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Microcystin and pyriproxyfen are toxic to early stages of development in *Rhamdia quelen*: An experimental and modelling study

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ABSTRACT

The recent increase of freshwater eutrophication has favored cyanobacteria blooms and consequently the increase of toxins such as microcystin-LR in aquatic environments, but few is know about the associated effect of toxin and other compounds. Pyriproxyfen is an insecticide indicated by WHO (World Health Organization) to control *Aedes aegypti* mosquito (vector of Dengue, Chikungunya and Zika diseases), however, the effects are not well described to non-target species, such as fish. The early life stages (ELS) of fish are more sensitive to chemical stress due to higher metabolic rate, immature immune system and high superficial area/volume ratio. In the current study, ELS of *R. quelen* a Neotropical fish were exposed to environmentally realistic concentrations of microcystin (1, 10 and 100 $\mu g\,L;^{-1}$ M1, M2 and M3 groups, respectively) from an algal extract, pyriproxyfen (1 and 10 $\mu g\,L^{-1}$, P1 and P2) and their association (co-exposure). The hatching, survival and larvae deformities were analyzed, and applied a mathematical model to evaluate the effects on the population size along further generations. Both compounds were toxic to embryos/larvae of fish, but the effects were more pronounced in M2, P1M2 and P2M1 for hatching and M2, P1M2, P2M1 and P1 for survival. Deformities prevailed in groups exposed to the chemicals at 48 hpf (hours post-fertilization) were suggestions of toxicological interaction in P1M2, P2M1 and P2M2 at 48 and 72 hpf. In 96 hpf, the levels of deformities were lower than in previous times. Model predicted population density over 100 years decreased to lower than 0.5 (50%) in all groups, except for P1M1, indicating risk of extinction. P1M2 had the worse results, followed by M2, P1M3 and P2M1. Cyanobacterial blooms can lead to microcystin-LR levels higher than M2 ($10\,\mu g\,L^{-1}$), and the suggestion of toxicological interaction with pyriproxyfen is relevant because both compounds may potentially coexist in aquatic environments. Finally, mathematical models may provide an ecological interpretation of the risk of exposure of fish.

1. Introduction

The increase of both global temperatures and human activities has led to the eutrophication of many water bodies, favoring the occurrence of cyanobacteria blooms (Puddick et al., 2014; U.S. EPA, 2015; Saraf et al., 2018) and the production of cyanotoxins such as microcystins (Wiegand and Pflugmacher, 2005). This is a cyclical heptapeptide with more than 100 congeners (Puddick et al., 2014).

Microcystin-LR is the most toxic microcystin (World Health Organization, 2003; Hilborn et al., 2005). Toxicity is due to inhibition of phosphatases (Carmichael, 1992), leading to severe hepatic damage in vertebrates after acute intoxication (Wiegand and Pflugmacher, 2005; Malbrouck and Kestemont, 2006; Pavagadhi and Balasubramanian, 2013). Microcystin-LR is also toxic to fish even under low concentrations at long-term exposure (Malbrouck and Kestemont, 2006) and uptake may occurs by food chain, gills and skin (Saraf et al., 2018).

Fish at early life stages (ELS) are more sensitive to chemical stress due to higher metabolic rate, immature immune system and skin, high superficial area/volume ratio and limited mobility on water column (Malbrouck and Kestemont, 2006; Saraf et al., 2018). Microcystin-LR effects on ELS of fish include reduction or absence of digestive system (Huynh-Delerme et al., 2005), reduction of the head size and the yolk sac, pericardial oedema (Liu et al., 2002), increased body and tail

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curvature (Wang et al., 2005), late hatching and decreased survival ratio (Malbrouck and Kestemont, 2006; Saraf et al., 2018). However, most of studies reviewed by Malbrouck and Kestemont (2006) used microinjection of purified microcystin into fertilized eggs due to decreased diffusion of microcystin through the chorion barrier of the embryo. Saraf et al. (2018) described a more realistic approach with water exposure of fish at ELS using cyanobacteria from blooms or laboratory.

Pyriproxyfen (2-[1-metil-2-(4-phenoxyfeniphenoxyfeni)etoxi]pyridine) is a wide spectrum insecticide, analogous to the juvenile hormone of insects, and acts on reproduction and development of insects, leading to death of pupa even at low concentrations (Sihuincha et al., 2005; Yapabandara and Curtis, 2004; Invest and Lucas, 2008). This molecule has been used in agriculture and, recently, in human drinking water (World Health Organization (WHO), 2008; Agência de Defesa Agropecuária do Paraná ADAPAR, 2