L}^{-1}$) it could cause a decline in the fish population.

Keywords: Avoidance, contamination gradient, non-forced exposure, *Poecilia reticulata*, spatial distribution, triclosan.

RESUMO

Triclosan (TCS) é um contaminante emergente de preocupação em estudos ambientais devido aos seus efeitos potenciais adversos sobre o comportamento dos peixes. Uma vez que a fuga demonstrou ser uma resposta comportamental relevante, nossos objetivos foram: (i) determinar se TCS é capaz de disparar uma resposta de fuga em *Poecilia reticulata*; (ii) prever o declínio imediato da população (PID) causado pela exposição ao TCS, integrando respostas de letalidade e fuga; e (iii) verificar a superestimação do risco quando a mortalidade é avaliada sob exposição forçada. Os peixes foram expostos ao TCS em um sistema de exposição forçada, para avaliar a mortalidade e em um gradiente TCS em um sistema de exposição não forçada (ENF). Dois cenários de ENF foram simulados: (# 1) um gradiente espacialmente permanente, incluindo concentrações baixas e altas; e (# 2) um cenário com altas concentrações, simulando uma descarga local. Os peixes evitaram concentrações de TCS tão baixas quanto $0.2 \mu\text{g}\cdot\text{L}^{-1}$ (evitação de 22%). A AC₅₀ obtida do cenário # 1 ($8.04 \mu\text{g}\cdot\text{L}^{-1}$) foi cerca de 15 vezes mais sensível do que a do cenário # 2 ($118.4 \mu\text{g}\cdot\text{L}^{-1}$). Em geral, até a maior concentração testada ($2000 \mu\text{g}\cdot\text{L}^{-1}$), o PID foi determinado pela fuga. A mortalidade da exposição forçada foi superestimada (48 h-

LC50 de 1650 mg·L⁻¹) em relação à ENF. A redução da mortalidade em um ambiente não forçado não implica um menor efeito, porque parte da população deverá desaparecer se movendo em direção a ambientes favoráveis. TCS é um potencial perturbador do meio ambiente, uma vez que em concentrações ambientalmente relevantes (<2 µg·L⁻¹) pode causar um declínio na população de peixes.

Palavras-Chave: Fuga, gradiente de contaminação, exposição não forçada, *Poecilia reticulata*, distribuição espacial, triclosan.

1. Introduction

Contaminants of emerging concern are synthetic or natural substances, as well as microorganisms, that are not routinely monitored, despite presenting potential risks to the environment or human health (USGS, 2016). Triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol) (TCS), a halogenated, non-ionic, phenolic compound, is added as a preservative or antiseptic agent to medical products such as hand disinfecting soaps, medical skin creams, and dental products (Jones et al., 2000). The global production of TCS, considered a contaminant of emerging concern, has now exceeded 1500 tons per year, with Europe being responsible for 350 tons of the total production (Singer et al., 2002; Dan and Hontela, 2011). Recently, the Food and Drug Administration (FDA) eliminated TCS from the list of substances permitted for use in non-prescribed antibacterial hand soaps and shower gels (FDA, 2016).

Although it is expected that the use of TCS will progressively decline, contamination by TCS is of concern, because even after passing through wastewater treatment plants, it has been detected in aquatic ecosystems. For example, it has been found in wastewater at concentrations in the range 0.07-14000 µg·L⁻¹ and in streams at between 0.05 and 2.3 µg·L⁻¹ (Kolpin et al., 2002; Lindstrom et al., 2002; McAvoy et al., 2002; Nakada et al., 2006; Okuda et al., 2008). Two studies in São Paulo State (Brazil) detected maximum TCS concentrations of 0.34 µg·L⁻¹ and 0.78 µg·L⁻¹ in the Atibaia River (Raimundo, 2011) and the Paraíba do Sul River (unpublished data: Daniel C.V.R. Silva), respectively.

TCS has shown toxic effects on behavior in different fish species and at different concentrations, including swimming speed in *Oryzias latipes* (at 170 µg·L⁻¹; Nassef et al., 2010); erratic swimming in *Oncorhynchus mykiss* (at 71 µg·L⁻¹; Orvos et al., 2002)

and *Danio rerio* (at 400-500 $\mu\text{g}\cdot\text{L}^{-1}$; Oliveira et al., 2009); swimming performance in *Pimephales promelas* (at 75 $\mu\text{g}\cdot\text{L}^{-1}$; Fritsch et al., 2013); alteration of patterns of diurnal activities (foraging success and predator avoidance) in *Gambusia holbrooki* (at 1, 10, and 100 $\mu\text{g}\cdot\text{L}^{-1}$; Melvin et al., 2016); and decreased aggression (ability to defend and hold a nest site) in *P. promelas* (at 0.56 $\mu\text{g}\cdot\text{L}^{-1}$ to 1.6 $\mu\text{g}\cdot\text{L}^{-1}$; Schultz et al., 2012). Although these data indicate the risks that TCS can pose to fish behavior, its effect on the spatial distribution of aquatic organisms remains unknown.

In studies of the impacts of contaminants on aquatic organisms, in which traditional responses such as survival, growth, reproduction, and behavioral endpoints are assessed, a forced exposure system is usually adopted, with the exposure of the organisms being restricted to a specific concentration of the contaminant or environmental sample. Although this approach can assist in predicting the effects in organisms following continuous exposure to contaminants, it fails in accurately predicting the ways that organisms would react under natural conditions where there is the possibility of moving to a more favorable environment (Moreira-Santos et al., 2008).

In a non-forced system, avoidance can be used as a sensitive and immediate response for assessment of the likely population displacement of aquatic organisms, triggered by chemical stress, and the consequent effects on the community structure and the ecosystem dynamics (Rosa et al., 2008, 2012; Araújo et al., 2016a; 2017). The main advantages of using non-forced avoidance assays are: (i) simulation of a more realistic scenario in which organisms (if they are able to move) are not forcedly and continuously exposed to a single concentration of a contaminant, enabling assessment of the contamination-driven spatial displacement when a contamination gradient is expected to occur; (ii) inclusion of the habitat selection concept in environmental risk assessment, changing the focus of toxic effects from the individual level (where organisms are not necessarily damaged) to environmental disturbance at the ecosystem level (loss of organisms by avoidance); and (iii) the ability to obtain an immediate and sensitive response. Avoidance should therefore be considered as a complementary approach to traditional sublethal and lethal assays, improving ecological relevance in the assessment of the environmental risks of contaminants (Moreira-Santos et al., 2008; Rosa et al., 2012).

In the present study, we hypothesized that a non-forced exposure simulating a contamination gradient could help in understanding how the spatial distribution of aquatic organisms could be affected by TCS, and that the population of the exposed organisms could immediately (in the very short term) decline at the local scale due to the avoidance of a contaminated environment. Therefore, our goals were as follows: (i) to determine whether TCS has the capacity to trigger an avoidance response in *Poecilia reticulata*, inducing its displacement to areas that are less contaminated; (ii) to predict the population immediate decline (PID) (proposed by Rosa et al., 2012) at the local scale, caused by exposure to TCS, by integrating both lethality and avoidance responses in a very short-term exposure; and (iii) to determine whether mortality might be overestimated when forced and continuous exposure is used to assess toxic effects.

Two different gradient scenarios with point source contamination were simulated: (#1) a spatially permanent and wider (higher concentration range) gradient including low and high concentrations (continuous discharges on a large spatial scale); and (#2) a scenario with high concentrations, simulating a spatially reduced local discharge event (sporadic discharges of untreated raw sewage; Singer et al., 2002). In the first scenario, mortality is not expected to occur and PID should be determined mainly by the avoidance response, due to the existence of very low concentrations that are expected to be non-lethal, towards which the organisms are likely to move. On the other hand, in the second and worse scenario, the gradient with very high concentrations could hinder or prevent the displacement of the organisms, so mortality could play a more important role than avoidance in the population decline. The fish *P. reticulata* (common name: guppy; family: Poeciliidae), a species found from Central America to Northwestern Brazil (UWI, 2016), was chosen as a test organism because it is easy and inexpensive to maintain in the laboratory and presents high sensitivity (Liguoro et al., 2012; Pelli and Connaughton, 2015). In addition, being a small fish, it is appropriate for use in non-forced avoidance assays (Moreira-Santos et al., 2008; Araújo et al., 2014a).

2. Materials and Methods

2.1. Test organisms

Fish were obtained from the *São Paulo Agency of Agribusiness and Technology* (Pindamonhangaba, Brazil) and prior to the assays were acclimatized under laboratory conditions in the ecotoxicology laboratory of the University of São Paulo (USP, Lorena, Brazil). The use of the fish was approved by the Ethics Committee on the Use of Animals (Protocol 236/2015 – IB-USP). All the animals used in this study were juveniles (males and females) no more than 3 months old and were of similar size (1.2 ± 0.3 cm). The use of older fish was avoided because after the third month, the females become mature and are sexually attractive to males, with the production of hormones (UWI, 2016), which could interfere in the fish dispersion pattern during the tests.

Prior to the tests, the fish were kept for 1 month in aquaria (60 L) that were constantly aerated using an air diffuser. The water employed was from an artesian well and was filtered through activated charcoal before use in the cultures. The pH, conductivity and dissolved oxygen (DO) values were near to 7, $120 \mu\text{S}\cdot\text{cm}^{-1}$ and $>6.0 \text{ mg}\cdot\text{L}^{-1}$, respectively. The culture water was renewed by 30% every week. Natural light was provided in the room, without direct exposure to the sun. Culture water quality parameters were monitored on a weekly basis and included water hardness and DO according to the CETESB – Companhia Ambiental do Estado de São Paulo (1978a; 1978b), as well as conductivity and pH (both measured with a Hanna model HI 9811-5 instrument). Before the experiments, the sensitivity of the fish was evaluated (in triplicates) by performing toxicity tests using potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) as a reference substance, at concentrations ranging from 10 to $300 \text{ mg}\cdot\text{L}^{-1}$, according to the ABNT – Associação Brasileira de Normas Técnicas (2011). The LC50 value (mean $\pm$ standard deviation; $N = 3$) was $37.50 \pm 8.41 \text{ mg}\cdot\text{L}^{-1}$. This value was within the upper and lower limits (63.80 and $21.0 \text{ mg}\cdot\text{L}^{-1}$) of the control chart for *P. reticulata* provided by the ecotoxicology laboratory of the *Engineering School of Lorena*.

2.2. Triclosan (TCS)

Before each toxicity and avoidance test, a stock solution of TCS ($\geq 97\%$, HPLC grade; Sigma-Aldrich) was prepared at a concentration of $2000 \mu\text{g}\cdot\text{L}^{-1}$ in distilled water. Methanol ($2 \text{ ml}\cdot\text{L}^{-1}$) was used as solubilizing agent in all the assays (control and treatments).

The dilutions used in the tests were made with culture water. The quantification of TCS was performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), at the Institute of Chemistry of the University of Campinas (Brazil), following the methodology described by Montagner et al. (2014). The equipment used was an Agilent model 1200 chromatograph equipped with a binary pump, an automatic injector, and a temperature-controlled column compartment. Chromatographic separation was achieved with a Zorbax SB-C18 column ($2.1 \times 30 \text{ mm}$, particle size of $3.5 \mu\text{m}$) at 25°C . The limits of detection and quantification were 4.3 and $14.3 \text{ ng}\cdot\text{L}^{-1}$, respectively ($r^2 = 0.993$).

2.3. Acute toxicity tests using forced exposure

Acute toxicity tests with the fish were based on ABNT – Associação Brasileira de Normas Técnicas (2011). The concentrations of TCS used in the tests ($0, 1000, 4000, 6000, 8000$, and $10000 \mu\text{g}\cdot\text{L}^{-1}$) were based on the results reported by Kim et al. (2009) for the fish *O. latipes*. The tests were performed in triplicates using 10 fish per aquarium (2 L), at a temperature of $23.0 \pm 0.4^\circ\text{C}$, with constant aeration (air diffuser) to maintain DO above $6 \text{ mg}\cdot\text{L}^{-1}$, and a photoperiod of 12:12 h (light:dark). The pH and conductivity were measured at the beginning (0 h) and end (48 h) of the experiment. The initial and final mean values were 8.0 ± 0.2 and 7.6 ± 0.4 for pH and $190 \pm 28.3 \mu\text{S}\cdot\text{cm}^{-1}$ and $153.3 \pm 8.2 \mu\text{S}\cdot\text{cm}^{-1}$ for conductivity (considering all the concentrations).

2.4. Non-forced exposure system

Non-forced avoidance tests were performed in a static multi-compartment system, as described by Araújo et al. (2014a). The system (Figure 1) was composed of seven interconnected compartments constructed from borosilicate glass bottles (VBTR), handcrafted in a high temperature furnace at 600°C , without the use of any

contaminating materials. The chambers were connected with 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 compartments were filled with 125 mL volumes of water containing TCS at different concentrations, so that the total system volume was 875 mL.

Figure 1. Schematic diagram of the static multi-compartment non-forced avoidance test system, with detail of one of the seven compartments (source: Araújo et al., 2014a).

2.5. Non-forced system validation

The first validation of the system ($N = 3$) consisted of confirming the stability of the linear gradient of the contaminant, in the absence of fish. For this, different concentrations (0, 17, 33, 50, 67, 83, and 100 $\text{mg}\cdot\text{L}^{-1}$) of analytical grade sodium chloride (NaCl) were prepared in distilled water. The NaCl concentrations were measured indirectly by conductivity (calibration curve: $r^2 = 0.9982$; $r = 0.9999$; $p = 0.000001$; $y = 2.8585 + 0.4436 * x$; $n = 7$) at 0 h (initial) and after 12 h (final). The initial concentrations (at 0 h) inside each compartment were (mean and standard deviation): 8.77 ± 2.6 , 17.65 ± 2.6 , 35.39 ± 2.6 , 53.13 ± 2.6 , 66.44 ± 2.6 , 84.19 ± 2.6 , and 101.93 ± 2.6 $\text{mg}\cdot\text{L}^{-1}$. Before dispensing the NaCl into the system, the compartments were isolated from each other using plasticine plugs wrapped in PVC film. After insertion of the different amounts of NaCl into the compartments, the plugs were removed, resulting in a linear gradient (this procedure was repeated for all the experiments in which a contamination gradient was simulated). Validation was performed in triplicates, in the dark and at 23 ± 1 °C.

Secondly, a system validation was carried out in the presence of fish ($N = 3$), with different concentrations of NaCl prepared in culture water. Measurements were

made in each chamber at 0 h and after 4 h. The initial concentrations (at 0 h) were (mean and standard deviation): 54.6 ± 2.6 , 70.9 ± 2.6 , 84.2 ± 2.6 , 93.1 ± 2.6 , 115.2 ± 2.6 , 128.5 ± 2.6 , and $137.4 \pm 2.6 \text{ mg}\cdot\text{L}^{-1}$. The experiment was conducted in the dark, at $23 \pm 1 \text{ }^{\circ}\text{C}$. The swimming movements of the fish was likely to cause faster mixing of the different NaCl concentrations, resulting in disruption of the gradient, so this validation was used to define the maximum exposure period (4 h, based on Araújo et al., 2014a). Three fish were used at each concentration, totaling 21 individuals in the system (3 fish * 7 compartments). The fish were transferred into the system when the gradient had been formed and immediately after removing the plugs. Conductivity measurements were used to indirectly determine the NaCl concentrations and evaluate the stability of the gradient.

Subsequently, a control distribution experiment was conducted ($N = 3$) in order to confirm that the fish did not exhibit a preference for any particular compartment. To this end, three organisms were transferred to each compartment containing only culture water (with no gradient) and the number of fish in each compartment was recorded after 4 h.

2.6. Avoidance tests with TCS

Avoidance tests with TCS were performed in a non-forced exposure system, simulating two distinct scenarios: (#1) a spatially permanent and wider (higher concentration range) gradient; and (#2) a scenario with high concentrations, simulating a spatially reduced local discharge event. The concentrations used were based on data published by Singer et al. (2002) indicating maximum TCS concentrations of $2.3 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ in streams and $14000 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ in effluents (liquid and sludge), and considering that the LC50 (the concentration that causes the death of 50% of a population) for *P. reticulata* is $1650\mu\text{g}\cdot\text{L}^{-1}$ (current study). The concentrations were as follows (in $\mu\text{g}\cdot\text{L}^{-1}$): 0 (control), 0.2, 2.0, 20, 200, 1000, and 2000 (scenario #1); and 0 (control), 100, 250, 500, 1000, 1500, and 2000 (scenario #2). The TCS concentrations found in the compartments at the end of the avoidance tests (after 4 h) were similar (variation less than 10%) to the nominal concentration gradient. Therefore, the nominal concentrations were used in the analyses.

In each test, three fish were used at each concentration, totaling 21 individuals in the system. The procedures used to dispense the TCS and fish into the system were as

described above. Measurements of conductivity, pH and DO were made at the beginning (0 h) and end (4 h) of each experiment. The tests were performed in triplicates, in the dark, at a temperature of 23 ± 1 °C.

2.7. Statistical analyses

The results (numbers of dead organisms) of the acute tests were analyzed using PriProbit software (Sakuma, 1998) to calculate the LC50 value and the corresponding confidence interval (CI).

One-way ANOVA followed by the Tukey test ($p < 0.05$) was employed to analyze the results of the validation tests (determination of the stability of the gradient in the absence and presence of fish, and determination of the random distribution of fish in the control culture) and the avoidance tests (distribution of organisms [in %] among the chambers).

For calculation of avoidance (%), the number of avoiders was determined for each compartment as the difference between the number of organisms expected (N_E) and the number of organisms observed (N_O): $\text{Avoiders} = N_E - N_O$. N_E was determined as described by Moreira-Santos et al. (2008): for the compartment with the highest concentration of the compound, N_E was equal to the number of fish introduced into the compartment at the beginning of the test; for the remaining compartments, N_E included the organisms initially introduced into the compartment, plus the organisms introduced into the adjacent compartment with higher concentration. Initially, as each compartment contained three fish, the N_E value was 3 for the most contaminated compartment (considered the first compartment). The N_E value for the adjacent compartment (second compartment) was 6 (3 organisms inserted in this compartment plus 3 organisms expected to move from the first compartment), while for the last (seventh, uncontaminated) compartment it was 21. Regarding the number of organisms observed for each concentration, it was considered as N_O the organisms found in that compartment and those found at higher concentrations. Finally, for the control compartment, because this compartment contained the water in which the organisms were cultured, no avoidance was expected; therefore, N_O was 21. The avoidance percentage for each compartment was calculated as follows: $(\text{Avoiders}/N_E) * 100$. The avoidance percentages for each concentration were used to obtain the AC50 values (concentration causing avoidance by 50% of the exposed organisms)

and the corresponding CI. These calculations were performed using the PriProbit software.

The avoidance and mortality percentages were subsequently integrated in order to calculate the PID_x (x in percent) induced by each TCS concentration that simultaneously caused a y mortality percentage (i.e., the 48-h LC_y) and a w avoidance percentage (i.e., the 4-h AC_w), as described by Rosa et al. (2012):

$$x = \left[1 - \left(1 - \frac{y}{100} \right) \cdot \left(1 - \frac{w}{100} \right) \right] \cdot 100$$

The calculation of PID was based on the premise that some of the fish first flee (avoidance %), and that mortality is then determined as a function of the remaining organisms (those that did not show avoidance). The PID_{50} values (the concentration causing a population immediate decline of 50% of the exposed organisms) and the corresponding CI were also obtained using the PriProbit software.

3. Results

3.1. Acute test

The percentages of deaths at the different TCS concentrations were 0% (control), 26% ($1000 \mu\text{g}\cdot\text{L}^{-1}$), 80% ($4000 \mu\text{g}\cdot\text{L}^{-1}$), and 100% (6000 , 8000 , and $10000 \mu\text{g}\cdot\text{L}^{-1}$). The 48-h LC_{50} (and CI) of the forced acute test was 1650 (1030 - 2300) $\mu\text{g}\cdot\text{L}^{-1}$ (Table 1). At the three highest concentrations, losses of agility and equilibrium were observed in some fish in the first 24 h.

3.2. Avoidance system validation

The results of the validation of the avoidance system using NaCl showed that in the absence of fish, the gradient was maintained after 12 h ($p < 0.05$; $F_{6,14} = 408$), since statistically significant differences were found among the concentrations in the compartments. After 12 h, the mean and standard deviation values for the NaCl concentrations in the validation test without fish were 10.3 ± 2.6 , 19.1 ± 2.6 , 38.3 ± 4.4 , 53.1 ± 2.6 , 66.4 ± 2.6 , 84.2 ± 2.6 , and $103.4 \pm 2.6 \text{ mg}\cdot\text{L}^{-1}$. Similarly, in the presence of fish, the gradient of NaCl was not disrupted after 4 h, as the NaCl concentrations in the compartments increased progressively and were significantly

different from each other ($p < 0.05$; $F_{6,14} = 245$): 57.6 ± 2.6 , 72.4 ± 5.1 , 84.2 ± 2.6 , 93.1 ± 2.6 , 115.2 ± 2.6 , 128.5 ± 2.6 , and $140.4 \pm 4.4 \text{ mg}\cdot\text{L}^{-1}$. The initial NaCl concentrations in both validation procedures are provided in Section 2.5.

In the control fish distribution experiment without TCS, a homogeneous distribution (with no preference for any compartment) of the organisms throughout the system was observed after 4 h ($p > 0.05$; $F_{6,14} = 0.72$). The mean distribution of the organisms ($\pm$ standard deviation) per compartment (1 to 7) was: 3.67 ± 1.53 , 2.67 ± 1.53 , 2.67 ± 2.08 , 2.33 ± 0.58 , 4.67 ± 3.79 , 1.67 ± 0.58 and 3.33 ± 3.06 .

3.3. Avoidance response and PID

The distributions of fish in the avoidance experiments with TCS are shown in Figure 2 for each concentration and both scenarios. The distribution of the organisms in the avoidance system was concentration-dependent: at $2000 \text{ }\mu\text{g}\cdot\text{L}^{-1}$, only one fish (1.59%) was found in both scenarios considering the three replicates (a total of 63 organisms), while at $0 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ (control) there were 21 organisms (33%) in scenario #1 and 34 organisms (54%) in scenario #2. As the TCS gradient in scenario #1 presented three concentrations lower than the lowest concentration of scenario #2, the difference in the distribution of fish in the control compartment, relative to the other compartments, tended to be less abrupt.

Figure 2. Mean percentages and standard deviations ($N = 3$) of organisms (the fish *Poecilia reticulata*) observed in each concentration after 4 h exposure to a gradient of triclosan in a non-forced system, under two different scenarios (see text for more

details): scenario #1 (left) and scenario #2 (right). Different letters indicate statistically significant differences (one-way ANOVA followed by Tukey's test).

The avoidance, mortality, and PID data (in %) for *P. reticulata* exposed to TCS gradients in scenarios #1 and #2 are shown in Figure 3. For the curves of PID and mortality, the percentages of dead organisms were calculated considering the non-forced system (avoidance system) and the forced system. In both test scenarios, avoidance was clearly concentration-dependent. Avoidance reached 90% at the concentration of 2000 $\mu\text{g}\cdot\text{L}^{-1}$, while at the lowest concentration (0.2 $\mu\text{g}\cdot\text{L}^{-1}$), in scenario #1, it did not exceed 22%.

In both scenarios, the PID curve followed the trend of the avoidance curve at concentrations below 1000 $\mu\text{g}\cdot\text{L}^{-1}$ (200 $\mu\text{g}\cdot\text{L}^{-1}$ for scenario #1 and 500 $\mu\text{g}\cdot\text{L}^{-1}$ for scenario #2), with mortality beginning to occur at higher concentrations. Mortality was not observed at any concentration in the non-forced system of scenario #1, although mortality rates of around 30 and 80% were expected when the organisms were forcedly exposed to TCS concentrations of 1000 and 2000 $\mu\text{g}\cdot\text{L}^{-1}$. In the case of scenario #2, the number of dead fish in the non-forced exposure system was reduced almost 3.5-fold (from 66% in the forced exposure to 19% in the non-forced exposure).

Figure 3. Concentration-response curves for the avoidance and mortality responses, and the estimated PID (population immediate decline) for *Poecilia reticulata* exposed to triclosan under two different scenarios (see text for more details): scenario #1 (left)

and scenario #2 (right). Mortality and PID were obtained from the results of tests using the forced and non-forced exposure systems.

3.3. AC50, LC50, and PID50

The AC50, LC50, and PID50 values are shown in Table 1 for both scenarios, considering mortality in the forced and non-forced exposure systems. The AC50 obtained from scenario #1 ($8.04 \mu\text{g}\cdot\text{L}^{-1}$) was about 15 times more sensitive than that from scenario #2 ($118.4 \mu\text{g}\cdot\text{L}^{-1}$). Comparison of the AC50 and LC50 values showed that the avoidance response was about 14 (scenario #2) to 200 (scenario #1) times more sensitive than mortality. Considering only the non-forced system data, the PID50 and AC50 values were similar, since mortality was not relevant at either concentration. In fact, the LC50 values ($>2000 \mu\text{g}\cdot\text{L}^{-1}$; Table 1) were not calculated for the non-forced exposure system, due to low (or absent) mortality (Figure 2).

Table 1. Values (in $\mu\text{g}\cdot\text{L}^{-1}$) of AC50, LC50, and PID50 (concentrations that cause avoidance, mortality, and population immediate decline, respectively, in 50% of the exposed organisms) for the fish *Poecilia reticulata* exposed to triclosan under two different scenarios and in two exposure systems: non-forced exposure (NFE) and forced exposure (FE).

Scenario	AC50 (NFE)	LC50 (NFE)	PID50 (NFE)	LC50 (FE)	PID50 (FE)
#1	8.04 (3.29-17.05)	>2000	8.04 (3.29-17.05)	1650 (1030-2300)	7.03 (1.97-19.19)
#2	118.4 (14.79- 244.2)	>2000	124.9 (15.97- 254.7)	1650 (1030-2300)	134.6 (56.0-217.3)

4. Discussion

4.1. Avoidance and mortality responses

The 48 h-LC50 value obtained here ($1650 \mu\text{g}\cdot\text{L}^{-1}$) was close to that found for *Xiphophorus helleri* (96 h-LC50: $1470 \mu\text{g}\cdot\text{L}^{-1}$; Liang et al., 2013), but higher than those for *D. rerio* (96 h-LC50: $340 \mu\text{g}\cdot\text{L}^{-1}$; Oliveira et al., 2009) and *O. latipes* (96 h-LC50: $600 \mu\text{g}\cdot\text{L}^{-1}$; Kim et al., 2009). Comparison of these concentrations with those

that actually occur in surface waters (a maximum of $2.3 \mu\text{g}\cdot\text{L}^{-1}$, according to Kolpin et al., 2002) showed that the LC50 was up to 717 times higher. High concentrations of TCS would only be likely to occur in two hypothetical situations: (i) disposal of untreated raw sewage, and (ii) problems with the removal of TCS in the wastewater treatment plants of industries that handle the product. Hence, the concentrations used to measure acute effects are not commonly expected and, therefore, lack environmental relevance.

The present study showed that the fish *P. reticulata* was able to detect a gradient of TCS and avoid levels considered potentially toxic. Therefore, the hypothesis that TCS affects spatial displacement in *P. reticulata* was supported, because the organisms moved towards uncontaminated compartments. At a concentration of $2.0 \mu\text{g}\cdot\text{L}^{-1}$, *P. reticulata* showed 38% avoidance (scenario #1), demonstrating that at low concentrations (close to environmental conditions), the organism detected TCS and escaped towards less contaminated habitats. Avoidance responses (not using a contamination gradient) of fish exposed to contaminants has been observed in several different species (Folmar, 1976; Smith and Bailey, 1990; Svecevičius, 1999; Richardson et al., 2001; Wells et al., 2004; see also the review by Araújo et al., 2016b). However, few studies have been based on non-forced exposure employing a contamination gradient, in which fish are simultaneously exposed to different concentrations, with the assessment of contamination-driven spatial displacement. *Oncorhynchus mykiss* showed high sensitivity in avoidance of metal contamination (Hansen et al., 1999a). Exposure of *D. rerio* to acid copper mine drainage effluent (Moreira-Santos et al., 2008) and the fungicide pyrimethanil (Araújo et al., 2014a) resulted in the organisms avoiding toxic concentrations and moving towards less contaminated environments. Tilapia fry (*Oreochromis* sp.) detected and avoided dangerous concentrations of an industrial effluent (Araújo et al., 2016a). The marine fish *Rachycentron canadum* avoided copper-contaminated environments (Araújo et al., 2016c).

The data obtained in this study, especially for scenario #1 (in which the lowest avoided TCS concentration was $0.2 \mu\text{g}\cdot\text{L}^{-1}$), indicated that concentrations causing avoidance in *P. reticulata* were lower than those that cause sublethal effects in other species of fish. For example, chronic effects such as cardiac problems were observed in *D. rerio* exposed to $400 \mu\text{g}\cdot\text{L}^{-1}$ of TCS (Saley et al., 2016); thyroid disruption

occurred in *Cyprinodon variegatus* at TCS concentrations of 20 and 100 $\mu\text{g}\cdot\text{L}^{-1}$ (Schnitzler et al., 2016); and deformation in the spine of *P. promelas* was recorded at 50 and 100 $\mu\text{g}\cdot\text{L}^{-1}$ of TCS (Salierno et al., 2016). The concentrations of TCS that triggered effective population displacement (AC50 in scenario #1: 8.04 $\mu\text{g}\cdot\text{L}^{-1}$) in *P. reticulata* were below the concentrations reported elsewhere that affected the swimming patterns of different fish species, including *O. latipes* (170.0 $\mu\text{g}\cdot\text{L}^{-1}$; Nassef et al., 2010), *O. mykiss* (71 $\mu\text{g}\cdot\text{L}^{-1}$; Orvos et al., 2002), *D. rerio* (400-500 $\mu\text{g}\cdot\text{L}^{-1}$; Oliveira et al., 2009), and *P. promelas* (75 $\mu\text{g}\cdot\text{L}^{-1}$; Fritsch et al., 2013).

4.2. Population immediate decline

Analysis of the escape responses in the two contamination scenarios (#1 and #2) showed that avoidance played a more important role in the population decline, for all concentrations, and was a determining factor in the PID calculation. Mortality only increased in importance from 1000 $\mu\text{g}\cdot\text{L}^{-1}$. At higher TCS concentrations, the effects on fish behavior and swimming patterns are expected to increase. Oliveira et al. (2009) observed that the swimming pattern of *D. rerio* became altered at TCS concentrations of 400 and 500 $\mu\text{g}\cdot\text{L}^{-1}$. The evidence suggests that fish can lose the ability to detect TCS and then move to areas with lower concentrations. The typical patterns of avoidance and mortality reported in the literature suggest that an initial avoidance response determines the population decline in the very short-term, while at higher concentrations the ability to avoid contamination becomes impaired (Rosa et al., 2012; Araújo et al., 2014b). Failures in the capacity to detect contamination and consequent loss (due to stupefaction or moribundity) of the ability to avoid toxic concentrations have been observed in cladocerans and copepods (Gutierrez et al., 2012), amphibians (Araújo et al., 2014b), and fishes (Jones, 1947; Hartwell et al., 1989). According to our data, stupeficient effects that prevent an avoidance response induced by TCS exposure are not expected to occur at environmentally relevant concentrations.

Two different ways were used to include the percentage of dead organisms in the PID calculation: (i) mortality data from the avoidance tests, and (ii) mortality data from the forced acute tests. Considering the non-forced exposure data, the PID caused by exposure to TCS was almost completely driven by the avoidance response. However, when the PID values were calculated considering the mortality from the

forced exposure, the results were overestimated (overestimation of the population downsizing caused by the mortality), since the mortality percentage was more pronounced under the forced exposure system. The ability to detect a contamination gradient and escape to more favorable habitats reduces the probability of suffering lethal effects as supposed in the forced acute tests. Notwithstanding, mortality data from forced exposure is valuable for understanding the toxicity potential of a given concentration, and is more realistic for scenarios where a contamination gradient is not expected. Furthermore, there is a high level of uncertainty in mortality data derived from a non-forced exposure, since dead organisms found in a given concentration cannot be unequivocally linked to that concentration, because they could have migrated from another concentration. Caution is required in the use of mortality data from a non-forced scenario for calculation of the PID, and such data should be employed to provide an overview of contamination effects at the ecosystem or landscape level, instead of at the individual level or at the local spatial scale.

4.3. Ecological relevance of avoidance response

It is evident that avoidance is an important response in situations where a contamination gradient occurs, since it can cause an immediate decline of the population at the local scale. According to Rosa et al. (2012), two distinct situations may occur in relation to avoidance: (i) in the case of avoidance near concentrations that exert sublethal effects (for example, on the birth rate), organisms will be able to escape before these effects are observed; (ii) if the escape only occurs near to lethal concentrations, forced exposure tests might overestimate the mortality, since only resistant organisms (those that do not die) are expected to be found in the environment.

The evaluation of contamination-driven spatial avoidance offers a complementary tool that can improve the ecological relevance of ecotoxicological risk assessment, providing crucial information concerning the ways that the presence of contaminants can affect the displacement and spatial distribution of species (Hansen et al., 1999b; Rosa et al., 2012; Araújo et al., 2016a, 2017). The relevance of this approach becomes more evident considering that avoidance is a fast response and occurs at low contaminant concentration levels at which lethal (or even sublethal) toxic effects are not expected to occur. From an ecosystem perspective, the

displacement of the population generates consequences that are as severe as mortality, since the population disappears (Moreira- Santos et al., 2008; Lopes et al., 2004).

5. Conclusions

The findings demonstrated that triclosan has the capacity to trigger an avoidance response in *P. reticulata*, inducing its displacement towards less contaminated areas. The observed response suggested that the population of *P. reticulata* could decrease at the local scale following exposure to environmentally relevant TCS concentrations lower than $2 \mu\text{g}\cdot\text{L}^{-1}$. The avoidance response was concentration-dependent and although it was not dependent on the intensity of the gradient, the gradient nonetheless influenced the AC50. The use of forced exposure could underestimate the environmental risks (population downsizing) of contamination, since it does not consider spatial avoidance as a potential response to the presence of a contaminant. On the other hand, it could overestimate lethal effects, due to the mandatory exposure of mobile organisms. Therefore, both types of exposure (forced and non-forced) should be used simultaneously in environmental risk assessments.

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4. Chapter II

Article under review (Aquatic Toxicology)

Bisphenol risk in fish exposed to a contamination gradient: triggering of spatial avoidance

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Abstract

Bisphenol A (BPA) is an emerging contaminant that is widely used in various industrial products. The environmental risk of BPA associated with sublethal effects in aquatic organisms is expected to occur at a concentration of around 500 $\mu\text{g}\cdot\text{L}^{-1}$, which is much higher than environmentally realistic concentrations found in water bodies (up to 0.41 $\mu\text{g}\cdot\text{L}^{-1}$). There is a lack of information concerning the way that a BPA contamination gradient could affect the spatial displacement of organisms by triggering avoidance responses. The hypothesis tested in this study is that in the presence of an environmentally realistic BPA contamination gradient, fish might be able to move to less contaminated areas, avoiding potential toxic effects due to continuous exposure. Therefore, the objectives of this work were: (i) to determine if

BPA could trigger an avoidance response in the freshwater fish *Poecilia reticulata*, inducing its displacement to less contaminated areas; (ii) to assess whether BPA-driven avoidance occurs at environmentally relevant concentrations; and (iii) to estimate the population immediate decline (PID) at the local scale, considering avoidance and mortality as endpoints. The sensitivity of the BPA-triggered avoidance response was compared with the ecotoxicological responses reported in other studies, and the risk was evaluated considering the concentrations indicated as safe by international agencies. Avoidance experiments were performed in a seven-compartment non-forced exposure system, in which a BPA contamination gradient was simulated. The results indicated that BPA triggered avoidance in *P. reticulata*. In a traditional forced acute toxicity test, lethal effects in 50% of the population occurred at a BPA concentration of $1,660 \mu\text{g}\cdot\text{L}^{-1}$, while in the non-forced system with a BPA concentration gradient, avoidance of 50% of the population occurred at a concentration four orders of magnitude lower ($0.20 \mu\text{g}\cdot\text{L}^{-1}$). This difference in sensitivity for exposure to BPA at environmentally relevant concentrations showed that PID was mainly determined by the avoidance response. From comparison of the data with other published studies, avoidance in *P. reticulata* populations is expected to occur at BPA concentrations below those that cause sublethal effects on fish and are considered safe by international agencies ($\leq 1 \mu\text{g}\cdot\text{L}^{-1}$). The approach used in the present study represents a valuable tool for use in environmental risk assessment strategies, providing a novel and ecologically relevant response that is complementary to traditional ecotoxicological tests.

Keywords: Avoidance, *Poecilia reticulata*, bisphenol, safe concentrations, non-forced exposure, population decline.

Resumo

Bisfenol A (BPA) é um contaminante emergente que é amplamente utilizado em vários produtos industriais. O risco ambiental de BPA associado aos efeitos subletal em organismos aquáticos pode ocorrer a uma concentração de cerca de $500 \mu\text{g}\cdot\text{L}^{-1}$, que é muito maior do que as concentrações ambientalmente realistas encontradas nos corpos de água (até $0.41 \mu\text{g}\cdot\text{L}^{-1}$). Existe uma falta de informações sobre a forma como um gradiente de contaminação de BPA pode afetar o

deslocamento espacial de organismos, desencadeando respostas de fuga. A hipótese testada neste estudo é que, na presença de um gradiente de contaminação BPA ambientalmente realista, os peixes podem se deslocar para áreas menos contaminadas, evitando potenciais efeitos tóxicos devido à exposição contínua. Portanto, os objetivos deste trabalho foram: (i) determinar se BPA poderia desencadear uma resposta de evitação no peixe de água doce *Poecilia reticulata*, induzindo seu deslocamento para áreas menos contaminadas; (ii) avaliar se a fuga orientada por BPA ocorre em concentrações ambientalmente relevantes; e (iii) estimar o declínio imediato da população (PID) na escala local, considerando a fuga e a mortalidade como respostas. A sensibilidade da resposta de evitação ao BPA foi comparada com as respostas ecotoxicológicas relatadas em outros estudos e o risco foi avaliado considerando as concentrações indicadas como seguras pelas agências internacionais. Os experimentos de evitação foram realizados em um sistema de exposição não forçada de sete compartimentos, no qual um gradiente de contaminação com BPA foi simulado. Os resultados indicaram que BPA desencadeou a fuga de *P. reticulata*. Em um teste tradicional de toxicidade aguda forçada, os efeitos letais em 50% da população ocorreram a uma concentração de BPA de $1.660 \mu\text{g}\cdot\text{L}^{-1}$, enquanto no sistema não forçado com gradiente de concentração de BPA, a fuga de 50% da população ocorreu a uma concentração de quatro ordens de grandeza inferior ($0.20 \mu\text{g}\cdot\text{L}^{-1}$). Esta diferença de sensibilidade para exposição ao BPA em concentrações ambientalmente relevantes mostrou que o PID foi determinado principalmente pela resposta de fuga. A partir da comparação dos dados com outros estudos publicados, prevê-se que a fuga em populações de *P. reticulata* ocorre em concentrações de BPA abaixo das que causam efeitos subletal em peixes e são consideradas seguras por agências internacionais ($\leq 1 \mu\text{g}\cdot\text{L}^{-1}$). A abordagem utilizada no presente estudo representa uma ferramenta valiosa para uso em estratégias de avaliação de risco ambiental, fornecendo uma resposta nova e ecologicamente relevante que é complementar aos testes ecotoxicológicos tradicionais.

Palavras-Chave: Fuga, *Poecilia reticulata*, bisfenol, concentrações seguras, exposição não forçada, declínio da população.

1. Introduction

The compound Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane) is widely found in aquatic environments, due to its use in the manufacture of many products including food and beverage packaging, flame retardants, adhesives, building materials, electronic components, and paper coatings (Fromme et al., 2002; Liu et al., 2017). In 1993, around 640,000 metric tons of BPA were produced worldwide, of which 0.017% was released as pollutants in the air, water bodies, and effluents (Staples et al., 1998). Delfosse et al. (2012) showed that the production of BPA exceeded 3 million tons per year. BPA has been found in drinking water after treatment, but at low concentrations ($0.003 \mu\text{g}\cdot\text{L}^{-1}$; Fan et al., 2013). However, in surface waters, BPA concentrations have typically been found in the range from 0.0005 to $0.41 \mu\text{g}\cdot\text{L}^{-1}$, while concentrations in effluents have been reported to vary from 0.018 to $0.702 \mu\text{g}\cdot\text{L}^{-1}$ (Fromme et al., 2002). Two more recent studies have shown average BPA concentrations of $0.026 \mu\text{g}\cdot\text{L}^{-1}$ in Taihu Lake in China (Liu et al., 2017) and $0.045 \mu\text{g}\cdot\text{L}^{-1}$ in the Paraíba do Sul River in Brazil (Silva et al., unpublished data).

The presence of BPA in water bodies is of concern, because the compound can cause lethal and sublethal toxic effects in aquatic organisms (Staples et al., 1998; Mathieu-Denoncourt et al., 2016). The mean lethal concentrations (LC_{50}) of BPA for aquatic invertebrates vary from 960 to $2,700 \mu\text{g}\cdot\text{L}^{-1}$, while the values for fish range from $6,800$ to $17,900 \mu\text{g}\cdot\text{L}^{-1}$. Chronic effects in aquatic organisms have been reported to occur at concentrations from 500 to $780 \mu\text{g}\cdot\text{L}^{-1}$ (Mathieu-Denoncourt et al., 2016). Chronic effects of BPA in fish include negative feedback on the gonadotropin-releasing hormone (GnRH) system in females (Qin et al., 2013), perturbation of molecular pathways (Chen et al., 2015), prevention of innate regenerative cell responses (such as in hair cells), and detrimental effects in sensory systems (Hayashi et al., 2015). The vast majority of research focusing on the adverse effects of BPA has involved the use of concentrations many times higher than found in natural water bodies. Although the “worst scenario” approach (using unrealistic concentrations of BPA) can help in predicting the potential long-term risk of BPA in the absence of any control strategy, the inability of ecotoxicological responses to detect toxicity at environmentally realistic concentrations prevents understanding of the actual risk of BPA.

A second problem (besides concentrations) that contributes to the lack of realism in ecotoxicological studies arises when exposure to contaminants is simulated assuming a homogeneous distribution of the contamination. In aquatic systems, contaminants often disperse, forming a dilution gradient, so the induced stress tends to be negatively correlated with the distance from the source of the contamination (Araújo et al., 2016a; López-Doval et al., 2017). In the case of mobile organisms, their displacement to locations distant from the contamination source prevents continuous exposure to contaminants. Therefore, the use of toxicity tests involving the long-term exposure of organisms under confined conditions, with no possibility of escape, could be considered unrepresentative of real exposure scenarios. In order to achieve better simulation of real contamination scenarios, where a contamination gradient is expected, a non-forced exposure system should preferably be employed (Moreira-Santos et al., 2008; Araújo et al., 2014, 2016; Silva et al., 2017). This new approach using a non-forced exposure system has been used in ecotoxicology as a complementary tool to analyze the ways that contaminants can affect the spatial distributions of organisms (Lopes et al., 2004). This changes the focus of environmental risk assessment from toxicity caused by continuous exposure at a specific level to contamination-driven habitat selection. In an experimental non-forced system with multiple compartments, organisms are free to move within a contamination gradient, hence simulating a heterogeneous aquatic environment. Evidence of contamination-driven avoidance has been found for different organisms exposed to various stress-inducing agents (see the review by Araújo et al., 2016), and studies have shown that before sublethal or lethal effects occur, organisms move towards more favorable habitats (Rosa et al., 2012; Araújo et al., 2014, Silva et al., 2017).

The hypothesis tested in the present study is that in heterogeneous environments with environmentally realistic BPA contamination gradients, fish might be able to rapidly move to less contaminated areas. Although such displacement is not considered a toxic effect, since the organisms escape from the contaminated region, contamination-driven habitat selection can have severe environmental consequences at the local scale, with declines in fish populations. Therefore, the objectives of this work were: (i) to determine whether BPA could trigger an avoidance response in the freshwater fish *Poecilia reticulata*, inducing its displacement to less contaminated

areas; (ii) to assess whether BPA-driven avoidance occurred at environmentally relevant concentrations; and (iii) to estimate the population immediate decline (PID) at the local scale (proposed by Rosa et al., 2012) caused by exposure to BPA, by integrating both mortality and avoidance responses during a short-term exposure. The sensitivity of the BPA-induced avoidance response was compared with other ecotoxicological responses reported elsewhere, and evaluation of risk was performed using the concentrations considered safe by international agencies.

The fish *P. reticulata* was selected as a test organism, since it is widely used in ecotoxicological tests, is easy to cultivate under laboratory conditions (De Liguoro et al., 2012; Pelli and Connaughton, 2015), and has shown avoidance behavior in the presence of contamination (Silva et al., 2017).

2. Materials and Methods

2.1. Test organism

A license for the use of *P. reticulata* was obtained from the Ethics Committee for the Use of Animals at the Institute of Biosciences of the University of São Paulo (Protocol 236/2015 - IB/USP). The fish were obtained from the Agency of Agribusiness and Technology (Pindaminhangaba, Brazil), where they were reared in 500 L tanks under natural conditions. Only fish up to 3 months of age and 1.2 ± 0.3 cm in size were selected. The organisms were then taken to the Ecotoxicology Laboratory of the University of São Paulo (USP, Lorena-SP, Brazil) and acclimated to the test conditions in 60 L aquaria for at least one week before the tests (OECD, 2000). During this time, the fish were fed with TetraMin flakes, and no mortality was observed. The culture water was obtained from a well and was filtered through a filter containing activated charcoal. The aquarium water was constantly aerated with an air diffuser and was changed by 30% every week. Dissolved oxygen (DO), pH, and conductivity were monitored daily (Hanna HI-9811-5 multi-parameter instrument and AlphaKit) and presented values of 6.4 ± 0.4 mg·L⁻¹, 7.0 ± 0.5 , and 120 ± 20 μS·cm⁻¹, respectively.

2.2. Bisphenol A (BPA)

For the acute toxicity and avoidance tests, stock solutions of BPA (99%, Sigma-Aldrich) were prepared in culture water at concentrations of 40,000 and 2,000

$\mu\text{g}\cdot\text{L}^{-1}$, respectively. Methanol ($4\text{ mL}\cdot\text{L}^{-1}$) was used as a solubilization agent in all the tests (controls and treatments). The concentrations used in the tests were prepared using culture water. Quantification of the target compound was performed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The analyses employed an Agilent 1200 chromatograph equipped with a binary pump, automatic injector, and thermostatically-controlled column compartment. Chromatographic separation was performed at $25\text{ }^{\circ}\text{C}$ using a Zorbax SB-C18 column ($2.1 \times 30\text{ mm}$, particle size of $3.5\text{ }\mu\text{m}$). The mobile phase consisted of ultrapure water (eluent A) and methanol (eluent B), previously filtered through membranes with $0.2\text{ }\mu\text{m}$ pore size. The eluent contained 0.01% ammonium hydroxide as an additive to favor the formation of ions. Detection and quantification employed an Agilent 6410B triple quadrupole mass spectrometer. The analytical curves were constructed using the peak areas obtained for different concentrations of the compound. The limits of detection and quantification were 6.4 and $21.2\text{ ng}\cdot\text{L}^{-1}$ ($r^2 = 0.991$), respectively. The concentrations employed in this work were checked at the beginning and end of the tests, as described above.

2.3. Acute lethal toxicity tests (forced exposure)

Forced static acute tests of BPA exposure were performed using a population of 10 fish per treatment ($n = 3$) in 1 L capacity aquaria. The animals were exposed for 72 h at a temperature of $23 \pm 2\text{ }^{\circ}\text{C}$, with a photoperiod of 12 h: 12h (light: dark), without feeding. A BPA stock solution ($40,000\text{ }\mu\text{g}\cdot\text{L}^{-1}$) was prepared on the same day as the tests, and the concentrations used in the experiments were 0 (control), 1,000, 5,000, 10,000, 20,000, and $40,000\text{ }\mu\text{g}\cdot\text{L}^{-1}$. The aquaria were constantly aerated in order to maintain a DO concentration of $>6\text{ mg}\cdot\text{L}^{-1}$. Measurements of pH, conductivity, and DO were made at the end of each test.

2.4. Non-forced exposure system

Avoidance tests were conducted in a multi-compartment system with dimensions similar to those described by Araújo et al. (2014b) (Figure 1). The system, constructed in borosilicate glass, consisted of seven 140 mL chambers interconnected using transparent and nontoxic silicone hoses, and each chamber was filled with 125 mL of solution. Before the tests with BPA, a control test ($n = 3$) was performed to

determine the non-preferential distribution of organisms in the absence of contaminants. In this test, all the chambers were filled with culture water and three fish were inserted per chamber, totaling 21 animals in the system. A homogeneous distribution of the fish was confirmed by observing their positions every 30 min during 2 h, using illumination from a red lamp to avoid interference of the observer in the displacement of the fish.

Figure 1. Static multi-compartment non-forced exposure system used in the avoidance tests. Detail is shown of one of the seven chambers (source: Araújo et al., 2014b).

2.5. Avoidance tests with BPA

The BPA concentrations used in the avoidance tests were prepared from the $2,000 \mu\text{g}\cdot\text{L}^{-1}$ stock solution on the same day as the tests. The highest concentration used in the system was based on the lethal response obtained in the forced test ($72 \text{ h-LC}_{50} = 1,660 \mu\text{g}\cdot\text{L}^{-1}$). Six concentrations were then prepared by dilution with culture water: 0 (control), 0.02, 0.2, 2, 20, 200, and $2,000 \mu\text{g}\cdot\text{L}^{-1}$. Briefly, the distribution of the concentrations in the system was obtained as follows: Plasticine plugs ($\varnothing = 1 \text{ cm}$) wrapped in PVC were inserted through the top openings of the chambers, and with the aid of a clamp were used to block the connections between adjacent chambers. Solutions of BPA at different concentrations were filled into the chambers, establishing a BPA gradient. Three fish were then transferred to each chamber, using a small net, followed by removal of the plugs to allow the fish to move freely along the system and select the most preferable chamber. The tests were performed in

triplicate, in the dark at a temperature of 23 ± 2 °C. The numbers of organisms in the chambers were observed every 30 min, with illumination from a red lamp.

2.6. Statistical analyses

Calculation of the concentration of BPA in the forced exposure system that caused 50% mortality after 72 h (72 h-LC₅₀ $\pm$ confidence interval (CI)) was performed using the PriProbit program (Sakuma, 1998).

In the non-forced approach, two-way ANOVA followed by Tukey's test ($\alpha = 0.05$) was employed to evaluate the distributions (in %) of the fish (arcsine transformed) in the control (using only culture water) and avoidance (with BPA gradient) tests. In both cases, the numbers of observation times ($n = 4$) and chambers/concentrations ($n = 7$) were fixed. If the observation time was a statistically significant factor ($p < 0.05$) for the fish distribution, then the avoidance results were based on the means (from the 3 replicates) for each time (30, 60, 90, and 120 min) separately; otherwise, if time was not a statistically significant factor ($p > 0.05$), the avoidance results were calculated from the mean values for the four times.

Quantification of the number of organisms that escaped (avoiders) from each concentration of BPA employed the equation described by Moreira-Santos et al. (2008):

$$\text{Avoiders} = N_E - N_O$$

where N_E is the expected number of organisms and N_O is the observed number of organisms. For the chamber with the highest BPA concentration, N_E was the number of organisms inserted at the beginning of the experiment (since no organism was expected to move from other chambers towards this one). For the other chambers, N_E was the number of organisms inserted at the beginning of the test plus the organisms inserted in the adjacent chamber with higher BPA concentration (assuming that the organisms would tend to move towards a less contaminated chamber). As each chamber contained three fish, the N_E values (from the most to least contaminated chamber) were 3, 6, 9, 12, 15, 18, and 21 (with the last value being for the control chamber). For a given concentration, N_O was determined considering the organisms inhabiting the studied chamber and the higher BPA concentration chambers. For example, for the highest concentration, N_O was the number of organisms observed in

that chamber; for the second most contaminated chamber (chamber #6), N_O was the organisms observed in that chamber and in the chamber with higher concentration (chamber #7); for the third most contaminated chamber (chamber #5), N_O was the organisms observed in that chamber and in the chambers with higher concentrations (chambers #6 and #7). The following equation was used to calculate the percentage avoidance for each concentration:

$$Avoidance (\%) = \left(\frac{Avoiders}{N_E} \right) \times 100.$$

The PID (population immediate decline) was calculated as described by Rosa et al. (2012):

$$PID (\%) = \left[1 - \left(1 - \frac{y}{100} \right) \cdot \left(1 - \frac{w}{100} \right) \right] \cdot 100$$

where the avoidance data (from the non-forced test) and the mortality data (from the acute forced test) were integrated in order to calculate, for each concentration, the PID (%) caused by the combination of a percentage y of mortality (LCy) and a percentage w of avoidance (ACw). In order to calculate the PID for the concentrations that were not tested in the acute forced test but were used in the avoidance test, mortality (%) data were estimated from the results of the acute forced test using the PriProbit program (Sakuma, 1998).

The BPA concentrations that triggered avoidance responses (after 2 h exposure) in 20, 50, and 80% (AC₂₀, AC₅₀, and AC₈₀, respectively $\pm$ confidence interval (CI)) of the fish population in the non-forced exposure system were determined using the PriProbit program (Sakuma, 1998).

3. Results

3.1. Acute lethal toxicity tests with BPA (forced exposure)

The lethal toxicity assays showed a marked concentration-response relationship, with 40% mortality in the fish population exposed to the lowest BPA concentration (1,000 $\mu\text{g}\cdot\text{L}^{-1}$) and 100% mortality at concentrations of 10,000 $\mu\text{g}\cdot\text{L}^{-1}$

and higher. The 72 h-LC₅₀ was 1,660 $\mu\text{g}\cdot\text{L}^{-1}$ and the corresponding confidence interval was 500-3,020 $\mu\text{g}\cdot\text{L}^{-1}$.

3.2. Distribution of organisms in avoidance tests

In the control distribution assay using culture water, the fish were displaced randomly along the system, with application of ANOVA revealing no significant differences in the percentages of organisms according to observation time ($F = 0.002$; $p = 1.00$; 3 replicates at 4 times) or chamber ($F = 1.455$; $p = 0.210$; 3 replicates at 4 times) (Table S1, Supplementary Material). The interaction between the two factors (time and chamber) was not statistically significant ($F = 0.830$; $p = 0.659$; 3 replicates at 4 times). The percentage distributions of the organisms in the chambers ranged from $12.69 \pm 1.29\%$ (chamber #6) to $16.26 \pm 0.79\%$ (chamber #7) (Figure 2).

Figure 2. Mean percentages and standard deviations ($n = 4$ observation times during 2 h) of the organisms in each chamber (control avoidance test) and for each concentration (BPA avoidance test). Different letters indicate statistically significant differences (two-way ANOVA followed by Tukey's test).

In tests with a BPA gradient, two-way ANOVA indicated that the distribution of organisms varied in relation to the concentration ($F = 100.415$; $p < 0.05$; 3

replicates at 4 times), but not in relation to the time ($F = 1.308$; $p = 0.281$; 3 replicates at 4 times), (Table S2, Supplementary Material), with a preferential distribution towards the less contaminated chambers (Figure 2). The interaction of the factors time and concentration was also statistically significant for the fish distribution ($F = 2.06$; $p < 0.05$; 3 replicates at 4 times). The mean distribution of organisms ($\pm$ standard deviation) was: $41.87 \pm 7.31\%$ ($0 \mu\text{g}\cdot\text{L}^{-1}$), $25.79 \pm 3.75\%$ ($0.02 \mu\text{g}\cdot\text{L}^{-1}$), $13.89 \pm 3.52\%$ ($0.2 \mu\text{g}\cdot\text{L}^{-1}$), $10.91 \pm 2.93\%$ ($2 \mu\text{g}\cdot\text{L}^{-1}$), $5.16 \pm 2.71\%$ ($20 \mu\text{g}\cdot\text{L}^{-1}$), $2.38 \pm 3.04\%$ ($200 \mu\text{g}\cdot\text{L}^{-1}$), and $0.00 \pm 0.00\%$ ($2,000 \mu\text{g}\cdot\text{L}^{-1}$).

3.3. Avoidance and PID

There was no mortality at any concentration during the avoidance tests. The avoidance percentage varied according to time ($F = 14.05$; $p < 0.05$; 3 replicates at 4 times) and concentration ($F = 216.06$; $p < 0.05$; 3 replicates at 4 times). The interaction between the two factors showed a weak effect ($F = 1.85$; $p < 0.05$; 3 replicates at 4 times) (Table S3, Supplementary Material). Since time was a significant factor in fish avoidance, the results were plotted considering the mean value ($n = 3$) for each time (30, 60, 90, and 120 min) (Figure 3). Two statistically similar groups were formed (group #1: 30 and 60 min; group #2: 90 and 120 min). There was a clear increase in the avoidance response in the second group (after 90 and 120 min), except for the highest concentration. The percentage avoidance values ($\pm$ standard deviations) according to concentration (0, 0.02, 0.2, 2, 20, 200, and 2,000 $\mu\text{g}\cdot\text{L}^{-1}$) for group #1 (mean values from 30 and 60 min) were $0.0 \pm 0.0\%$, $25.0 \pm 4.6\%$, $42.2 \pm 10.0\%$, $56.9 \pm 12.3\%$, $72.2 \pm 15.3\%$, $83.3 \pm 10.5\%$, and $100.0 \pm 0.0\%$. For group #2 (mean values from 90 and 120 min), the values were $0.0 \pm 0.0\%$, $37.0 \pm 12.5\%$, $63.3 \pm 11.0\%$, $76.4 \pm 6.3\%$, $90.7 \pm 8.4\%$, $100.0 \pm 0.0\%$, and $100.0 \pm 0.0\%$.

Figure 3. Concentration-response curves for the avoidance responses of *P. reticulata* exposed to a BPA gradient, for four different observation times (30, 60, 90, and 120 min). Different letters indicate statistically significant differences (two-way ANOVA followed by Tukey's test) among the concentrations (see the points in the graph) and the times (see the legend).

The concentrations that caused avoidance in 20, 50, and 80% of the fish population (AC_{20} , AC_{50} , and AC_{80}) were 0.003 (CI: 0.0003-0.01) $\mu\text{g}\cdot\text{L}^{-1}$, 0.154 (CI: 0.04-0.37) $\mu\text{g}\cdot\text{L}^{-1}$, and 6.55 (CI: 2.71-20.31) $\mu\text{g}\cdot\text{L}^{-1}$, respectively.

No dead fish were found in the non-forced system, so the PID was calculated using the percentage of dead organisms predicted from the forced exposure. Since mortality was not expected, the PID curve (Figure 4) followed almost the same pattern as the avoidance curve, especially for the lowest concentrations (mortality was only predicted to occur at the two highest concentrations). The PID values calculated from the mortality and avoidance data obtained in the forced and non-forced assays are detailed in the Supplementary Material (Table S4). The concentrations that caused PID of 20, 50, and 80% of the fish population (PID_{20} , PID_{50} , and PID_{80}) were 0.004 (CI: 0.0009-0.01) $\mu\text{g}\cdot\text{L}^{-1}$, 0.2 (CI: 0.009-0.4) $\mu\text{g}\cdot\text{L}^{-1}$, and 9.99 (CI: 5.05-22.95) $\mu\text{g}\cdot\text{L}^{-1}$, respectively.

Figure 4. Concentration-response curves for avoidance (data from non-forced exposure), mortality (estimated from forced exposure), and PID (considering the avoidance and mortality responses) for *P. reticulata* exposed to BPA.

4. Discussion

The 72 h-LC₅₀ value of 1,660 µg·L⁻¹ obtained for the fish *P. reticulata* in the forced test with BPA was lower than the values reported for other fish species such as *Xiphophorus helleri* (96 h-LC₅₀ = 17,930 µg·L⁻¹; Kwak et al., 2001), *Oryzias latipes* (72 h-LC₅₀ = 6,800 µg·L⁻¹; Kashiwada et al., 2002), and *Danio rerio* (72 h-LC₅₀ = 15,710 µg·L⁻¹; Chan and Chan, 2012). According to the review by Mathieu-Denoncourt et al. (2016), the BPA LC₅₀ range for freshwater fish is between 6,800 and 17,900 µg·L⁻¹. It can be seen that *P. reticulata* was more sensitive to BPA, compared to the other fish, showing that it is a good bioindicator for use in environmental risk assessments.

Several studies have described chronic effects of BPA in fish. Kinnberg and Toft (2003) exposed *P. reticulata* for 30 days to BPA concentrations ranging from 5 to 5000 µg·L⁻¹ and observed alterations in testicular structures at higher concentrations. Chen et al. (2015) exposed males and females of *D. rerio* to BPA at 0.228 µg·L⁻¹ for 150 days and observed changes in the proportion of males and females, together with decreases in sperm production, density, and quality in males. Little and Seebacher (2015) exposed *D. rerio* to BPA at 20 µg·L⁻¹ during 21 days and observed a reduction in swimming performance, effects on the heart rate and muscles, and altered gene expression. Huang et al. (2017) analyzed the effects of BPA at

concentrations between 50 and 1,600 $\mu\text{g}\cdot\text{L}^{-1}$ on the reproduction and development of *Oryzias melastigma* and found that for exposure periods from 6 days (embryos: 2 days post-fertilization) to 4 months (adults), there was alteration of the heartbeat in embryos, reduction of body size in females, alteration in mature ovarian follicles, and reduction of testosterone in males. Comparison of these results with the avoidance responses suggests that BPA can trigger an avoidance response at concentrations much lower ($0.02 \mu\text{g}\cdot\text{L}^{-1}$) than those considered to be sub-lethally toxic towards fish, reinforcing the notion that avoidance is an early warning response used to prevent exposure to contaminants and consequent toxic effects (Araújo et al., 2016a). Additionally, although the distribution of the organisms for the first two observation times (30 and 60 min) differed from the distribution for the last two periods (90 and 120 min, when the avoidance percentages tended to increase), it was shown that the avoidance response was very fast, with 100% of the organisms avoiding the highest concentration ($2,000 \mu\text{g}\cdot\text{L}^{-1}$) in the first 30 min.

Concentrations at which adverse effects on fish are detected are not usually found in surface waters, where values have been reported to range from 0.0005 to $0.41 \mu\text{g}\cdot\text{L}^{-1}$ (Fromme et al., 2002). Even in regions close to effluent discharges, BPA concentrations are expected to be low, since the removal of BPA at effluent treatment plants can be as high as 99.6% (Luo et al., 2014). Different safe concentrations of BPA, below which toxicity in aquatic organisms is not expected, have been established for different regions/countries, including the European Union ($1.5 \mu\text{g}\cdot\text{L}^{-1}$), Canada ($0.175 \mu\text{g}\cdot\text{L}^{-1}$), and Japan ($1.6 \mu\text{g}\cdot\text{L}^{-1}$) (USEPA, 2010). From comparison of these concentration values with those that cause sub-lethal effects in fish, it can be seen that forced tests use concentrations higher than levels considered safe, so toxicity would normally be expected to occur. On the other hand, the present results showed that at environmentally relevant BPA concentrations (as low as $0.02 \mu\text{g}\cdot\text{L}^{-1}$), there were significant avoidance responses (25 to 37%) in *P. reticulata*. Even when environmentally relevant concentrations are used in ecotoxicological studies (such as in the study by Chen et al., 2015), simulation of a homogeneous and static environment employing a forced exposure test is not necessarily relevant. In water bodies, especially those with large water volumes and/or high flows, the formation of contamination gradients can occur. For instance, López-Doval et al. (2017) showed the formation of gradients in the concentrations of chemical compounds in the

Guarapiranga reservoir (Brazil), where BPA concentrations varied temporally and spatially during the dry and rainy seasons, with a range from 0.113 to 0.345 $\mu\text{g}\cdot\text{L}^{-1}$. Therefore, the measurement of responses that can indicate the existence of risks in the absence of visible toxic effects at the individual level, focusing on the spatial distribution of the organism (such as spatial avoidance in a non-forced exposure system), is highly relevant and can improve understanding of the risk associated with the presence of BPA in the environment.

Some other recent studies with fish have adopted the non-forced exposure approach, using simulated contamination gradients. Silva et al. (2017) exposed *P. reticulata* to a gradient of triclosan. Moreira-Santos et al. (2008) and Araújo et al. (2014a) evaluated the avoidance behavior of *D. rerio* exposed to gradients of copper mine effluent and the fungicide pyrimethanil, respectively. Araújo et al. (2016b) reported avoidance behavior in *Oreochromis* sp. exposed to fish processing plant effluent. In all these studies, the fish populations moved to areas that were less contaminated.

At environmentally relevant concentrations ($<1 \mu\text{g}\cdot\text{L}^{-1}$), under non-forced exposure, BPA elicited avoidance responses in *P. reticulata*. The concentration that caused 50% avoidance in the exposed population ($\text{AC}_{50} = 0.2 \mu\text{g}\cdot\text{L}^{-1}$) was 15 times lower than the values established as safe by the European Union ($1.5 \mu\text{g}\cdot\text{L}^{-1}$) and Japan ($1.6 \mu\text{g}\cdot\text{L}^{-1}$) (USEPA, 2010). If the AC_{20} (estimated from the avoidance data using the PriProbit software) is taken to be a potentially safe concentration to prevent BPA-driven avoidance, a value of $0.004 \mu\text{g}\cdot\text{L}^{-1}$ is obtained, which is lower than the concentration of $0.175 \mu\text{g}\cdot\text{L}^{-1}$ considered safe by the Canadian government (USEPA, 2010).

For determination of the PID in a non-forced exposure scenario, the number of dead organisms should be obtained in avoidance tests employing a non-forced system. However, no mortality was observed in the non-forced exposure system. There are two possible reasons for such an absence of mortality, namely the short duration of the assay and the fact that it was a non-forced exposure. Even when mortality at the concentrations used in the avoidance tests was estimated, the data indicated that no lethal effects would be expected at any of the concentrations tested. According to the data, a population decline of 50% (PID_{50}) would be expected at $0.154 \mu\text{g}\cdot\text{L}^{-1}$ of BPA, which is an ecologically relevant concentration that lies within the expected range

(<0.4 $\mu\text{g}\cdot\text{L}^{-1}$) for water bodies (Fromme et al., 2002). The estimation of mortality using traditional acute toxicity tests can be a useful way to predict contamination-driven population declines. However, Silva et al. (2017) showed that the lethal effects towards *P. reticulata* due to exposure to triclosan in forced tests overestimated the mortality that would actually occur in an ecosystem where exposure to contaminants is avoidable due to fish evasion. In summary, even at environmentally relevant concentrations, BPA has the capacity to trigger an avoidance response in *P. reticulata*, causing displacement of the fish to less contaminated areas and consequent population decline at the local scale. Comparison of the results obtained here with the BPA values considered safe by international agencies showed that avoidance would be expected to occur at lower concentrations, before detection of any acute or chronic effects. The approach used in the present study seems to offer an important tool for use in environmental risk assessment strategies, providing a novel and ecologically relevant response that is complementary to traditional ecotoxicological tests.

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5. Chapter III

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Habitat fragmentation caused by contaminants: Atrazine as a chemical barrier
isolating fish populations

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Abstract

Information on how atrazine can affect the spatial distribution of organisms is non-existent. As this effect has been observed for some other contaminants, we hypothesized that atrazine-containing leachates/discharges could trigger spatial avoidance by the fish *Poecilia reticulata* and form a chemical barrier isolating upstream and downstream populations. Firstly, guppies were exposed to an atrazine gradient in a non-forced exposure system, in which organisms moved freely among

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the concentrations, to assess their ability to avoid atrazine. Secondly, a chemical barrier formed by atrazine, separating two clean habitats (extremities of the non-forced system), was simulated to assess whether the presence of the contaminant could prevent guppies from migrating to the other side of the system. Fish were able to avoid atrazine contamination at environmentally relevant concentrations ($0.02 \mu\text{g}\cdot\text{L}^{-1}$), below those described to cause sub-lethal effects. The AC_{50} (atrazine concentration causing avoidance to 50% of the population) was $0.065 \mu\text{g}\cdot\text{L}^{-1}$. The chemical barrier formed by atrazine at $150 \mu\text{g}\cdot\text{L}^{-1}$ (concentration that should produce an avoidance around 82%) caused a reduction in the migratory potential of the fish by 47%; while the chemical barrier at $1058 \mu\text{g}\cdot\text{L}^{-1}$ (concentration that produces torpidity) caused a reduction in the migratory potential of the fish by 91%. Contamination by atrazine, besides driving the spatial distribution of fish populations, has potential to act as a chemical barrier by isolating fish populations. This study includes a novel approach to be integrated in environmental risk assessment schemes to assess high-tier contamination effects such as habitat fragmentation and population displacement and isolation.

Keywords: avoidance; contamination gradient; guppy; habitat disturbance; non-forced exposure; population isolation.

Resumo

A informação sobre como a atrazina pode afetar a distribuição espacial dos organismos é inexistente. Como este efeito foi observado para alguns outros contaminantes, a hipótese é de que os lixiviados / descargas contendo atrazina poderiam desencadear evitação espacial pelo peixe *Poecilia reticulata* e formar uma barreira química que isolasse as populações a montante e a jusante. Em primeiro lugar, os guppies foram expostos a um gradiente de atrazina em um sistema de exposição não forçada, em que os organismos se moviam livremente entre as concentrações, para avaliar sua capacidade de evitar a atrazina. Em segundo lugar, foi simulada uma barreira química formada pela atrazina, separando dois habitats limpos (extremidades do sistema não forçado), para avaliar se a presença do contaminante poderia evitar que os guppies migrassem para o outro lado do sistema. Os peixes foram capazes de evitar a contaminação da atrazina em concentrações

ambientalmente relevantes ($0.02 \mu\text{g}\cdot\text{L}^{-1}$), abaixo das descritas que podem causar efeitos sub-letais. A AC_{50} (concentração de atrazina que causa a evitação a 50% da população) foi de $0.065 \mu\text{g}\cdot\text{L}^{-1}$. A barreira química formada pela atrazina a $150 \mu\text{g}\cdot\text{L}^{-1}$ (concentração que deveria produzir uma evasão em torno de 82%) provocou uma redução no potencial migratório dos peixes em 47%; enquanto a barreira química a $1058 \mu\text{g}\cdot\text{L}^{-1}$ (concentração que produz torpidez) provocou uma redução do potencial migratório dos peixes em 91%. A contaminação pela atrazina, além de promover a distribuição espacial das populações de peixes, tem potencial para atuar como barreira química ao isolar as populações de peixes. Este estudo inclui uma nova abordagem a ser integrada em esquemas de avaliação de risco ambiental para avaliar efeitos de contaminação de alto nível, como a fragmentação do habitat e o deslocamento da população e seu isolamento.

Palavras-chave : fuga; gradiente de contaminação; guppy; perturbação de habitat; exposição não forçada; isolamento da população

1. Introduction

Habitat fragmentation consists in a discontinuity of the habitat; caused mainly by physical, physical-chemical and biological barriers, either due to natural causes or human interference, that prevents contact between adjacent populations to some extent (Fahrig, 2003; Fuller et al., 2015; Haddad et al., 2015). Although population isolation due to a naturally fragmented habitat can be considered an intrinsic component of the landscape, when the fragmentation is produced by human intervention, loss of biodiversity and changes in ecological interactions are expected to occur (Fuller et al., 2015; Haddad et al., 2015). Human-driven habitat fragmentation (dams, culverts, channels, thermal or chemical contamination, presence of exotic species, etc.) particularly in aquatic ecosystems such as rivers, can create a barrier breaking the connectivity between up and downstream habitats and totally or partially prevent the displacement of individuals between those habitats (Braulik et al., 2014; Fuller et al., 2015). Although contamination has been included as a potential factor to cause habitat fragmentation in aquatic systems (Fuller et al., 2015), the contaminant-driven chemical barrier effect, as far as we are aware of, has not, so far, been taken into account in environmental risk assessment (ERA) schemes.

The inclusion of the concept of habitat fragmentation within an ecotoxicological context for aquatic environments has been prevented probably for conceptual and methodological reasons. Conceptually, the ecotoxicological tests used in ERA schemes are almost exclusively focused on bioaccumulation and on the lethal or sub-lethal toxic effects that chemical compounds can exert on individuals, assuming a direct and continuous exposure for a short- or long-term. Therefore, the environmental risk is directly and exclusively linked to how organisms physiologically respond to or accumulate contaminants. Due to this conceptual limitation, ecotoxicological tests are, in general, methodologically restricted to forcedly exposing organisms to contaminants and waiting for a response (effect). Later, these effects are extrapolated to biological organization levels such as community and ecosystem to check for possible changes in population size, in population performance and traits, in community structure and, finally, in ecosystem functioning. Although direct toxic effects are important and expected to occur in field situations, other important questions neglected in typical ERA schemes remain unanswered: (1) how can contaminants influence the spatial distribution of the populations? and (2) how can contaminants contribute to the loss of habitat continuity (habitat fragmentation)?

Although the traditional exposure system used in ecotoxicological tests does not allow the assessment of the behaviour of the organisms within a spatial dimension with different contamination levels, a novel approach using a non-forced exposure system has been employed in ecotoxicology as a complementary tool to assess how contaminants determine the spatial distribution of organisms (Lopes et al., 2004). Instead of being continuously exposed to a given sample or concentration of a compound, organisms are exposed to a contamination gradient with several concentrations in a free-choice, multi-compartmented, non-forced exposure system. Results obtained up to date confirm that fish present the ability to detect and spatially avoid different contaminants such as: metals (Svecevičius, 1999; Hartwell et al., 1989), domestic and industrial effluents (Smith and Bailey, 1990), ammonia and low dissolved oxygen (Richardson et al., 2001), and agrochemicals (Folmar, 1976) including atrazine (Tierney et al., 2007); thus preventing their long-term exposure and the resulting physiologically toxic effects and bioaccumulation (see review by Araújo et al., 2016a for examples of avoidance response by other organisms). From these

findings, it was hypothesized that if a contaminant at a given concentration triggers a spatial avoidance by x% (e.g. 40%) of the population, that same concentration will allow a colonization by 100-x% (e.g. 60%) of that same population (R Ribeiro, M Moreira-Santos and CVM Araújo, data not published). Therefore, the colonization of a recovering contaminated habitat can be predicted from the intensity of spatial avoidance. Applying this reasoning to up and downstream populations isolated by contamination (habitat chemical fragmentation), if contamination occurs at levels avoidable by organisms, then it is expectable that populations would be partially isolated due to the chemical barrier created by the contaminant, preventing the flux of individuals. This innovative approach of attributing a role in habitat fragmentation to contaminants provides, to ERA schemes, a novel approach not exclusively focused on the toxicity at the individual level, but also on the effects at the ecosystem and landscape levels, such as population isolation and habitat discontinuity.

The hypothesis underlying the present study was the logical deduction that a contaminant concentration provoking a 100% spatial avoidance (AC_{100}) would act as a full chemical barrier in-between adjacent undisturbed habitats, leading to a complete spatial break-up of a previously continuous population into two isolated ones. Extending this idea to other possible stressor intensities, AC_x would prevent x% of organisms to flow between the separated habitats. To test this hypothesis, which was the aim of the present study, the herbicide atrazine and the model freshwater fish *Poecilia reticulata* (guppy) were chosen. Our aims were: first, to investigate whether guppies were able to detect and avoid atrazine and, if so, to what extent atrazine determines their spatial distribution; second, to explore the potential role of atrazine as a chemical barrier contributing to the fragmentation of habitats by simulating a point source atrazine discharge into an aquatic system at a laboratory small spatial scale.

Atrazine was chosen as the test compound because it is one of the most common herbicides used to control weeds and grasses in several crops (Graymore et al., 2001; Kannan et al., 2006; Jablonowski et al., 2011; Botelho et al., 2015). In addition, atrazine has generated controversial opinions about its environmental danger (Van Der Kraak et al., 2014); consequently, it has been totally banned in the European Union since 2003 (Sass and Colangelo, 2006; Fingler et al., 2017), but it is still used in a large part of the world (USGS, 2000; Kannan et al., 2006; Jablonowski et al.,

2011; Botelho et al., 2015). Effects on fish behaviour (including avoidance responses) have been widely described (Steinberg et al., 1995; Graymore et al., 2001; Tierney et al., 2007; Pérez et al., 2013; Yan et al., 2015). The easy-to-culture guppy is a model organism in the fields of ecology, evolution and ethology and has already been shown to spatially avoid chemical contamination (Silva et al., 2017). Therefore, the fish *P. reticulata* was chosen as the test organism for the present study.

2. Materials and Methods

2.1. Test organisms

Fish (less than 3-months old and with total length of 1.2 ± 0.3 cm), provided by the *São Paulo Agency of Agribusiness and Technology* (Pindamonhangaba, SP, Brazil), were acclimated (12:12h light:dark regime, temperature of 22 to 24 °C) to the testing conditions in the ecotoxicology laboratory of the University of São Paulo (USP, Lorena, Brazil) for at least one week prior to the tests, during which time no mortalities were registered (OECD, 2000). During acclimation, the fish were kept in 60 L aquaria with water from an activated charcoal-filtered artesian well. Fish were fed (TetraMin Flakes) daily during the acclimation period, but no food was provided 24 h prior to the start and during experiments. Cultures were constantly aerated using an air diffuser and the water was renewed by 30% every week. The pH (around 7.0), conductivity (around $120 \mu\text{S}\cdot\text{cm}^{-1}$) and dissolved oxygen (DO; $>6.0 \text{ mg}\cdot\text{L}^{-1}$) values were monitored daily. The use of the fish was approved by the Ethics Committee on the Use of Animals (Protocol 236/2015 – IB-USP).

2.2. Atrazine

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine; Pestanal, analytical **standard, Sigma-Aldrich, 99.1% purity**) was used in the tests; its solubility under the testing conditions was of $34.7 \text{ mg}\cdot\text{L}^{-1}$. A stock solution of $10 \text{ mg}\cdot\text{L}^{-1}$ of atrazine (total volume of 2 L) was prepared in culture water. This suspension was sonicated during 30 min until total dissolution. The dilutions to prepare the test solutions were made with the fish culture water. Atrazine concentrations used in the avoidance and chemical barrier tests were prepared in two separate sets, by serial dilutions starting from the stock solution (e.g. in the chemical barrier test, the concentration of $1000 \mu\text{g}\cdot\text{L}^{-1}$ was prepared by diluting 10 times the

stock solution and the concentration of $100 \mu\text{g}\cdot\text{L}^{-1}$ by diluting 10 times that of $1000 \mu\text{g}\cdot\text{L}^{-1}$). Atrazine concentrations in each compartment were checked at the end of all tests (pool of the four replicates) to verify initial nominal concentration. The mean values between the nominal and actual atrazine concentrations at the beginning and at the end of the avoidance tests, respectively, were used for calculations (see Supplementary Material), as we assumed that those were the concentrations to which organisms were exposed during the tests. The quantification of atrazine was performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) after solid phase extraction of the samples using OASIS HLB cartridges 6cc, 500 mg (Waters, Milford, MA, USA), at the Institute of Chemistry of the University of Campinas (Brazil), following the methodology described by Montagner et al. (2014). The equipment used was an Agilent (Santa Clara, CA, USA) model 1200 chromatograph equipped with a binary pump, an automatic injector, and a temperature-controlled column compartment. Chromatographic separation was achieved with a Zorbax (Agilent) SB-C18 column (2.1 x 30 mm, particle size of $3.5 \mu\text{m}$) at 25°C . The limits of detection and quantification of the method were 0.3 and $0.9 \text{ ng}\cdot\text{L}^{-1}$, respectively ($r^2 = 0.998$). For more details, see Supplementary Material.

2.3. Non-forced exposure system

The avoidance tests were performed in a static, free-choice and non-forced exposure system composed of seven tandem interconnected compartments (Figure 1; Araújo et al., 2014). Each compartment was constructed from borosilicate glass bottles (VBTR) connected with sections of nontoxic transparent silicon hose. The total length of the system was 105 cm. During tests, the compartments were filled with 125 mL of test solution consisting either of fish culture water (control) or atrazine-contaminated culture water at different concentrations totalling a volume of 875 mL in the entire system.

Figure 1. Diagram of the static multi-compartment non-forced avoidance test system, highlighting one of the seven compartments (from Araújo et al., 2014). A plug and the pin used to isolate the compartments are also represented.

2.4. Avoidance test

A control of the avoidance test, with four replicates (two temporal replicates repeated twice), was performed to disprove any preference or avoidance behaviour for any compartment of the system. For this, the non-forced exposure system was filled with culture water (125 mL in each compartment) and three organisms were transferred to each compartment of the system (Araújo et al., 2014). Then, the number of fish in each compartment was recorded at each 30 min during 3 h (at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 h).

For the avoidance test with atrazine, seven concentrations were tested in four replicates (with an experimental design similar to that of the control test). Nominal concentrations at the start of the test were 0, 0.01, 0.02, 0.1, 1.0, 10, and 100 $\mu\text{g}\cdot\text{L}^{-1}$, and actual concentrations at the end of the test, used for calculation, were 0.002 (considered control), 0.02, 0.02, 0.3, 1.9, 11.2, and 105 $\mu\text{g}\cdot\text{L}^{-1}$. To avoid mixing of test solutions between concentrations in adjacent compartments at the beginning of the tests, before dispensing the test solutions into the system, the compartments were isolated from each other using plasticine plugs wrapped in Parafilm film. Three fish were introduced into each concentration (seven compartments in each system; totalling 21 individuals). Then, the plugs were carefully and immediately removed by using a pin (Figure 1). Exposure lasted 3 h and observations were made at each 30 min. This short exposure period was used because previous experiments indicated

that avoidance is a very fast response and because the continuous swimming of the fish could increase the mixing of the atrazine concentrations, disrupting the gradient (Araújo et al., 2014; Silva et al., 2017). Both control and avoidance tests were performed at a temperature of 22 to 24 °C, in the dark. Observations were made using a red light to avoid the observer influencing the organisms' displacement. Conductivity, pH and DO values were checked at the beginning (0 h) and end (3 h) of both the control and avoidance tests and no variability was observed.

2.5. Chemical barrier tests

Two chemical barrier tests were performed independently using two atrazine concentrations (initial nominal concentrations: 100 and 1000 $\mu\text{g}\cdot\text{L}^{-1}$), prepared as described-above. These concentrations were chosen as previous results had showed that the former causes an avoidance of almost the entire population (around 80%) and the latter causes torpidity (the loss of the ability to move), preventing the avoidance response. The actual atrazine concentrations at the end of the tests (pool of four replicates) were 150 and 1058 $\mu\text{g}\cdot\text{L}^{-1}$. For these tests, the distribution of culture water and atrazine-contaminated test solutions in the free-choice non-forced system was different relative to that in the avoidance test: the culture water was put in the first two compartments (compartments #1 and #2, considered the downstream zone) and in the last two compartments (compartments #6 and #7, considered the upstream zone), and the atrazine test solution (150 or 1058 $\mu\text{g}\cdot\text{L}^{-1}$) was placed in the three central compartments (#3, #4 and #5). The procedure used to dispose atrazine and culture water into the system was modified from that previously described: only the connections between compartments #2 and #3 and between compartments #5 and #6 were isolated with the plugs. Afterwards, 21 organisms were introduced in compartment #1 and the plugs were immediately removed. A control tests were also carried out to verify the distribution of the organisms during the chemical barrier tests in the absence of contamination. For this test, the system was filled only with culture water and 21 organisms were equally all introduced in the first compartment. The exposure lasted 3 h and observations were made at each 30 min for both the control and chemical barrier tests. The tests were also performed in quadruplicate (two temporal replicates repeated twice), at a temperature of 22 to 24 °C, in the dark (as mentioned above, a red light was used during observations). Conductivity, pH and

DO values were checked at the beginning (0 h) and end (3 h) of each test and no variability was observed.

2.6. Statistical analyses

A two-way ANOVA (on arcsine transformed data and with observation times and compartments as fixed factors) followed by a chi-square test comparing the expected distribution (12 organisms per compartment) with the observed distribution (average of organisms during the six observation periods after pooling the four replicates) was used in the control of the avoidance test. The purpose was to check the absence of differences among the six observation times (allowing their pooling for further statistical analysis) and the uniform distribution of fish in the control for the avoidance test, proving the absence of preference for any compartment. Although chi-square is a non-parametric test, it was applied to check absence of preference among compartments (distribution of the fish in the control of the avoidance test is random) because it is one of the most robust tests to determine significant differences between expected and observed frequencies (Zar, 1996). In the avoidance test with atrazine, a two-way ANOVA was employed to check for differences in organism distribution among the seven different concentrations during the exposure, followed by a Tukey test.

To calculate avoidance (%), the number of avoiders was determined for each compartment as the difference between the expected (N_E) and the observed numbers (N_O): Avoiders = $N_E - N_O$. The N_E was determined as described by Moreira-Santos et al. (2008): for the compartment with the highest concentration of the compound, N_E was equal to the number of fish introduced into the compartment at the beginning of the test. For the remaining compartments, N_E included the organisms initially introduced into the compartment, plus the organisms introduced into the adjacent compartment(s) with higher concentration(s). Initially, as each compartment contained three fish, for the most contaminated compartment $N_E = 3$. For the adjacent compartment $N_E = 6$, while for the last compartment $N_E = 21$. Regarding the number of observed organisms (N_O) for each concentration, it represented the organisms found in that compartment and those found at higher concentrations; for the compartment with no atrazine no avoidance was expected, as $N_O = 21$. The avoidance percentage for each compartment was calculated as follows: $(\text{Avoiders}/N_E) \times 100$.

The avoidance percentages for each concentration were used to obtain the AC_{50} values (concentration causing avoidance by 50% of the exposed organisms) and the corresponding 95% confidence interval (CI). These calculations were performed using the PriProbit software (Sakuma, 1998).

The organism percentages in the two chemical barrier tests and the respective controls were determined per compartment (#1 to #7) and per zone (downstream, chemical barrier and upstream). Two-way ANOVA (on arcsine transformed data and with observation times and compartments as fixed factors) followed by Tukey tests were used to determine statistically significant differences in the distribution of organisms among compartments (#1 to #7) at all observation moments. One-way ANOVA followed by a Tukey test was then performed to detect statistically significant differences among the percentages of organisms (data pooled from the six observation moments, as no significant differences were found across observation times) of the three (control and the two treatments with atrazine) chemical barrier tests for each compartment and for each zone. From the difference between the organisms' distribution in the control test without atrazine and in the chemical barrier tests, two parameters were calculated. First, the emigration inhibition (%), which is the relative reduction, compared to the control, in the number of organisms that left the downstream zone (considering organisms that colonized compartments #3 to #7, i.e., that moved from the downstream zone). Second, the crossing inhibition (%), which is the relative reduction, compared to the control, in the number of organisms that attained the upstream zone (only organisms in compartments #6 and #7 were considered). This parameter measures the efficacy of the chemical barrier in preventing the organisms from crossing it.

3. Results

3.1. Avoidance test

Mortality was found neither in the control nor in the avoidance test with atrazine. In the control for the avoidance test, the fish distribution was constant (Supplementary Material - Table S3) during the exposure period (ANOVA: $F = 0.126$; $p = 0.99$; $n = 84$) and homogeneous, with no preference for any compartment (chi-square: $p > 0.30$). The two-way ANOVA with the avoidance test data indicated that the percentage of organisms in each compartment did not vary with time ($F =$

0.623; $p = 0.68$; $n = 84$), but it varied with concentration ($F = 65.7$; $p < 0.001$; $n = 84$) (see distribution of organisms and statistical analyses in Supplementary Material - Tables S4 to S6), with a preferential distribution towards the uncontaminated compartment, where around 50% of the organisms were found (Figure 2). Around 15% of the organisms were found in the compartments with the concentration ($0.02 \mu\text{g}\cdot\text{L}^{-1}$), and from there on 7.9, 6.7, 5.2, and 2.8% of organisms were found at 0.3, 1.9, 11.2, and $105 \mu\text{g}\cdot\text{L}^{-1}$, respectively. Atrazine triggered avoidance in a concentration-dependent manner (Figure 2). The calculated AC_{50} was 0.065 (CI: $0.021 - 0.150$) $\mu\text{g}\cdot\text{L}^{-1}$.

Figure 2. Mean percentage ($\pm$ standard deviation) of organisms (after pooling the six sequential observations) distributed along the system in the avoidance tests with atrazine (black circles) and in the control (containing only culture water; white circles) and avoidance (in %; white crossed squares), with respective standard deviation, of the fish *Poecilia reticulata* caused by an atrazine gradient in a non-forced exposure system. Two X-axis are presented: with the atrazine concentrations (for the avoidance tests with atrazine) and with the number of compartments (for the control test with no atrazine). Different letters indicate statistically significant

differences (Tukey test: $p < 0.05$) for distribution of the organisms in avoidance test. Letters are not presented for the control as there were no statistical differences (see description of results).

3.2. Chemical barrier tests

No mortality was observed in the tests (control and chemical barrier with atrazine at 150 and 1058 $\mu\text{g}\cdot\text{L}^{-1}$). Distribution of organisms per compartment is presented in Supplementary Material (Tables S7 to S9). Results of three two-way ANOVA tests for the distribution of the organisms showed that the times of observation did not influence the response in the tests ($F = 0.159, 0.296$ and 0.182 , respectively; p values > 0.9 ; $n = 84$) (Supplementary Material - Tables S10, S11 and S12, respectively). The percentages of the distribution of the organisms (pooled data from six sequential observations: 0.5 to 3.0 h) during the chemical barrier treatments and the respective control are presented both per compartment and per zone in Figures 3A and 3B, respectively.

In general, for the same compartment, the percentage of organisms in the control test was statistically different (Tukey test: $p < 0.05$) from either of the two chemical barrier treatments, as it was lower and higher in the downstream and upstream zones, respectively, indicating that more fish left the downstream zone in the control than in either of the two chemical barrier treatments and more fish were able to reach upstream in the control at 150 $\mu\text{g}\cdot\text{L}^{-1}$, than in the chemical barrier treatment with 1058 $\mu\text{g}\cdot\text{L}^{-1}$ atrazine (Figure 3A).

Figure 3. Mean percentage ($\pm$ standard deviation) of organisms (after pooling the six sequential observations; $n=6$) distributed along the seven compartments (A) and along the three zones of the system (B) during the control test (containing only culture water) and in the two tests with chemical barriers (CB) of atrazine at 150 and 1058 $\mu\text{g}\cdot\text{L}^{-1}$. Downstream (the two first compartments) represents the first zone with culture water in which organisms were introduced; CB (three compartments) represents the chemical barrier zone containing either an atrazine concentration of 150 $\mu\text{g}\cdot\text{L}^{-1}$ (CB – 150) or 1058 $\mu\text{g}\cdot\text{L}^{-1}$ (CB – 1058); Upstream (the two last compartments) represents the zone containing culture water after the chemical barrier. Different letters indicate statistically significant differences (Tukey test; $p<0.05$) among tests for each compartment and for each zone.

The latter pattern was more evident when the percentage of organisms was compared per zone (Figure 3B), where the control differed significantly from the two chemical barrier tests both downstream and upstream, though significant differences between both chemical barrier treatments were only found downstream and in the chemical barrier zone.

From the organism distribution across the three zones, the emigration inhibition and crossing inhibition were calculated (Table 1). Results showed that the effects of the chemical barrier on fish population were more severe the higher the

atrazine concentration. Compared with the results of the avoidance test, an atrazine concentration of $105 \mu\text{g}\cdot\text{L}^{-1}$ caused an avoidance of 80.6% (standard deviation: $\pm 17.2\%$) that is statistically (unpaired t test with Welch correction; Welch's approximate $t = 3.429$ and two-tailed $p = 0.009$) higher than the effect (around 51%) caused as a chemical barrier (for concentration of $150 \mu\text{g}\cdot\text{L}^{-1}$). Extrapolating beyond the tested range (from the avoidance test percentages and using PriProbit), the avoidance intensity for a concentration of $1058 \mu\text{g}\cdot\text{L}^{-1}$, would be around 86.2% (standard deviation: $\pm 13.0\%$), which is statistically (unpaired t test with Welch correction; Welch's approximate $t = 1.447$ and two-tailed $p = > 0.05$) similar to the value of 94.2% observed for the chemical barrier effect.

Table 1. Effects of the chemical barrier due to contamination by atrazine (at 150 and $1058 \mu\text{g}\cdot\text{L}^{-1}$) on the emigration and crossing inhibition of the population of the fish *Poecilia reticulata*.

Effects of the chemical barrier (%)	$150 \mu\text{g}\cdot\text{L}^{-1}$	$1058 \mu\text{g}\cdot\text{L}^{-1}$
Emigration inhibition (population that did not cross the contamination)	47.5 ± 20.7	91.8 ± 10.9
Crossing inhibition (reduction in the migratory potential)	51.4 ± 11.8	94.2 ± 3.8

4. Discussion

Studies of atrazine in aquatic ecosystems from different regions have shown values ranging from less than 0.001 up to $1000 \mu\text{g}\cdot\text{L}^{-1}$ (Graymore et al., 2001; USGS, 2000; Battaglin et al., 2009; Sangchan et al., 2014; Botelho et al., 2015; Sousa et al., 2016; Fingler et al., 2017; Starr et al., 2017). Therefore, the results of the present study showed that atrazine, at environmentally relevant concentrations, is a contaminant capable of triggering the avoidance response in the fish *P. reticulata*. The ability to detect and avoid contaminants has been observed for different fish species exposed to metals (Svecevičius, 1999; Hartwell et al., 1989), domestic and industrial effluents (Smith and Bailey, 1990), ammonia and low dissolved oxygen (Richardson et al., 2001), and different agrochemicals (Folmar, 1976) including atrazine (Tierney et al., 2007). Moreover, inside a free-choice, non-forced exposure system with a contamination gradient, avoidance has been similarly described for

Danio rerio exposed to copper, acid mine drainage (Moreira-Santos et al., 2008) and the fungicide pyrimethanil (Araújo et al., 2014) and *Oreochromis* sp. exposed to fish processing plant effluents (Araújo et al., 2016b). It has been observed that the uptake of atrazine by fish occurs quickly, in the first 2 h of exposure (Yan et al., 2015); therefore, under natural conditions, as avoidance is a very fast response (30 min in the present study), fish might avoid the uptake of atrazine due to continuous exposure by swimming to less disturbed habitats, thus reducing the expected toxicity at the individual level (Saglio and Trijasse, 1998). Consequently, this avoidance behaviour could determine, to some extent, the spatial distribution of the fish in natural systems.

In the present study, an avoidance response was observed at concentrations as low as $0.02 \mu\text{g}\cdot\text{L}^{-1}$ (avoided by 40% of the exposed *P. reticulata* population), demonstrating it to be a highly sensitive response; indeed almost all the population (80%) avoided atrazine at a concentration of $105 \mu\text{g}\cdot\text{L}^{-1}$. When compared to lethal responses, the death expected of 50% of the population (LC_{50}) for many fish species was estimated to occur at much higher concentrations: the LC_{50} (exposure time was not indicated) of atrazine was 9.37 and $6.37 \text{ mg}\cdot\text{L}^{-1}$ for *O. niloticus* and *Chrysichthyes auratus*, respectively (Hussein et al., 1996), the 96-h LC_{50} for *Channa punctatus* was $42.3 \text{ mg}\cdot\text{L}^{-1}$ (Nwani et al., 2010), for *BrachyDanio rerio* it was $29.1 \text{ mg}\cdot\text{L}^{-1}$ (Yan et al., 2015) and $15.6 \text{ mg}\cdot\text{L}^{-1}$ (Wang et al., 2017), and for *Cyprinus carpio* it was and $2.14 \text{ mg}\cdot\text{L}^{-1}$ (Xing et al., 2015). The results cited, including the no-observed-effective concentration (NOEC) of $3 \text{ mg}\cdot\text{L}^{-1}$ estimated for lethality (Yan et al., 2015), are around two orders of magnitude higher than the 3-h AC_{50} : $0.065 \mu\text{g}\cdot\text{L}^{-1}$ and could prevent avoidance behaviour due to the torpidity effect (which was observed in our laboratory at $1 \text{ mg}\cdot\text{L}^{-1}$; results not shown).

Regarding sub-lethal effects, it is recognized that atrazine exposure can produce different effects in fish such as: reduction in plasma protein in *O. mykiss* as of $50 \mu\text{g}\cdot\text{L}^{-1}$ and depression of blood leucocrit in *Galaxias maculatus* exposed to 3 and $50 \mu\text{g}\cdot\text{L}^{-1}$ (Davies et al., 1994), renal alterations in *O. mykiss* with $5 \mu\text{g}\cdot\text{L}^{-1}$ exposure (Fischer-Scherl et al., 1991), accelerated respiration and increased rate of gill cover movements in *O. niloticus* and *C. auratus* at $3 \text{ mg}\cdot\text{L}^{-1}$ (Hussein et al., 1996), and changes in the detoxication system of *D. rerio* as of $1 \text{ mg}\cdot\text{L}^{-1}$ (Wiegand et al., 2001). More recently, other effects caused by atrazine exposure have been described: DNA damage and enzymatic stress in *D. rerio* at 2.5, 5 and $10 \text{ mg}\cdot\text{L}^{-1}$ (Zhu

et al., 2011), multiple effects of histopathological, biochemical and physiological character in *Rhamdia quelen* at $2 \mu\text{g}\cdot\text{L}^{-1}$ (Mela et al., 2013), induction of micronuclei and nuclear abnormalities in erythrocytes of *D. rerio* at $0.5 \mu\text{g L}^{-1}$ (Botelho et al., 2015), disruption in the immune response of *C. carpio* from $4 \mu\text{g}\cdot\text{L}^{-1}$ (Xing et al., 2015) and of *D. rerio* from $30 \mu\text{g}\cdot\text{L}^{-1}$ (Liu et al., 2017), and inhibition of acetylcholinesterase (AChE) in *D. rerio* as of $30 \mu\text{g}\cdot\text{L}^{-1}$ (Liu et al., 2016). When these data are contrasted with the avoidance response observed in the present study, this latter response seems to be triggered at much lower concentrations, which indicates that avoidance might prevent (after very short-term exposure) any succeeding toxic effects. Considering the concentrations of the above-mentioned responses, in the range of 1 to $10 \text{ mg}\cdot\text{L}^{-1}$ of atrazine, the expected avoidance would be close to 70% (60 to 80%), whereas for atrazine concentrations within 0.1 and $1 \text{ mg}\cdot\text{L}^{-1}$ the intensity of avoidance would still be around 55% (50 to 60%).

Even for studies focusing on behavioural alterations in forced exposure conditions (responses evaluated for a series of independent concentrations), the responses measured were only altered at atrazine concentrations higher than those causing avoidance in the present study. For instance, a slowing down of reflexes and swimming movements were recorded in *O. niloticus* and *C. auratus* at 3 and $6 \text{ mg}\cdot\text{L}^{-1}$ (Hussein et al., 1996); hypoactivity was observed in *C. carpio* exposed to relatively lower atrazine concentrations ($4 \mu\text{g}\cdot\text{L}^{-1}$, Xing et al., 2015); at $1 \mu\text{g}\cdot\text{L}^{-1}$ atrazine reduced aggressive mating signals in males of *P. reticulata* (Shenoy, 2012); and at still lower concentrations ($0.5 \mu\text{g}\cdot\text{L}^{-1}$) atrazine caused burst swimming reactions in *C. auratus* (Saglio and Trijasse, 1998). Even for the lowest concentration reported here, the expected avoidance, according to the results of the present study, would be higher than 50%. Although atrazine impairs the swimming pattern of fish, due to its effects on the sensory organs and nervous system (Graymore et al., 2001; Tierney et al., 2007), such effects are only expected when the possibility of escaping is prevented by the very high concentrations causing torpidity (in a preliminary study we detected torpidity in *P. reticulata* exposed to $10 \text{ mg}\cdot\text{L}^{-1}$ of atrazine; see also the examples with other contaminants and organisms described by Araújo et al., 2016a). In y-mazes exposure experiments (countercurrent olfactometer), for instance, it was shown that exposure to atrazine (1, 10 and $100 \mu\text{g}\cdot\text{L}^{-1}$) affected neurophysiological responses and the behavioral responses based on olfaction, reducing thus the preference response to

the amino acid L-histidine and causing alterations in the fish activity (Tierney et al., 2007).

Besides triggering avoidance, it was shown in the present study that atrazine can cause a habitat fragmentation by forming a chemical barrier that isolates fish populations. This effect has not, until now, been explored in ERA schemes, which have particularly focused their approach on toxic effects at the individual level. However, apart from being a potentially toxic compound (as discussed above), our results add a novel deleterious role of atrazine (which can most probably be applied to other contaminants) as an environmental disturber and fragmentor, by breaking the habitat connectivity in aquatic ecosystems. The problems of human-driven habitat discontinuity in aquatic ecosystems have been mainly discussed associated to the construction of physical barriers that, in the long term, can provoke a reduction in biodiversity (up to the level of a loss in genetic variability), changes in migratory patterns, an increase in the population vulnerability when restricted to small habitable patches (specially in downstream populations), and impairment in ecosystem functions (Braulik et al., 2014; Fuller et al., 2015). However, typical problems linked to chemical barriers, such as the depletion of oxygen and hot water discharges, have also been studied (Fuller et al., 2015): for instance, poor water quality acted as a migration barrier for the upstream movement of *Alosa fallax fallax* in the River Scheldt (West Europe) (Maes et al., 2008) and water physical-chemical characteristics (e.g., conductivity, pH, DO, turbidity, acidity, the clearwater-blackwater gradient) constituted a chemical barrier for many fish species in the Orinoco and Amazon basins (Winemiller et al., 2008). It is important, therefore, to include this novel approach to the chemical barrier effect when ecological risks of contaminants are evaluated. To know the threshold concentration of any contaminant that creates a habitat fragmentation is as important to know as the threshold of lethal and sub-lethal effects at the individual level.

Results of the present study suggest that environmentally relevant concentrations can be a risk for an ecosystem by affecting the spatial distribution of the organisms and causing a chemical barrier effect. Our results also suggest that the analysis of the maximum allowable toxicant concentrations for atrazine of 3 to 10 $\mu\text{g}\cdot\text{L}^{-1}$, derived from physiological responses of fish species (Davies et al., 1994), and 17.9 $\mu\text{g}\cdot\text{L}^{-1}$, derived from effects in oxygen production and the ability of communities

(bacteria, fungi, protozoa, algae, and micrometozoans) to sequester magnesium and calcium (Pratt et al., 1998), should be considered more prudently as the expected avoidance at those concentrations would be: 67.5, 72.5 and 74.5%, respectively. Since atrazine presents moderate solubility and can persist for many months in the aquatic ecosystems (Graymore et al., 2001; Kannan et al., 2006; Jablonowski et al., 2011), the risk may be prolonged over time, making atrazine a contaminant of great environmental concern.

Finally, the approach used in the current study has showed that avoidance is a very fast response, capable of identifying potentially toxic environments after only very short-term exposures (3 h and even at a shorter time – 30 min – if one considers that there were no statistically significant differences among observation moments). Regarding the logical deduction that a contaminant concentration provoking a 100% spatial avoidance (AC_{100}) would act as a full chemical barrier between adjacent undisturbed habitats, our results have showed that the intensity of population isolation was significantly different from those expected from the avoidance results. It is possible that the spatial dimension of the chemical barrier in the non-forced exposure system plays an important role for organisms deciding whether to explore or not and cross the chemical barrier: organisms might explore the chemical barrier more than was expected from the results of the avoidance tests if the spatial extension to be crossed is short.

5. Conclusions

The fish *P. reticulata* was able to detect and avoid atrazine at environmentally relevant concentrations. Compared to lethal and many sub-lethal responses published in the literature, avoidance was shown to occur at much lower concentrations and in a much shorter exposure period. Contamination by atrazine can influence the spatial distribution of the fish populations. Moreover, atrazine can cause a habitat fragmentation by forming a chemical barrier that can totally or partially isolate populations. Finally, it is possible to conclude that the non-forced exposure system can help to understand how contaminants can affect the spatial distribution of organisms, even when toxic effects, at the individual level, are not expected to occur, providing a novel and pertinent approach to be employed in ERA schemes.

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6. Chapter IV

This article will soon be submitted to the Science of the Total
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Influence of interspecific interactions on avoidance response to contamination

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Abstract

The entry of contaminants into water bodies can reduce the quality of water and sediment, causing disturbances to the community structure and the entire balance of the system. The biological consequences resulting from the exposure to contaminants are commonly attributed to damage caused to individuals, including lethal and sublethal effects. However, some studies have indicated that organisms are able to

detect and avoid contamination, preventing toxic effects. Regarding interspecific interactions, one species may affect the distribution of another species by competition for food, space, or other factors intrinsic to each population. 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 displacement, the main question addressed in the present study is: Does interspecific interaction between the freshwater fishes *Danio rerio* (zebrafish) and *Poecilia reticulata* (guppy) change their patterns of contamination avoidance? The hypothesis adopted was that the spatial distributions of both fish populations exposed to a contamination gradient would be affected by interspecific interactions and that the less aggressive species would be displaced towards previously avoided contaminated habitats. Copper (Cu) was selected as the test contaminant, due to its worldwide importance as a pollutant and the adverse effects that it can cause in fish. The fishes *P. reticulata* and *D. rerio* were selected as test organisms. Non-forced avoidance tests were performed in a static free-choice exposure system composed of seven interconnected chambers. In order to determine whether the avoidance response to copper of one species was affected by the presence of the other species, rather than being influenced by the population densities, two distinct approaches were used: (1) monospecific tests, in which only one species was present in the system, at two different population densities; and (2) multispecific tests, in which both species were tested simultaneously in the system. In the control monospecific tests with *P. reticulata* and *D. rerio*, and in the control multispecific test considering *D. rerio*, the distribution of organisms was random, while in the control multispecific test considering *P. reticulata*, the fish distribution showed a weak statistical difference among the chambers. In the monospecific tests with a copper gradient, the different concentrations led to fish avoidance (with migration to less contaminated areas), while in multispecific tests with a copper gradient, *P. reticulata* influenced the dispersion of *D. rerio* (with a reduction in migration potential). This study shows the importance of understanding the interactions among fish in contaminated areas, and the way that one species can prejudice the avoidance behavior of another species.

Keywords: Avoidance, guppy, zebrafish, interspecific interaction, behavioral change

Resumo

A entrada de contaminantes em corpos d'água pode reduzir a qualidade da água e dos sedimentos, causando distúrbios na estrutura da comunidade e de todo o equilíbrio do sistema. As consequências biológicas resultantes da exposição a contaminantes são comumente atribuídas a danos causados a indivíduos, incluindo efeitos letais e sub-letais. No entanto, alguns estudos indicam que os organismos são capazes de detectar e evitar a contaminação, evitando efeitos tóxicos. Em relação às interações interespecíficas, uma espécie pode afetar a distribuição de outra espécie pela competição por alimentos, espaço ou outros fatores intrínsecos a cada população. Portanto, considerando que (i) o peixe pode evitar a contaminação, movendo-se espacialmente para habitats menos contaminados, e (ii) a contaminação pode afetar o comportamento dos peixes em termos de agressividade, competição e deslocamento, a principal questão abordada no presente estudo é: A interação interespecífica entre os peixes de água doce *Danio rerio* (peixe zebra) e *Poecilia reticulata* (guppy) altera seus padrões de fuga à contaminação? A hipótese adotada foi que as distribuições espaciais de ambas as populações de peixes expostas a um gradiente de contaminação seriam afetadas por interações interespecíficas e que as espécies menos agressivas seriam deslocadas para habitats contaminados previamente evitados. O cobre (Cu) foi selecionado como contaminante de teste, devido à sua importância mundial como poluente e os efeitos adversos que pode causar aos peixes. Os peixes *P. reticulata* e *D. rerio* foram selecionados como organismos teste. Os testes de evitação não forçada foram realizados em um sistema estático de exposição de livre escolha composto por sete câmaras interligadas. Para determinar se a resposta de evitação ao cobre de uma espécie foi afetada pela presença da outra espécie, em vez de ser influenciada pelas densidades populacionais, utilizaram-se duas abordagens distintas: (1) testes monoespecíficos, em que apenas uma espécie estava presente no sistema, em duas densidades populacionais diferentes; e (2) testes multiespecíficos, em que ambas as espécies foram inseridas simultaneamente no sistema. Nos testes controle monoespecíficos com *P. reticulata* e *D. rerio* e no teste controle multispecífico considerando *D. rerio*, a distribuição de organismos foi aleatória, enquanto no teste controle multispecífico considerando *P. reticulata*, a distribuição de peixes apresentou uma fraca diferença estatística entre as câmaras. Nos testes monoespecíficos com gradiente de cobre, as diferentes concentrações levaram à fuga

dos peixes (com migração para áreas menos contaminadas), enquanto em testes multispecíficos com gradiente de cobre, *P. reticulata* influenciou a dispersão de *D. rerio* (com redução no potencial de migração). Este estudo mostra a importância de compreender as interações entre peixes em áreas contaminadas e a forma como uma espécie pode prejudicar o comportamento de fuga de outra espécie.

Palavras chave: Fuga; guppy; zebrafish; interação interespecífica; alteração comportamental

1. Introduction

The entry of contaminants into water bodies can reduce the quality of water and sediment, causing disturbances to the community structure and the entire equilibrium of the system (Crévecœur et al., 2011; Kuzmanovi et al., 2016). The biological consequences resulting from the exposure to contaminants are commonly related to damage caused to individuals, including lethal and sublethal effects (Martinez-Haro et al., 2015). Generally, these effects induced by contamination are studied by means of ecotoxicological tests with forced exposure, which assume a direct and continuous exposure to contaminants. However, although less frequent, some earlier studies have indicated that organisms are able to detect and avoid contamination, thereby preventing toxic effects. Folmar (1976) exposed the fish *Salmo gairdneri* (rainbow trout) to nine herbicides in a bi-compartmentalized system and observed the frequency with which the fish stayed in the clean or contaminated 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 were prevented by the avoidance response to contamination.

Recently, a new approach using a free-choice, multi-compartmented, non-forced system was applied in ecotoxicological studies to examine the avoidance response and the way that contaminants affected the spatial distribution of individuals (Lopes et al., 2004). This non-forced exposure approach aims at simulating a contamination gradient so that the effects are no longer measured in individuals, but instead on their spatial distribution. This expands the concept of environmental disturbance beyond toxicity at the organism level. Assessment of the avoidance

response in a multi-compartmented exposure system enables an analysis based on the migration of a population from a contaminated habitat towards an uncontaminated (or less contaminated) one. Evidence of contamination-driven spatial displacement (avoidance response) in non-forced exposure systems has been described for organisms including fish (Moreira-Santos et al., 2008; Araújo et al., 2014; Silva et al., 2017), amphibians (Vasconcelos et al., 2016), and invertebrates (Lopes et al., 2004; Araújo et al., 2016) exposed to different contaminants: pyrimethanil (fungicide), triclosan (bactericide), abamectin (biopesticide), copper, and effluent from a tuna fish processing plant.

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 determines 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 when the environment was shaded, the avoidance response was suppressed. 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 process of 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 (immediate importance attractive factor) in the avoidable habitat can change the spatial distribution pattern.

Interspecific interaction can involve the effect of one species on the distribution of another by competition for food, space, or other factors intrinsic to each population (Begon et al., 2007). Studies have demonstrated that changes in the community structure can be expected when different populations coinhabit. For

example, 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, with the presence of rainbow trout resulting in significant reductions in these variables, as well as changes in spatial orientation (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 interspecific avoidance (Saraux et al., 2014).

In the presence of contaminants, changes are expected to occur in the patterns of interaction (such as competition and aggressiveness) among fish populations (Lingaraja and Bhushana, 1979; Clotfelter and Rodriguez, 2006). 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 displacement, the main question addressed in the present study was: Does interspecific interaction between the freshwater fishes *Danio rerio* (zebrafish) and *Poecilia reticulata* (guppy) change their patterns of avoidance to contamination? The hypothesis adopted was that the spatial distributions of these two fish populations exposed to a contamination gradient would be affected by interspecific interactions and that the less aggressive species would be displaced towards previously avoided contaminated habitats.

Copper (Cu) was selected as a test contaminant due to its worldwide importance as a pollutant and the adverse effects that it can cause in fish. Such effects include 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). The fishes *P. reticulata* and *D. rerio* were selected as test organisms as 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; Silva et al., 2017).

2. Materials and Methods

2.1 Test organisms

Individuals of the fishes *P. reticulata* and *D. rerio* (less than 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 (in 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. The 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 ad libitum with commercial fish food flakes (Tetramin[®]), until 24 h prior to the experiments.

The pH (7.0 ± 0.5), conductivity ($120 \pm 20 \mu\text{S}\cdot\text{cm}^{-1}$) and dissolved oxygen ($6.4 \pm 0.4 \text{ mg}\cdot\text{L}^{-1}$) 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 $23 \pm 2 \text{ }^{\circ}\text{C}$. Before the experiments, the sensitivity of the fish was analyzed by performing toxicity tests using potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) as a reference substance, at concentrations ranging from 10 to $300 \text{ mg}\cdot\text{L}^{-1}$, following the OECD guidelines (OECD, 2000). The LC_{50} values (mean $\pm$ standard deviation; $n = 3$) were $41.66 \pm 14.17 \text{ mg}\cdot\text{L}^{-1}$ (*P. reticulata*) and 127.3 ± 19.53 (*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 $63.8 \text{ mg}\cdot\text{L}^{-1}$ and 75.8 to $167.0 \text{ mg}\cdot\text{L}^{-1}$, respectively).

2.2 Copper

Metallic copper powder (99.99%, VETEC/Sigma) was used in the avoidance tests. All the concentrations employed were prepared with culture water. The concentrations were checked at the end of the tests ($n = 3$). The samples were stored

at 4 °C, in the dark, for a maximum of 1 week, and were then sent cooled in thermally insulated packages for analysis of the effective concentrations. A stock copper solution was prepared by dissolution in sulfuric acid (H₂SO₄) (95-99%, VETEC/Sigma). Controls were prepared with sulfuric acid in the same proportions as the Cu treatments. The copper concentrations were determined in the Chemical Analysis Laboratory of Universidade Federal Fluminense (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 0.100 g quantity of copper was dissolved in a minimum volume of HNO₃ (65%, Sigma) and diluted to 100 mL with ultrapure water from a Milli-Q Direct system. This solution was diluted to obtain copper standard solutions in the concentration range from 0 to 1000 µg·L⁻¹. The linear equation of the standard curve was $y = 9.5 \times 10^{-5}x - 6.4 \times 10^{-4}$ ($r^2 = 0.999$).

2.3 Non-forced exposure system

Non-forced avoidance tests were performed in a static free-choice exposure system composed of seven interconnected chambers (Figure 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.

Figure 1. Schematic diagram of the static multi-compartmented non-forced avoidance test system, highlighting one of the seven compartments (from Araújo et al., 2014).

2.4 Avoidance tests

In order to determine if the copper avoidance response of one species was affected by the presence of the other species, rather than by the population density, two distinct approaches were adopted: (1) monospecific tests, in which only one species was introduced into the system, at two different population densities, and (2) multispecific tests, in which both species were tested simultaneously in the system. 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 preferences for any of the seven chambers. In the monospecific approach, 14 fish were used (2 fish in each chamber), while in the multispecific approach, 14 fish were used in total, with 7 organisms of each species being inserted in 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. In all the tests, the coefficients of variation of the Cu concentrations were below 10%, so the nominal concentrations were employed in the calculations and for construction of the graphs. Tests using the monospecific approach were performed with 7 and 14 fish per system, while the multispecific approach employed 14 fish (7 fish of each species per system). In each system, the same Cu gradient was prepared from a 100,000 $\mu\text{g}\cdot\text{L}^{-1}$ Cu stock solution, with nominal concentrations in the chambers of 0, 11, 17, 29, 40,

55, and 70 $\mu\text{g}\cdot\text{L}^{-1}$, hence forming a Cu gradient. Prior to insertion of the culture water and the aliquots of Cu solution, plasticine plugs wrapped in PVC were inserted between adjacent chambers, using a clamp, hence blocking the connections between them and preventing mixing of the different 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 Cu 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.

2.5 Statistical analyses

The percentage of organisms in each chamber was arcsine transformed and analyzed using two-way ANOVA followed by Tukey's test ($\alpha = 0.05$) to determine whether the distribution of the fish varied according to exposure time and chamber (both considered as fixed factors). The distributions of the organisms were obtained for the different observation times, except when time was not a statistically significant factor, in which case the mean value for the six observation times was used. In the avoidance tests with a Cu 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: $\text{Avoiders} = N_E - N_O$, 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: $(\text{Avoiders}/N_E) * 100$. 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 per species, totaling 14 fish per system) experiments, in order to determine whether the distributions occurred in a random fashion. In the monospecific tests with *P. reticulata*, the fish distribution was random (Figure 2-A), with no statistically significant differences according to time ($F = 0.52$, $p > 0.05$) or among the chambers ($F = 0.635$, $p > 0.05$) (Tables S1 and S2, Supplementary Material). The same pattern was observed for *D. rerio* (time: $F = 0.110$, $p > 0.05$; chambers: $F = 0.535$, $p > 0.05$) (Tables S3 and S4, Supplementary Material).

In the multispecific tests (Figure 2-B), the data were treated considering the species individually and in combination (Table S5, Supplementary Material). In the case of *P. reticulata*, the fish distribution differed statistically among chambers ($F = 5.514$, $p < 0.05$), but not according to time ($F = 0.033$, $p > 0.05$) (Table S6, Supplementary Material), while for *D. rerio* there were no statistically significant differences for either of the fixed factors (chambers: $F = 1.750$, $p > 0.05$; time: $F = 0.037$, $p > 0.05$) (Table S7, Supplementary Material). Considering the distribution of both species together, there was a statistically significant difference among the chambers ($F = 3.891$, $p < 0.05$), but not according to time ($F = 0.037$, $p > 0.05$) (Table S8,

Supplementary Material). The interaction of the two fixed factors (time and chamber) was not statistically significant in any control test.

Figure 2. (A) Distributions (in %) of *P. reticulata* and *D. 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 average distributions of the fish species individually and in combination.

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 Cu contamination gradient, in order to evaluate the effect of the population density on the distribution of the organisms. For *P. reticulata* (Tables S9 and S10, Supplementary Material), in the first scenario (7 fish), the distribution of the organisms was influenced by the different copper concentrations ($F = 184.843$, $p < 0.05$) and time ($F = 8.237$, $p < 0.05$) (Figure 3-A; Table S11, Supplementary Material). However, the interaction of the two fixed factors was not statistically significant. The avoidance mean (in %) and standard deviation values for each treatment (0, 11, 17, 29, 40, 55, and $70 \mu\text{g}\cdot\text{L}^{-1}$) were 0.0 ± 0.0 , 29.0 ± 11.0 , 54.0 ± 20.0 , 75.0 ± 21.0 , 91.0 ± 19.0 , 97.0 ± 12.0 , and 100.0 ± 0.0 , respectively. In the second scenario (14 fish), only the concentrations influenced the distribution ($F = 107.081$, $p < 0.05$) (Figure 3-B; Table S12, Supplementary Material). Irrespective of the population density, the organisms tended to avoid the contaminated chambers,

moving towards the control chamber. The avoidance mean (in %) and standard deviation values for each Cu concentration were 0.0 ± 0.0 , 41.67 ± 10.39 , 52.78 ± 8.0 , 64.58 ± 7.80 , 77.78 ± 4.97 , 95.83 ± 6.97 , and 97.22 ± 6.80 , respectively.

Figure 3. (A) Avoidance (in %) of *P. reticulata* in the monospecific test (7 fish) with a Cu gradient. The lines show the mean distributions of the fish at different times. Different letters indicate statistically significant differences. (B) Avoidance (in %) of *P. reticulata* in the monospecific test (14 fish) with a Cu gradient. Different letters indicate statistically significant differences.

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 (Figures 4-A and 4-B; Tables S13 and S14, Supplementary Material). *D. rerio* showed similar gradient-dependent distribution patterns in both scenarios, with the concentration and time being statistically significant factors (for 7 fish: $F = 5.998$, $p < 0.05$ (concentration), and $F = 276.148$, $p < 0.05$ (time); for 14 fish: $F = 720.120$, $p < 0.05$ (concentration), and $F = 3.418$, $p < 0.05$ (time)) (Tables S15 and S16, Supplementary Material).

The avoidance mean (in %) and standard deviation values for each Cu concentration (0, 11, 17, 29, 40, 55, and 70 $\mu\text{g}\cdot\text{L}^{-1}$) were 0.0 ± 0.0 , 22.22 ± 9.90 , 43.33 ± 10.29 , 65.28 ± 15.19 , 87.04 ± 20.26 , 97.22 ± 11.79 , and 100.0 ± 0.0 ,

respectively (7 fish), and 0.0 ± 0.0 , 22.69 ± 4.78 , 36.11 ± 6.12 , 50.69 ± 4.87 , 67.59 ± 4.18 , 98.61 ± 3.40 , and 100.0 ± 0.0 , respectively (14 fish).

Figure 4. Avoidance (in %) of *D. 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 Cu gradient. Different letters in the time legend and above the lines indicate statistically significant differences.-

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) (Table S17, Supplementary Material). In all the scenarios and for both species, only the concentrations had a statistically significant effect on the fish distribution (*P. reticulata* - 7 fish: $F = 25.814$, $p < 0.05$; *D. rerio* - 7 fish: $F = 9.159$, $p < 0.05$; *P. reticulata* + *D. rerio* - 14 fish: $F = 24.735$, $p < 0.05$). The time and the interaction between the effects of time and chamber did not show any statistically significant effects on the fish distribution (Figure 5; Tables S18-S20, Supplementary Material).

Figure 5. Avoidance (in %) by *P. reticulata* and *D. rerio*, individually and in combination, in the multispecific test with 14 fish exposed to a Cu gradient.

The avoidance mean (in %) and standard deviation values for each Cu concentration (0, 11, 17, 29, 40, 55, and 70 $\mu\text{g}\cdot\text{L}^{-1}$) were 0.0 ± 0.0 , 15.74 ± 10.19 , 27.78 ± 18.09 , 40.28 ± 20.69 , 59.26 ± 18.14 , 86.11 ± 19.48 , and 94.44 ± 13.61 , respectively, for *P. reticulata*. For *D. rerio*, the values were 0.0 ± 0.0 , 0.0 ± 0.0 , 0.0 ± 0.0 , 1.39 ± 3.40 , 12.96 ± 16.36 , 22.22 ± 29.19 , and 44.44 ± 34.43 , respectively. For the combined population (*P. reticulata* + *D. rerio*), the values were 0.0 ± 0.0 , 3.24 ± 3.69 , 3.33 ± 4.22 , 10.42 ± 9.03 , 29.63 ± 16.63 , 48.61 ± 19.31 , and 69.44 ± 19.48 , respectively.

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 *P. reticulata* and *D. rerio*, 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 Cu, with decreases in avoidance of 4.45 and

3.94 times, respectively, comparing the AC₂₀ (14 fish) and AC₅₀ (14 fish) values for the monospecific test with the AC₂₀ and AC₅₀ values for the multispecific test.

Table 1. AC₂₀, AC₅₀, and AC₈₀ values (in µg·L⁻¹, concentrations triggering avoidance in 20, 50, and 80% of the exposed population) for *P. reticulata* and *D. rerio* in the monospecific (Mono.) and multispecific (Multi.) tests.

Test	Species	Scenario	AC ₂₀	AC ₅₀	AC ₈₀
Mono.	<i>P. reticulata</i>	7 fish	9.36 (7.56 - 10.97)	16.37 (14.45 - 18.18)	28.61 (25.83 - 32.20)
		14 fish	6.99 (2.85 - 10.64)	15.68 (10.15 - 20.28)	35.13 (27.51 - 50.69)
	<i>D. rerio</i>	7 fish	11.30 (8.57 - 13.63)	19.20 (16.36 - 21.96)	32.64 (28.40 - 38.92)
		14 fish	12.41 (5.31 - 17.65)	22.38 (14.92 - 30.04)	40.38 (30.08 - 71.29)
Multi.	<i>P. reticulata</i>		14.76 (9.34 - 19.12)	28.01 (22.24 - 34.56)	53.18 (42.04 - 78.66)
	<i>D. rerio</i>		50.33 (46.58 - 54.00)	75.66 (68.86 - 86.87)	113.74 (96.80 - 146.96)
	Both species		31.72 (24.34 - 37.82)	55.38 (46.54 - 71.51)	96.70 (74.15 - 162.23)

4. Discussion

4.1. Distribution control in the absence of a contamination gradient

In the monospecific tests in the absence of contamination, the distributions of the organisms within the system were 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. In other studies using the non-forced exposure system, similar responses were 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).

In the multispecific tests without a contamination gradient, *P. reticulata* showed a tendency to aggregate, which was not observed for *D. rerio*. Since this trend was not observed in the monospecific tests, the response of *P. reticulata* was probably the result of interspecific interaction. In a study with two species of trout, brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*), in the presence of brook trout, the brown trout were forced to move to other resting areas, due to competition for space along a stretch of the Au Sable River (Michigan, USA) (Fausch and White, 1981). According to Chapman (1966), it is likely that fish have minimum spatial

requirements that are related to the size of the individual fish, visual isolation, and maintenance of distance, 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 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 in many cases, 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). It can therefore be seen that both species studied here commonly form shoals, but that there are no specific patterns in terms of the number of organisms or the type of behavior. During the adaptation of the organisms in the laboratory, it was observed that both species sometimes formed small shoals, while in other cases the fish were randomly dispersed in the aquaria (such as in the control monospecific distribution tests).

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, density 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 $11 \mu\text{g}\cdot\text{L}^{-1}$ of copper and 14 organisms, where the average guppy avoidance percentage during the test (41.67%) was almost 2-fold higher than the value obtained for zebrafish (22.69%), while a 1.46-fold higher value was obtained at a copper concentration of $17 \mu\text{g}\cdot\text{L}^{-1}$. 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, with the avoidance being concentration dependent. Both species are able to detect pollutants and escape to cleaner areas. Araújo et al. (2014b) exposed zebrafish to a fungicide (pyrimethanil) gradient, while Silva et al. (2017) analyzed the escape response of *P. reticulata* exposed to the compound triclosan. In both cases, the organisms were able to detect the compounds present at environmentally relevant concentrations and move to less contaminated areas.

In acute tests, it has been found that *D. rerio* is more sensitive to copper, compared to *P. reticulata*, with concentrations of Cu lethal to 50% of the populations (LC_{50}) being $145.4 \mu\text{g}\cdot\text{L}^{-1}$ for *P. reticulata* (Shuhaimi-Othman et al., 2010) and $61 \mu\text{g}\cdot\text{L}^{-1}$ for *D. rerio* (Abdel-moneim et al., 2015). The present work revealed a possible relationship between Cu toxicity and detection and avoidance of the metal by *D. rerio*. The zebrafish were more susceptible to Cu (which was more toxic to *D. rerio* than to *P. reticulata*) 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 Cu in monospecific tests and found dose/avoidance responses very similar to those observed here, with AC_{50} values ranging from 16.37 to $22.38 \mu\text{g}\cdot\text{L}^{-1}$, compared to AC_{50} of $\sim 16 \mu\text{g}\cdot\text{L}^{-1}$ obtained in the present work.

From comparison of the avoidance responses to Cu 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 $15 \mu\text{g}\cdot\text{L}^{-1}$. Vieira et al. (2009) reported that *Pomatoschistus microps* exposed to $50 \mu\text{g}\cdot\text{L}^{-1}$ Cu 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* for 21 days to $45 \mu\text{g}\cdot\text{L}^{-1}$ of Cu 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 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*, with such responses decreasing in the presence of another species. 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 reversed the competitive outcome between the two species, with this reversal being accentuated in degraded habitats, hence changing the pattern of aggression between the two species. The behavioral modifications suggested that elevated CO_2 could change the ways that species interact with each other, hence altering the processes that shape communities and ecosystems.

The short duration (3 h) of exposure to low concentrations of Cu (up to $70 \mu\text{g}\cdot\text{L}^{-1}$) may not have contributed to the behavioral changes in the two species (due to

physiological or neurological effects), since a relationship between dose and response was only observed after longer exposure times, according to the data for sublethal effects of Cu (Section 4.2). However, each organism influenced the dispersion of the other. It appears that the copper avoidance reaction of *P. reticulata* was less influenced by the presence of the other species, so contamination was the most important factor affecting the displacement of this species. In the case of *D. rerio*, the presence of *P. reticulata* had an effect on its spatial distribution that was more significant, relative to the contamination to which it was exposed. 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 were able to detect Cu and move to a less contaminated area. In the multispecific tests, even in the presence of copper, which caused avoidance in *D. rerio*, the presence of *P. reticulata* affected the distribution of the former species, reducing its migration potential.

This study shows the importance of understanding the interactions among fish in contaminated areas and the way that one species may prejudice another, when avoidance behavior could provide aquatic organisms with a means of escaping from stressful conditions in polluted environments.

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7. General Conclusions

Habitat disturbance due to contamination can restrict habitable areas, modify migratory mechanisms and cause instability for the community dynamics. Thus, this study contributed in different ways to the understanding of the influence of the aquatic pollutants on the dispersion and habitat selection by the fish *P. reticulata* and *D. rerio*. The methodology used, simulating a contamination gradient, makes it possible to create environments similar to those that would occur in any water body, in which the pollutant load is reduced as it distances from its generating source. Several different approaches were worked on in this study, where we could understand various patterns of dispersion of organisms when exposed to different scenarios, such as:

- Gradients of pollution
- Chemical Barriers
- Interspecific interactions

Thus, we will answer the questions asked in the "General Introduction" section, separated by each chapter:

➤ Chapter I:

- How can different contamination scenarios (spatially permanent gradient and a local discharge) influence the avoidance response in fish?

The avoidance response was concentration-dependent and although it was not dependent on the intensity of the gradient, the gradient nonetheless influenced the AC₅₀.

- Can the results (mortality) obtained in forced tests be considered overestimated, since fish in real conditions can move to less contaminated areas?

The use of forced exposure could underestimate the environmental risks (population downsizing) of contamination, since it does not consider spatial avoidance as a potential response to the presence of a

contaminant. On the other hand, it could overestimate lethal effects, due to the mandatory exposure of mobile organisms.

- What is the expected population immediate decline in environmentally relevant concentrations?

The observed response suggested that the population of *P. reticulata* could decrease at the local scale following exposure to environmentally relevant TCS concentrations lower than 2 mg·L⁻¹.

🐟 Chapter II:

- How environmentally relevant is the avoidance response to fish and how safe are the limits set by international agencies for the concentrations of pollutants found in surface waters when studying the avoidance response?

In summary, even at environmentally relevant concentrations, BPA has the capacity to trigger an avoidance response in *P. reticulata*, causing displacement of the fish to less contaminated areas and consequent population decline at the local scale. Comparison of the results obtained here with the BPA values considered safe by international agencies showed that avoidance would be expected to occur at lower concentrations, before detection of any acute or chronic effects.

🐟 Chapter III

- Can contaminants cause habitat fragmentation, producing a chemical barrier to the flow of individuals?

Contamination by atrazine can influence the spatial distribution of the fish populations. Moreover, atrazine can cause a habitat fragmentation by forming a chemical barrier that can totally or partially isolate populations.

➤ Chapter IV

- How can species density and interaction influence the avoidance response in environments where there are gradients of contamination by aquatic pollutants?

In the multispecific tests (with two species in the same system), even in the presence of copper, which caused avoidance in *D. rerio*, the presence of *P. reticulata* affected the distribution of the former specie, reducing its migration potential.

So what is the overall importance of avoidance?

- Its allows organisms to reduce or eliminate the toxic effects of contaminants
- Its poses a serious threat to communities due to the marked emigration of organisms

The occurrence of contaminant avoidance may have immediate and wider ecological implications (ecosystem level) than predicted for a sub-lethal response (focus on the individual). The preference or avoidance of stressors can be determinant for the occurrence of species that inhabit contaminated ecosystems and should be taken into account aspects such as competition, selective predation, availability of resources, etc. Therefore, is inevitable to endorse the recommendation for including avoidance as a complementary endpoint within the ecotoxicological line-of-evidence of environmental risk analysis schemes, at least at higher tiers, along with sub-lethal chronic tests.

8. Supplementary Material

(Chapter II)

Table S1. Two-way ANOVA results for the distribution of organisms (dependent variable) in the control test with culture water. Chambers and observation times were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	0.007	3	0.002	0.002	1.000
Chambers	12.783	6	2.130	1.455	0.210
Time * Chambers	21.878	18	1.215	0.830	0.659
Error	81.995	56	1.464		

Table S2. Two-way ANOVA results for the percentage of organisms (dependent variable) during the BPA gradient avoidance test. Concentrations and observation times were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	89.877	3	29.959	1.308	0.281
Concentrations	13804.551	6	2300.758	100.415	0.000
Time * Concentrations	827.159	18	45.953	2.006	0.025
Error	1283.098	56	22.912		

Table S3. Two-way ANOVA results for the avoidance response (dependent variable) of *Poecilia reticulata* exposed to a BPA gradient. Concentrations and observation times were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	2147.775	3	715.925	14.050	0.000
Concentrations	66059.764	6	11009.961	216.067	0.000
Time * Concentrations	1701.599	18	94.533	1.855	0.041
Error	2853.548	56	50.956		

Table S4. Percentages (n = 3) of avoidance (in the non-forced system), mortality (in the non-forced (NF) and forced (F) systems), and PID (population immediate decline), taking into account the dead animals in the forced (PID-F) and nonforced (PID-NF) systems, for each BPA concentration tested.

Concentration	Avoidance (%)		Mortality (NF)		Mortality (F)		*PID-F		PID-NF	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.02	29.9	6.3	0.0	0.0	0.0	0.0	29.9	6.3	29.9	6.3
0.2	51.1	8.6	0.0	0.0	0.0	0.0	51.1	8.6	51.1	8.6
2	65.7	8.5	0.0	0.0	0.0	0.0	65.7	8.5	65.7	8.5
20	81.2	5.4	0.0	0.0	0.0	0.0	81.2	5.4	81.2	5.4
200	90.7	1.6	0.0	0.0	3.3	0.6	91.0	1.6	90.7	1.6
2000	100.0	0.0	0.0	0.0	57.0	6.0	100.0	0.0	100.0	0.0

* For calculation of the PID-F (with forced mortality), an estimate was made using the LC₅₀ result to predict the percentages of dead animals for the same concentrations used in the non-forced system.

(Chapter III)

Section: Atrazine analysis

The SPE extraction was performed with 500 mL of sample from each compartment with concentrations 0.001, 0.01, 0.1 $\mu\text{g}\cdot\text{L}^{-1}$ OASIS HLB 500 mg, 6cc cartridges conditioned with 4 mL of methanol, 4 mL of acetonitrile and 4 mL of ultrapure water. Atrazine was eluted of the solid phase using 4 mL of methanol and 4 mL of acetonitrile. The eluate was completely dried with nitrogen and reconstituted with 0.5 mL of the solution, which is the initial mobile phase composition for the chromatographic analysis, i.e., 70:30 (v:v) H_2O :MeOH. The samples from containing concentrations of 1, 10, 100 $\mu\text{g}\cdot\text{L}^{-1}$ were diluted 50:50 (v:v) sample:ultrapure water before analyses by LC-MS/MS. The LC-MS/MS analysis was performed using an Agilent 1200 Series LC system coupled to an Agilent 6410 triple quadrupole mass spectrometer with an electrospray ionization source (ESI). The software MassHunter was used to control the instrument and to evaluate the chromatographic and mass data. The chromatographic separation was performed in a thermostatted column compartment (TCC G1316A) at 25 °C, using a reversed phase Zorbax SB-C18 column (2.1 x 30 mm, particle size of 3.5 μm) from Agilent Technologies and carried out with isocratic elution using ultrapure water (A), with 0.1% formic acid, and methanol (B). The solvents used as the mobile phase were filtered through 0.2 μm nylon membranes (Sigma Aldrich, Steinheim, Germany). It took 3 minutes for the isocratic separation with a proportion of 70% water and 30% methanol. The injection volume was 10 μL . After the chromatographic separation, the atrazine was ionized using an electrospray ionization source (ESI) operating in the positive ion mode. The following parameters were adjusted to maximize ionization: drying gas flow rate of 10 $\text{L}\cdot\text{min}^{-1}$, drying gas temperature of 350 °C, nebulizing gas pressure at 20 psi, and capillary voltage of 4000 V. Nitrogen was used as collision gas. Multiple reaction monitoring (MRM) transitions were employed for confirmation and quantification of the target compound (Table S1). A table indicating the nominal (at the beginning of the test) and actual (at the end of the avoidance test) atrazine concentrations (values in $\mu\text{g}\cdot\text{L}^{-1}$) is provided below (Table S2). The limits of detection and quantification of the method were 0.3 and 0.9 $\text{ng}\cdot\text{L}^{-1}$, respectively ($r^2 = 0.998$; Figure S1).

Table S1: Transitions and the respective collision energies (CE) selected for the quantification of atrazine employing de MRM mode.

Compound	Polarity	Fragmentor (V)	Precursor (m/z)	Quantification ion		Confirmation ion	
				m/z	CE (V)	m/z	CE (V)
Atrazine	+	100	216.2	174.1	15	103.9	15

*m/z $\pm$ 0.1

Table S2: Initial (nominal) and final (actual) atrazine concentrations in the avoidance and chemical barrier tests.

Atrazine concentrations in avoidance tests	
Initial (nominal)	Final
Control	0.002
0.01	0.02
0.02	0.02
0.1	0.3
1	1.9
10	11.2
100	105
Atrazine concentrations in chemical barrier tests with 100	
Control (<LoD)	<LoD
100	150
Control (<LoD)	<LoD
Atrazine concentrations in chemical barrier tests with 1000	
Control (<LoD)	<LoD
1000	1058
Control (<LoD)	<LoD

LoD: Limit of detection (0.3 ng·L⁻¹).

Figure S1: Calibration curve for atrazine analysis.

Section: Results

Table S3: Distribution of the organisms in the control of avoidance test.

Replicate	Compartment						
	#1	#2	#3	#4	#5	#6	#7
Organisms introduced initially in each compartment							
#1	3	3	3	3	3	3	3
#2	3	3	3	3	3	3	3
#3	3	3	3	3	3	3	3
#4	3	3	3	3	3	3	3
Organisms recorded per compartment after 0.5 h							
#1	6	6	2	3	2	2	0
#2	14	3	1	1	1	1	0
#3	9	5	2	2	2	1	0
#4	10	5	2	2	1	1	0
Organisms recorded per compartment after 1.0 h							
#1	3	2	2	2	4	4	4
#2	4	5	1	0	3	4	4
#3	3	1	2	4	2	4	5
#4	3	2	2	3	2	5	4
Organisms recorded per compartment after 1.5 h							
#1	3	3	2	1	2	5	5
#2	5	2	5	3	2	1	3
#3	3	3	2	0	3	5	5
#4	4	3	3	2	2	4	3

Organisms recorded per compartment after 2.0 h							
#1	4	2	2	2	2	5	4
#2	3	3	2	3	0	5	5
#3	2	4	2	0	3	5	5
#4	4	2	1	2	1	6	5
Organisms recorded per compartment after 2.5 h							
#1	5	2	1	2	3	3	5
#2	5	1	4	1	2	4	4
#3	4	3	1	2	0	6	5
#4	5	2	2	2	2	4	4
Organisms recorded per compartment after 3.0 h							
#1	4	3	1	3	4	2	4
#2	5	2	4	2	2	2	4
#3	4	2	2	2	3	3	5
#4	5	2	1	3	3	3	4
Mean number of organisms recorded per compartment (n=6; means of the six observation periods)							
Mean	3.79	2.67	2.08	2.04	2.25	4.00	4.17
SD	0.68	0.61	0.47	0.43	0.57	0.89	0.52

Table S4: Distribution of the organisms in the avoidance test.

Replicate	Concentration ($\mu\text{g}\cdot\text{L}^{-1}$)						
	0.0 (control)	0.002	0.020	0.3	1.9	11.2	105
Organisms introduced initially in each compartment							
#1	3	3	3	3	3	3	3
#2	3	3	3	3	3	3	3
#3	3	3	3	3	3	3	3
#4	3	3	3	3	3	3	3
Organisms recorded per compartment after 0.5 h							
#1	6	6	2	3	2	2	0
#2	14	3	1	1	1	1	0
#3	9	5	2	2	2	1	0
#4	10	5	2	2	1	1	0
Organisms recorded per compartment after 1.0 h							
#1	6	6	3	2	2	1	1
#2	14	3	1	1	1	1	0
#3	7	7	3	1	1	1	1
#4	10	5	2	2	1	1	0
Organisms recorded per compartment after 1.5 h							
#1	15	2	1	1	1	1	0
#2	15	2	2	1	1	0	0
#3	8	3	8	2	0	0	0
#4	15	2	2	1	1	0	0

Organisms recorded per compartment after 2.0 h							
#1	11	1	1	1	3	3	1
#2	10	2	2	2	2	2	1
#3	4	3	9	3	1	0	1
#4	11	2	2	2	2	1	1
Organisms recorded per compartment after 2.5 h							
#1	11	4	1	2	2	1	0
#2	10	1	1	1	2	2	4
#3	4	3	11	1	1	1	0
#4	11	3	1	2	2	1	1
Organisms recorded per compartment after 3.0 h							
#1	13	1	1	1	1	2	2
#2	10	2	3	2	2	1	1
#3	4	3	11	2	0	1	0
#4	12	2	2	2	2	1	0
Mean number of organisms recorded per compartment (n=6; means of the six observation periods)							
Mean	10.00	3.17	3.08	1.67	1.42	1.08	0.58
SD	1.63	1.45	0.92	0.30	0.44	0.44	0.52

Table S5: Number of expected (N_E) and observed (N_O) organisms in the avoidance test.

Replicates		N_E : Expected Organisms					
#1	21	18	15	12	9	6	3
#2	21	18	15	12	9	6	3
#3	21	18	15	12	9	6	3
#4	21	18	15	12	9	6	3
Replicates		N_O : Observed organisms after 0.5 h					
#1	21	15	9	7	4	2	0
#2	21	7	4	3	2	1	0
#3	21	12	7	5	3	1	0
#4	21	11	6	4	2	1	0
Replicates		N_O : Observed organisms after 1.0 h					
#1	21	15	9	6	4	2	1
#2	21	7	4	3	2	1	0
#3	21	14	7	4	3	2	1
#4	21	11	6	4	2	1	0
Replicates		N_O : Observed organisms after 1.5 h					
#1	21	6	4	3	2	1	0
#2	21	6	4	2	1	0	0
#3	21	13	10	2	0	0	0
#4	21	6	4	2	1	0	0

Replicates		N _O : Observed organisms after 2.0 h					
#1	21	10	9	8	7	4	1
#2	21	11	9	7	5	3	1
#3	21	17	14	5	2	1	1
#4	21	10	8	6	4	2	1
Replicates		N _O : Observed organisms after 2.5 h					
#1	21	10	6	5	3	1	0
#2	21	11	10	9	8	6	4
#3	21	17	14	3	2	1	0
#4	21	10	7	6	4	2	1
Replicates		N _O : Observed organisms after 3.0 h					
#1	21	8	7	6	5	4	2
#2	21	11	9	6	4	2	1
#3	21	17	14	3	1	1	0
#4	21	9	7	5	3	1	0
Mean number of observed organisms (n=6; means of the six observation periods)							
Mean	21.0	11.0	7.8	4.8	3.1	1.7	0.6
SD	0.0	1.6	1.9	1.5	1.3	0.9	0.5

Table S6: Two-way ANOVA results for percentage of organisms (dependent variable) in the avoidance test with atrazine. Concentration and observation moment (time) were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	164.076	5	32.815	0.623	0.682
Concentration	20752.538	6	3458.756	65.706	0.000
Time * concentration	2139.511	30	71.317	1.355	0.126
Error	6632.656	126	52.640		

Table S7: Distribution of organisms in the control (with no atrazine) of the chemical barrier test.

Replicate	Compartment						
	#1	#2	#3	#4	#5	#6	#7
Organisms introduced initially in each compartment							
#1	21	0	0	0	0	0	0
#2	21	0	0	0	0	0	0
#3	21	0	0	0	0	0	0
#4	21	0	0	0	0	0	0
Organisms recorded per compartment after 0.5 h							
#1	6	3	3	3	4	1	1
#2	3	5	6	0	3	0	4
#3	15	1	4	0	1	0	0
#4	15	2	3	0	0	0	1
Organisms recorded per compartment after 1.0 h							
#1	5	3	3	4	3	0	3
#2	4	3	5	3	0	2	4
#3	11	1	2	2	1	3	3
#4	14	3	3	0	1	0	0
Organisms recorded per compartment after 1.5 h							
#1	7	3	1	4	2	2	2
#2	7	4	6	0	1	1	2
#3	14	0	2	1	1	0	3
#4	17	0	0	1	1	1	1
Organisms recorded per compartment after 2.0 h							
#1	12	2	3	4	0	0	0
#2	8	8	0	0	2	0	3
#3	11	1	0	0	1	3	5
#4	13	0	0	0	1	2	5
Organisms recorded per compartment after 2.5 h							
#1	11	0	2	3	1	1	3
#2	9	4	4	1	2	0	1
#3	4	5	0	0	2	2	8
#4	13	0	0	0	1	2	5
Organisms recorded per compartment after 3.0 h							
#1	11	1	1	2	3	2	1
#2	11	3	2	1	0	0	4
#3	5	5	0	0	1	2	8
#4	13	0	0	0	1	1	6

Table S8: Distribution of organisms in the chemical barrier test with 150 µg L⁻¹ atrazine.

arazine.

Replicate	Compartment						
	#1	#2	#3	#4	#5	#6	#7
	Downstream zone		Chemical barrier zone			Upstream zone	
Organisms introduced initially in each compartment							
#1	21	0	0	0	0	0	0
#2	21	0	0	0	0	0	0
#3	21	0	0	0	0	0	0
#4	21	0	0	0	0	0	0
Organisms recorded per compartment after 0.5 h							
#1	19	1	0	1	0	0	0
#2	4	13	1	0	0	0	3
#3	16	0	2	3	0	0	0
#4	19	0	2	0	0	0	0
Organisms recorded per compartment after 1.0 h							
#1	19	1	1	0	0	0	0
#2	15	3	0	2	0	0	1
#3	9	5	3	2	1	0	1
#4	17	1	1	1	0	0	1
Organisms recorded per compartment after 1.5 h							
#1	20	0	0	1	0	0	0
#2	13	4	0	1	0	0	3
#3	12	2	0	2	1	1	3
#4	15	3	1	0	0	0	2
Organisms recorded per compartment after 2.0 h							
#1	20	0	0	0	0	0	1
#2	12	2	2	0	0	1	4
#3	14	0	0	2	1	0	4
#4	16	2	0	0	0	0	3
Organisms recorded per compartment after 2.5 h							
#1	17	0	1	1	0	0	2
#2	13	2	2	0	0	1	3
#3	14	1	0	1	1	0	4
#4	16	1	1	0	0	0	3
Organisms recorded per compartment after 3.0 h							
#1	13	4	0	2	1	1	0
#2	8	6	1	0	3	0	3
#3	14	2	0	0	3	1	1
#4	15	1	0	0	0	2	3

Table S10: Two-way ANOVA results for percentage of organisms (dependent variable) in chemical barrier control test. Compartment and observation moment (time) were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	92.513	5	18.503	0.159	0.977
Compartment	19927.779	6	3321.297	28.629	0.000
Time * compartment	2900.409	30	96.680	0.833	0.713
Error	14617.570	126	116.012		

Table S11 Two-way ANOVA results for percentage of organisms (dependent variable) in chemical barrier control test with atrazine a 150 µg·L⁻¹. Compartment and observation moment (time) were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	134.823	5	26.965	0.296	0.915
Compartment	51479.556	6	8579.926	94.084	0.000
Time * compartment	2867.860	30	95.595	1.048	0.412
Error	11490.445	126	91.194		

Table S12: Two-way ANOVA results for number of organisms (dependent variable) in chemical barrier control test with atrazine a 1058 µg·L⁻¹. Compartment and observation moment (time) were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	28.776	5	5.755	0.182	0.969
Compartment	111014.809	6	18502.468	585.795	0.000
Time * compartment	1702.435	30	56.748	1.797	0.014
Error	3979.740	126	31.585		

(Chapter IV)

Table S1: Distribution of *Poecilia reticulata* in the control test (monospecific – 14 fish), mean (n=3) and standard deviation (SD).

Replicates	Chambers						
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
	Organisms introduced initially in each compartment						
#1	3	3	3	3	3	3	3
#2	3	3	3	3	3	3	3
#3	3	3	3	3	3	3	3
Mean	3.0	3.0	3.0	3.0	3.0	3.0	3.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Organisms recorded per compartment after 0.5 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	1	3	1	1	3	2	3
#2	3	3	2	2	1	1	2
#3	2	0	3	3	3	1	2
Mean	2.0	2.0	2.0	2.0	2.3	1.3	2.3
SD	1.0	1.7	1.0	1.0	1.2	0.6	0.6
Organisms recorded per compartment after 1.0 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	2	3	2	1	2	2	2
#2	1	2	3	2	2	2	2
#3	2	2	2	1	3	2	2
Mean	1.7	2.3	2.3	1.3	2.3	2.0	2.0
SD	0.6	0.6	0.6	0.6	0.6	0.0	0.0
Organisms recorded per compartment after 1.5 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	1	3	2	1	2	3	2
#2	2	3	3	2	1	2	1
#3	3	2	2	1	2	1	3
Mean	2.0	2.7	2.3	1.3	1.7	2.0	2.0
SD	1.0	0.6	0.6	0.6	0.6	1.0	1.0
Organisms recorded per compartment after 2.0 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	2	4	1	2	2	1	2
#2	3	1	2	1	1	3	3
#3	2	2	2	3	3	1	1
Mean	2.3	2.3	1.7	2.0	2.0	1.7	2.0
SD	0.6	1.5	0.6	1.0	1.0	1.2	1.0
Organisms recorded per compartment after 2.5 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	2	3	3	1	2	2	1
#2	2	2	2	3	3	1	1
#3	2	2	2	1	3	2	2

Mean	2.0	2.3	2.3	1.7	2.7	1.7	1.3
SD	0.0	0.6	0.6	1.2	0.6	0.6	0.6
Organisms recorded per compartment after 3.0 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	3	2	1	2	1	2	3
#2	2	3	1	3	1	2	2
#3	3	1	1	2	2	2	3
Mean	2.7	2.0	1.0	2.3	1.3	2.0	2.7
SD	0.6	1.0	0.0	0.6	0.6	0.0	0.6

Table S2: Two-way ANOVA results for percentage of *Poecilia reticulata* (dependent variable) in the control test with culture water. Chambers and observation moment (time) were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	6.787	5	1.357	0.052	0.998
Chambers	99.613	6	16.602	0.635	0.702
Time * Chambers	706.275	30	23.542	0.901	0.616
Error	2194.850	84	26.129		

Table S3: Distribution of *Danio rerio* in the control test (monospecific – 14fish), mean (n=3) and standard deviation (SD).

Replicates	Chambers						
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
Organisms introduced initially in each compartment							
#1	3	3	3	3	3	3	3
#2	3	3	3	3	3	3	3
#3	3	3	3	3	3	3	3
Mean	3.0	3.0	3.0	3.0	3.0	3.0	3.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Organisms recorded per compartment after 0.5 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	3	2	2	3	2	1	1
#2	3	0	2	0	3	3	3
#3	1	1	3	2	1	4	2
Mean	2.3	1.0	2.3	1.7	2.0	2.7	2.0
SD	1.2	1.0	0.6	1.5	1.0	1.5	1.0
Organisms recorded per compartment after 1.0 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	3	3	2	3	1	2	0
#2	2	2	2	2	2	3	1
#3	2	3	1	2	1	2	3

Mean	2.3	2.7	1.7	2.3	1.3	2.3	1.3
SD	0.6	0.6	0.6	0.6	0.6	0.6	1.5
Organisms recorded per compartment after 1.5 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	3	3	2	2	2	1	1
#2	2	1	2	1	2	3	3
#3	2	2	1	3	3	1	2
Mean	2.3	2.0	1.7	2.0	2.3	1.7	2.0
SD	0.6	1.0	0.6	1.0	0.6	1.2	1.0
Organisms recorded per compartment after 2.0 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	3	2	2	3	2	1	1
#2	2	3	2	2	1	1	3
#3	3	2	1	1	1	3	3
Mean	2.7	2.3	1.7	2.0	1.3	1.7	2.3
SD	0.6	0.6	0.6	1.0	0.6	1.2	1.2
Organisms recorded per compartment after 2.5 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	2	2	1	2	2	2	3
#2	3	3	2	1	2	1	2
#3	1	1	2	3	2	3	2
Mean	2.0	2.0	1.7	2.0	2.0	2.0	2.3
SD	1.0	1.0	0.6	1.0	0.0	1.0	0.6
Organisms recorded per compartment after 3.0 h							
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
#1	2	3	1	2	3	1	2
#2	1	3	3	2	1	2	2
#3	3	3	1	2	1	2	2
Mean	2.0	3.0	1.7	2.0	1.7	1.7	2.0
SD	1.0	0.0	1.2	0.0	1.2	0.6	0.0

Table S4: Two-way ANOVA results for percentage of *D. rerio* (dependent variable) in the control test with cultive water. Chambers and observation moments were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	19.762	5	3.952	0.110	0.990
Chambers	115.071	6	19.178	0.535	0.780
Time * Chambers	938.287	30	31.276	0.872	0.655
Error	3011.632	84	35.853		

Table S5: Distribution of *P. reticulata* (P.r.) and *D. rerio* (D.r.) in the control test (multispecific – 14 fish), mean (n=3) and standard deviation (SD).

Replicates	Chambers						
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
	Organisms introduced initially in each compartment						
#1 P.r.	1	1	1	1	1	1	1
#1 D.r.	1	1	1	1	1	1	1
TOTAL	2	2	2	2	2	2	2
#2 P.r.	1	1	1	1	1	1	1
#2 D.r.	1	1	1	1	1	1	1
TOTAL	2	2	2	2	2	2	2
#3 P.r.	1	1	1	1	1	1	1
#3 D.r.	1	1	1	1	1	1	1
TOTAL	2	2	2	2	2	2	2
Mean (P.r.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mean (D.r.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mean (Total)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Replicates	Chambers						
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
	Organisms recorded per compartment after 0.5 h						
#1 P.r.	2	0	0	1	1	2	1
#1 D.r.	2	1	0	1	1	1	1
TOTAL	4	1	0	2	2	3	2
#2 P.r.	2	2	1	0	1	1	0
#2 D.r.	1	2	2	0	1	1	0
TOTAL	3	4	3	0	2	2	0
#3 P.r.	2	1	0	2	1	1	0
#3 D.r.	1	2	1	1	1	1	0
TOTAL	3	3	1	3	2	2	0
Mean (P.r.)	2.0	1.0	0.3	1.0	1.0	1.3	0.3
SD	0.0	1.0	0.6	1.0	0.0	0.6	0.6
Mean (D.r.)	1.3	1.7	1.0	0.7	1.0	1.0	0.3
SD	0.6	0.6	1.0	0.6	0.0	0.0	0.6
Mean (Total)	3.3	2.7	1.3	1.7	2.0	2.3	0.7
SD	0.6	1.5	1.5	1.5	0.0	0.6	1.2

Replicates	Chambers						
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
	Organisms recorded per compartment after 1.0 h						
#1 P.r.	2	0	1	1	0	2	1
#1 D.r.	1	0	1	1	1	2	1
TOTAL	3	0	2	2	1	4	2
#2 P.r.	2	2	1	0	1	1	0

#2 D.r.	1	2	1	1	1	1	0
TOTAL	3	4	2	1	2	2	0
#3 P.r.	2	1	1	1	1	0	1
#3 D.r.	2	2	1	1	0	0	1
TOTAL	4	3	2	2	1	0	2
Mean (P.r.)	2.0	1.0	1.0	0.7	0.7	1.0	0.7
SD	0.0	1.0	0.0	0.6	0.6	1.0	0.6
Mean (D.r.)	1.3	1.3	1.0	1.0	0.7	1.0	0.7
SD	0.6	1.2	0.0	0.0	0.6	1.0	0.6
Mean (Total)	3.3	2.3	2.0	1.7	1.3	2.0	1.3
SD	0,6	2,1	0,0	0,6	0,6	2,0	1,2

Replicates	Chambers						
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
	Organisms recorded per compartment after 1.5 h						
#1 P.r.	2	1	0	0	1	2	1
#1 D.r.	1	1	0	1	1	2	1
TOTAL	3	2	0	1	2	4	2
#2 P.r.	2	1	2	0	1	0	1
#2 D.r.	2	1	1	1	1	0	1
TOTAL	4	2	3	1	2	0	2
#3 P.r.	2	1	1	1	1	0	1
#3 D.r.	1	1	2	2	0	0	1
TOTAL	3	2	3	3	1	0	2
Mean (P.r.)	2.0	1.0	1.0	0.3	1.0	0.7	1.0
SD	0.0	0.0	1.0	0.6	0.0	1.2	0.0
Mean (D.r.)	1.3	1.0	1.0	1.3	0.7	0.7	1.0
SD	0.6	0.0	1.0	0.6	0.6	1.2	0.0
Mean (Total)	3.3	2.0	2.0	1.7	1.7	1.3	2.0
SD	0.6	0.0	1.7	1.2	0.6	2.3	0.0

Replicates	Chambers						
	#C1	#C2	#C3	#C4	#C5	#C6	#C7
	Organisms recorded per compartment after 2.0 h						
#1 P.r.	2	0	0	1	2	1	1
#1 D.r.	1	1	0	1	1	2	1
TOTAL	3	1	0	2	3	3	2
#2 P.r.	2	1	1	1	1	1	0
#2 D.r.	1	2	1	1	1	0	1
TOTAL	3	3	2	2	2	1	1
#3 P.r.	2	2	0	0	1	2	0
#3 D.r.	1	1	1	0	1	2	1
TOTAL	3	3	1	0	2	4	1
Mean (P.r.)	2.0	1.0	0.3	0.7	1.3	1.3	0.3
SD	0.0	1.0	0.6	0.6	0.6	0.6	0.6
Mean (D.r.)	1.0	1.3	0.7	0.7	1.0	1.3	1.0
SD	0.0	0.6	0.6	0.6	0.0	1.2	0.0

Mean (Total)	3.0	2.3	1.0	1.3	2.3	27	1.3
SD	0.0	1.2	1.0	1.2	0.6	1.5	0.6
Chambers							
Replicates	#C1	#C2	#C3	#C4	#C5	#C6	#C7
Organisms recorded per compartment after 2.5 h							
#1 P.r.	2	2	1	1	0	0	1
#1 D.r.	1	0	1	1	0	2	2
TOTAL	3	2	2	2	0	2	3
#2 P.r.	1	2	1	1	1	1	0
#2 D.r.	1	1	2	2	0	1	0
TOTAL	2	3	3	3	1	2	0
#3 P.r.	2	1	1	1	0	2	0
#3 D.r.	2	1	1	1	0	1	1
TOTAL	4	2	2	2	0	3	1
Mean (P.r.)	1.7	1.7	1.0	1.0	0.3	1.0	0.3
SD	0.6	0.6	0.0	0.0	0.6	1.0	0.6
Mean (D.r.)	1.3	0.7	1.3	1.3	0.0	1.3	1.0
SD	0.6	0.6	0.6	0.6	0.0	0.6	1.0
Mean (Total)	3.0	2.3	2.3	2.3	0.3	2.3	1.3
SD	1.0	0.6	0.6	0.6	0.6	0.6	1.5
Chambers							
Replicates	#C1	#C2	#C3	#C4	#C5	#C6	#C7
Organisms recorded per compartment after 3.0 h							
#1 P.r.	2	1	2	0	1	0	1
#1 D.r.	1	0	1	1	2	1	1
TOTAL	3	1	3	1	3	1	2
#2 P.r.	1	2	1	1	1	1	0
#2 D.r.	1	1	1	2	1	1	0
TOTAL	2	3	2	3	2	2	0
#3 P.r.	2	1	1	1	0	1	1
#3 D.r.	2	1	1	1	0	1	1
TOTAL	4	2	2	2	0	2	2
Mean (P.r.)	1.7	1.3	1.3	0.7	0.7	0.7	0.7
SD	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Mean (D.r.)	1.3	0.7	1.0	1.3	1.0	1.0	0.7
SD	0.6	0.6	0.0	0.6	1.0	0.0	0.6
Mean (Total)	3.0	2.0	2.3	2.0	1.7	1.7	1.3
SD	1.0	1.0	0.6	1.0	1.5	0.6	1.2

Table S6: Two-way ANOVA results for percentage of *P. reticulata* (dependent variable) in the control test with cultivate water (multispecific – 7 fish). Chambers and observation moments were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	19.786	5	3.957	0.033	0.999
Chambers	3937.570	6	656.262	5.514	0.000
Time * Chambers	3380.525	30	112.684	0.947	0.553
Error	9997.722	84	119.021		

Table S7: Two-way ANOVA results for percentage of *D. rerio* (dependent variable) in the control test with cultivate water (multispecific – 7 fish). Chambers and observation moments were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	19.786	5	3.957	0.037	0.999
Chambers	1115.345	6	185.891	1.750	0.120
Time * Chambers	3016.364	30	100.545	0.946	0.553
Error	8924.563	84	106.245		

Table S8: Two-way ANOVA results for percentage of *P. reticulata* and *D. rerio* (dependent variable) in the control test with cultivate water (multispecific – 14 fish). Chambers and observation moments were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	15.020	5	3.004	0.037	0.999
Chambers	1886.365	6	314.394	3.891	0.002
Time * Chambers	2047.617	30	68.254	0.845	0.692
Error	6786.533	84	80.792		

Table S9: Distribution of *P. reticulata* in the avoidance test with Cu (monospecific – 7 fish), mean (n=3) and standard deviation (SD).

Replicates	Concentrations ($\mu\text{g}\cdot\text{L}^{-1}$)						
	0 (control)	11	17	29	40	55	70
Organisms introduced initially in each compartment							
#1	3	3	3	3	3	3	3
#2	3	3	3	3	3	3	3
#3	3	3	3	3	3	3	3
Mean	3.0	3.0	3.0	3.0	3.0	3.0	3.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Organisms recorded per compartment after 0.5 h							

	0	11	17	29	40	55	70
#1	2	1	1	1	1	1	0
#2	3	2	1	1	0	0	0
#3	2	2	1	1	1	0	0
Mean	2.3	1.7	1.0	1.0	0.7	0.3	0.0
SD	0.6	0.6	0.0	0.0	0.6	0.6	0.0
Organisms recorded per compartment after 1.0 h							
	0	11	17	29	40	55	70
#1	2	2	2	1	0	0	0
#2	2	2	2	1	0	0	0
#3	3	1	1	1	1	0	0
Mean	2.3	1.7	1.7	1.0	0.3	0.0	0.0
SD	0.6	0.6	0.6	0.0	0.6	0.0	0.0
Organisms recorded per compartment after 1.5 h							
	0	11	17	29	40	55	70
#1	2	2	2	1	0	0	0
#2	2	2	2	1	0	0	0
#3	3	1	1	1	1	0	0
Mean	2.3	1.7	1.7	1.0	0.3	0.0	0.0
SD	0.6	0.6	0.6	0.0	0.6	0.0	0.0
Organisms recorded per compartment after 2.0 h							
	0	11	17	29	40	55	70
#1	3	2	1	1	0	0	0
#2	3	3	1	0	0	0	0
#3	3	2	1	1	0	0	0
Mean	3.0	2.3	1.0	0.7	0.0	0.0	0.0
SD	0.0	0.6	0.0	0.6	0.0	0.0	0.0
Organisms recorded per compartment after 2.5 h							
	0	11	17	29	40	55	70
#1	3	2	2	0	0	0	0
#2	3	3	1	0	0	0	0
#3	2	2	2	1	0	0	0
Mean	2.7	2.3	1.7	0.3	0.0	0.0	0.0
SD	0.6	0.6	0.6	0.6	0.0	0.0	0.0
Organisms recorded per compartment after 3.0 h							
	0	11	17	29	40	55	70
#1	4	1	1	1	0	0	0
#2	4	3	0	0	0	0	0
#3	3	3	1	0	0	0	0
Mean	3.7	2.3	0.7	0.3	0.0	0.0	0.0
SD	0.6	1.2	0.6	0.6	0.0	0.0	0.0

Table S10: Distribution of *P. reticulata* in the avoidance test with Cu (monospecific – 14 fish), mean (n=3) and standard deviation (SD).

Replicates	Concentrations ($\mu\text{g}\cdot\text{L}^{-1}$)						
	0 (control)	11	17	29	40	55	70
Organisms introduced initially in each compartment							
#1	3	3	3	3	3	3	3
#2	3	3	3	3	3	3	3
#3	3	3	3	3	3	3	3
Mean	3.0	3.0	3.0	3.0	3.0	3.0	3.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Organisms recorded per compartment after 0.5 h							
	0	11	17	29	40	55	70
#1	7	5	1	1	0	0	0
#2	3	3	3	3	2	0	0
#3	5	2	2	2	2	1	0
Mean	5.0	3.3	2.0	2.0	1.3	0.3	0.0
SD	2.0	1.5	1.0	1.0	1.2	0.6	0.0
Organisms recorded per compartment after 1.0 h							
	0	11	17	29	40	55	70
#1	8	2	2	1	1	0	0
#2	6	2	2	2	2	0	0
#3	4	3	3	2	2	0	0
Mean	6.0	2.3	2.3	1.7	1.7	0.0	0.0
SD	2.0	0.6	0.6	0.6	0.6	0.0	0.0
Organisms recorded per compartment after 1.5 h							
	0	11	17	29	40	55	70
#1	11	1	1	1	0	0	0
#2	8	2	2	1	1	0	0
#3	4	3	2	2	1	1	1
Mean	7.7	2.0	1.7	1.3	0.7	0.3	0.3
SD	3.5	1.0	0.6	0.6	0.6	0.6	0.6
Organisms recorded per compartment after 2.0 h							
	0	11	17	29	40	55	70
#1	11	2	1	0	0	0	0
#2	8	2	2	1	1	0	0
#3	6	2	2	2	2	0	0
Mean	8.3	2.0	1.7	1.0	1.0	0.0	0.0
SD	2.5	0.0	0.6	1.0	1.0	0.0	0.0
Organisms recorded per compartment after 2.5 h							
	0	11	17	29	40	55	70
#1	10	1	1	1	1	0	0
#2	7	2	2	2	1	0	0
#3	6	2	2	2	2	0	0
Mean	7.7	1.7	1.7	1.7	1.3	0.0	0.0
SD	2.1	0.6	0.6	0.6	0.6	0.0	0.0

	Organisms recorded per compartment after 3.0 h						
	0	11	17	29	40	55	70
#1	9	2	1	1	1	0	0
#2	7	2	2	2	1	0	0
#3	6	3	3	1	1	0	0
Mean	7.3	2.3	2.0	1.3	1.0	0.0	0.0
SD	1.5	0.6	1.0	0.6	0.0	0.0	0.0

Table S11: Two-way ANOVA results for avoidance (%) of *P. reticulata* (dependent variable) in the test with Cu (monospecific – 7 fish). Concentrations and observation moments were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	4406.772	5	881.354	8.237	0.000
Concentrations	118669.659	6	19778.277	184.84	0.000
Time *	4031.585	30	134.386	1.256	0.208
Concentrations				3	
Error	8988.040	84	107.000		

Table S12: Two-way ANOVA results for avoidance (%) of *P. reticulata* (dependent variable) in the test with Cu (monospecific – 14 fish). Concentrations and observation moments were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	1193.712	5	238.742	1.574	0.176
Concentrations	97478.850	6	16246.475	107.081	0.000
Time *	1861.645	30	62.055	0.409	0.996
Concentrations					
Error	12744.578	84	151.721		

Table S13: Distribution of *D. rerio* in the avoidance test with Cu (monospecific – 7 fish), mean (n=3) and standard deviation (SD).

Replicates	Concentrations ($\mu\text{g}\cdot\text{L}^{-1}$)						
	0 (control)	11	17	29	40	55	70
	Organisms introduced initially in each compartment						
#1	3	3	3	3	3	3	3
#2	3	3	3	3	3	3	3
#3	3	3	3	3	3	3	3
Mean	3.0	3.0	3.0	3.0	3.0	3.0	3.0

SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Organisms recorded per compartment after 0.5 h						
	0	11	17	29	40	55	70
#1	2	2	2	1	0	0	0
#2	2	2	1	1	1	0	0
#3	3	1	1	1	1	0	0
Mean	2.3	1.7	1.3	1.0	0.7	0.0	0.0
SD	0.6	0.6	0.6	0.0	0.6	0.0	0.0
	Organisms recorded per compartment after 1.0 h						
	0	11	17	29	40	55	70
#1	4	1	1	1	0	0	0
#2	2	2	2	1	0	0	0
#3	3	2	1	1	0	0	0
Mean	3.0	1.7	1.3	1.0	0.0	0.0	0.0
SD	1.0	0.6	0.6	0.0	0.0	0.0	0.0
	Organisms recorded per compartment after 1.5 h						
	0	11	17	29	40	55	70
#1	2	2	1	1	1	0	0
#2	2	1	1	1	1	1	0
#3	2	2	2	1	0	0	0
Mean	2.0	1.7	1.3	1.0	0.7	0.3	0.0
SD	0.0	0.6	0.6	0.0	0.6	0.6	0.0
	Organisms recorded per compartment after 2.0 h						
	0	11	17	29	40	55	70
#1	2	2	1	1	1	0	0
#2	2	2	2	1	0	0	0
#3	2	2	1	1	1	0	0
Mean	2.0	2.0	1.3	1.0	0.7	0.0	0.0
SD	0.0	0.0	0.6	0.0	0.6	0.0	0.0
	Organisms recorded per compartment after 2.5 h						
	0	11	17	29	40	55	70
#1	2	2	2	1	0	0	0
#2	2	2	2	1	0	0	0
#3	3	2	1	1	0	0	0
Mean	2.3	2.0	1.7	1.0	0.0	0.0	0.0
SD	0.6	0.0	0.6	0.0	0.0	0.0	0.0
	Organisms recorded per compartment after 3.0 h						
	0	11	17	29	40	55	70
#1	3	2	1	1	0	0	0
#2	2	2	2	1	0	0	0
#3	2	2	2	1	0	0	0
Mean	2.3	2.0	1.7	1.0	0.0	0.0	0.0
SD	0.6	0.0	0.6	0.0	0.0	0.0	0.0

Table S14: Distribution of *D. rerio* in the avoidance test with Cu (monospecific – 14 fish), mean (n=3) and standard deviation (SD).

Replicates	Concentrations ($\mu\text{g}\cdot\text{L}^{-1}$)						
	0 (control)	11	17	29	40	55	70
Organisms introduced initially in each compartment							
#1	3	3	3	3	3	3	3
#2	3	3	3	3	3	3	3
#3	3	3	3	3	3	3	3
Mean	3.0	3.0	3.0	3.0	3.0	3.0	3.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Organisms recorded per compartment after 0.5 h							
	0	11	17	29	40	55	70
#1	6	2	2	2	2	0	0
#2	5	3	2	2	2	0	0
#3	4	3	3	2	2	0	0
Mean	5.0	2.7	2.3	2.0	2.0	0.0	0.0
SD	1.0	0.6	0.6	0.0	0.0	0.0	0.0
Organisms recorded per compartment after 1.0 h							
	0	11	17	29	40	55	70
#1	4	3	3	2	2	0	0
#2	6	2	2	2	2	0	0
#3	4	4	2	2	2	0	0
Mean	4.7	3.0	2.3	2.0	2.0	0.0	0.0
SD	1.2	1.0	0.6	0.0	0.0	0.0	0.0
Organisms recorded per compartment after 1.5 h							
	0	11	17	29	40	55	70
#1	5	3	2	2	2	0	0
#2	8	2	2	1	1	0	0
#3	4	4	2	2	2	0	0
Mean	5.7	3.0	2.0	1.7	1.7	0.0	0.0
SD	2.1	1.0	0.0	0.6	0.6	0.0	0.0
Organisms recorded per compartment after 2.0 h							
	0	11	17	29	40	55	70
#1	6	3	2	2	1	0	0
#2	4	3	3	2	2	0	0
#3	4	3	3	2	2	0	0
Mean	4.7	3.0	2.7	2.0	1.7	0.0	0.0
SD	1.2	0.0	0.6	0.0	0.6	0.0	0.0
Organisms recorded per compartment after 2.5 h							
	0	11	17	29	40	55	70
#1	4	3	3	2	2	0	0
#2	3	3	3	3	2	0	0
#3	5	3	2	2	2	0	0
Mean	4.0	3.0	2.7	2.3	2.0	0.0	0.0
SD	1.0	0.0	0.6	0.6	0.0	0.0	0.0

	Organisms recorded per compartment after 3.0 h						
	0	11	17	29	40	55	70
#1	4	3	3	2	2	0	0
#2	3	3	3	2	2	1	0
#3	6	2	2	2	2	0	0
Mean	4.3	2.7	2.7	2.0	2.0	0.3	0.0
SD	1.5	0.6	0.6	0.0	0.0	0.6	0.0

Table S15: Two-way ANOVA results for avoidance (%) of *D. rerio* (dependent variable) in the Cu test (monospecific – 7 fish). Concentrations and observation moments were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	2191.923	5	438.385	5.998	0.000
Concentrations	121093.207	6	20182.201	276.148	0.000
Time *	2569.160	30	85.639	1.172	0.281
Concentrations					
Error	6139.112	84	73.085		

Table S16: Two-way ANOVA results for avoidance (%) of *D. rerio* (dependent variable) in the test with Cu (monospecific – 14 fish). Concentrations and observation moments were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	446.895	5	89.379	3.418	0.007
Chambers	112995.117	6	18832.520	720.120	0.000
Time * Chambers	402.857	30	13.429	.513	0.979
Error	2196.762	84	26.152		

Table S17: Distribution of *P. reticulata* (P.r.) and *D. rerio* (D.r.) in the avoidance test with Cu (multispecific – 14 fish), mean (n=3) and standard deviation (SD).

Replicates	Concentrations ($\mu\text{g}\cdot\text{L}^{-1}$)						
	0	11	17	29	40	55	70
	Organisms introduced initially in each compartment						
#1 P.r.	1	1	1	1	1	1	1
#1 D.r.	1	1	1	1	1	1	1
TOTAL	2	2	2	2	2	2	2
#2 P.r.	1	1	1	1	1	1	1
#2 D.r.	1	1	1	1	1	1	1

TOTAL	2	2	2	2	2	2	2
#3 P.r.	1	1	1	1	1	1	1
#3 D.r.	1	1	1	1	1	1	1
TOTAL	2	2	2	2	2	2	2
Mean (P.r.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mean (D.r.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mean (Total)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chambers							
	0	11	17	29	40	55	70
Organisms recorded per compartment after 0.5 h							
#1 P.r.	3	1	2	0	0	0	1
#1 D.r.	0	0	0	2	3	0	2
TOTAL	3	1	2	2	3	0	3
#2 P.r.	3	3	1	0	0	0	0
#2 D.r.	0	0	3	3	1	0	0
TOTAL	3	3	4	3	1	0	0
#3 P.r.	2	2	0	0	1	2	0
#3 D.r.	0	1	3	3	0	0	0
TOTAL	2	3	3	3	1	2	0
Mean (P.r.)	2.7	2.0	1.0	0.0	0.3	0.7	0.3
SD	0.6	1.0	1.0	0.0	0.6	1.2	0.6
Mean (D.r.)	0.0	0.3	2.0	2.7	1.3	0.0	0.7
SD	0.0	0.6	1.7	0.6	1.5	0.0	1.2
Mean (Total)	2.7	2.3	3.0	2.7	1.7	0.7	1.0
SD	0.6	1.2	1.0	0.6	1.2	1.2	1.7
Chambers							
	0	11	17	29	40	55	70
Organisms recorded per compartment after 1.0 h							
#1 P.r.	0	1	2	4	0	0	0
#1 D.r.	2	1	0	0	2	0	2
TOTAL	2	2	2	4	2	0	2
#2 P.r.	1	1	1	3	1	0	0
#2 D.r.	0	0	3	0	1	3	0
TOTAL	1	1	4	3	2	3	0
#3 P.r.	2	1	1	0	2	1	0
#3 D.r.	0	1	3	3	0	0	0
TOTAL	2	2	4	3	2	1	0
Mean (P.r.)	1.0	1.0	1.3	2.3	1.0	0.3	0.0
SD	1.0	0.0	0.6	2.1	1.0	0.6	0.0
Mean (D.r.)	0.7	0.7	2.0	1.0	1.0	1.0	0.7
SD	1.2	0.6	1.7	1.7	1.0	1.7	1.2
Mean (Total)	1.7	1.7	3.3	3.3	2.0	1.3	0.7

SD	0.6	0.6	1.2	0.6	0.0	1.5	1.2
Chambers							
	0	11	17	29	40	55	70
Organisms recorded per compartment after 1.5 h							
#1 P.r.	2	1	2	0	2	0	0
#1 D.r.	0	0	0	3	1	3	0
TOTAL	2	1	2	3	3	3	0
#2 P.r.	1	1	1	0	3	1	0
#2 D.r.	0	0	0	4	0	3	0
TOTAL	1	1	1	4	3	4	0
#3 P.r.	2	2	1	2	0	0	0
#3 D.r.	0	0	0	1	3	3	0
TOTAL	2	2	1	3	3	3	0
Mean (P.r.)	1.7	1.3	1.3	0.7	1.7	0.3	0.0
SD	0.6	0.6	0.6	1.2	1.5	0.6	0.0
Mean (D.r.)	0.0	0.0	0.0	2.7	1.3	3.0	0.0
SD	0.0	0.0	0.0	1.5	1.5	0.0	0.0
Mean (Total)	1.7	1.3	1.3	3.3	3.0	3.3	0.0
SD	0.6	0.6	0.6	0.6	0.0	0.6	0.0
Chambers							
	0	11	17	29	40	55	70
Organisms recorded per compartment after 2.0 h							
#1 P.r.	2	3	2	0	0	0	0
#1 D.r.	1	0	1	1	2	0	2
TOTAL	3	3	3	1	2	0	2
#2 P.r.	3	0	0	3	1	0	0
#2 D.r.	0	1	5	0	0	0	1
TOTAL	3	1	5	3	1	0	1
#3 P.r.	2	2	0	3	0	0	0
#3 D.r.	0	0	0	1	3	3	0
TOTAL	2	2	0	4	3	3	0
Mean (P.r.)	2.3	1.7	0.7	2.0	0.3	0.0	0.0
SD	0.6	1.5	1.2	1.7	0.6	0.0	0.0
Mean (D.r.)	0.3	0.3	2.0	0.7	1.7	1.0	1.0
SD	0.6	0.6	2.6	0.6	1.5	1.7	1.0
Mean (Total)	2.7	2.0	2.7	2.7	2.0	1.0	1.0
SD	0.6	1.0	2.5	1.5	1.0	1.7	1.0
Chambers							
	0	11	17	29	40	55	70
Organisms recorded per compartment after 2.5 h							
#1 P.r.	2	3	0	1	1	0	0
#1 D.r.	0	0	3	0	2	1	1
TOTAL	2	3	3	1	3	1	1
#2 P.r.	1	0	0	4	2	0	0
#2 D.r.	0	1	2	0	0	3	1

TOTAL	1	1	2	4	2	3	1
#3 P.r.	2	2	3	0	0	0	0
#3 D.r.	0	0	1	3	2	1	0
TOTAL	2	2	4	3	2	1	0
Mean (P.r.)	1.7	1.7	1.0	1.7	1.0	0.0	0.0
SD	0.6	1.5	1.7	2.1	1.0	0.0	0.0
Mean (D.r.)	0.0	0.3	2.0	1.0	1.3	1.7	0.7
SD	0.0	0.6	1.0	1.7	1.2	1.2	0.6
Mean (Total)	1.7	2.0	3.0	2.7	2.3	1.7	0.7
SD	0.6	1.0	1.0	1.5	0.6	1.2	0.6
Chambers							
	0	11	17	29	40	55	70
Organisms recorded per compartment after 3.0 h							
#1 P.r.	4	0	2	1	0	0	0
#1 D.r.	0	1	0	3	1	1	1
TOTAL	4	1	2	4	1	1	1
#2 P.r.	0	2	1	0	4	0	0
#2 D.r.	2	0	0	5	0	0	0
TOTAL	2	2	1	5	4	0	0
#3 P.r.	3	1	3	0	0	0	0
#3 D.r.	0	0	0	4	2	1	0
TOTAL	3	1	3	4	2	1	0
Mean (P.r.)	2.3	1.0	2.0	0,3	1,3	0.0	0.0
SD	2.1	1.0	1.0	0,6	2,3	0.0	0.0
Mean (D.r.)	0.7	0.3	0.0	4.0	1.0	0.7	0.3
SD	1.2	0.6	0.0	1.0	1.0	0.6	0.6
Mean (Total)	3.0	1.3	2.0	4.3	2.3	0.7	0.3
SD	1.0	0.6	1.0	0.6	1.5	0.6	0.6

Table S18: Two-way ANOVA results for avoidance (%) of *P. reticulata* (dependent variable) in the test with Cu (multispecific – 7 fish). Concentrations and observation moments were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	4694.608	5	938.922	1.426	0.223
Concentrations	101973.090	6	16995.515	25.81	0.000
Time *	13193.027	30	439.768	0.668	0.893
Concentrations				4	
Error	55304.800	84	658.390		

Table S19: Two-way ANOVA results for avoidance (%) of *D. rerio* (dependent variable) in the test with Cu (multispecific – 7 fish). Concentrations and observation moments were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	5285.875	5	1057.175	1.339	0.256
Concentrations	43376.772	6	7229.462	9.159	0.000
Time *	16512.414	30	550.414	0.697	0.866
Concentrations					
Error	66305.304	84	789.349		

Table S20: Two-way ANOVA results for avoidance (%) of *P. reticulata* and *D. rerio* (dependent variable) in the test with Cu (multispecific – 14 fish). Concentrations and observation moments were considered fixed factors.

Source of variation	Sum of squares	Degrees of freedom	Mean square	<i>F</i>	<i>p</i>
Time	3290.339	5	658.068	1.599	0.169
Concentrations	61071.094	6	10178.516	24.735	0.000
Time *	9931.345	30	331.045	0.804	0.745
Concentrations					
Error	34565.806	84	411.498		

9. Biography

I am graduated in Biology, UNIFATEA, Lorena-SP (2007), specialist in Environmental Engineering, Engineering School of Lorena-USP (2009), Lorena-SP, and MSc in Sciences: Ecology, University of São Paulo (Institute of Biosciences), São Paulo-SP (2013).

The most relevant publications i have are:

Published articles:

Silva, D. C. V. R.; Araújo, C. V. M. ; López-Doval, J. C. ; Neto, M. B. ; Paiva, T. C. B.; Silva, F. T.; Pompêo, M. L. M. . Potential effects of triclosan on spatial displacement and local population decline of the fish *Poecilia reticulata* using a non-forced system. Chemosphere, v. 184, p. 329-336, 2017.

Cardoso-Silva, Sheila; **Silva, Daniel Clemente Vieira Rêgo**; Lage, Fernanda; Paiva, Teresa Cristina Brazil; Moschini-Carlos, Viviane; Rosa, André Henrique; Pompêo, Marcelo . Metals in sediments: bioavailability and toxicity in a tropical reservoir used for public water supply. Environmental Monitoring and Assessment, v. 188, p. 188: 310, 2016.

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Silva, D. C. V. R.; Queiroz, L. G. ; Cardoso-Silva, S. ; Fernandes, J. G.; Alaminio, D. A.; Paiva, T. C. B. ; pômpeo, M. M. L. Evaluation of the efficiency of a trophic state index in determining the water quality of public water supply reservoirs. Engenharia Sanitária e Ambiental, 2017.

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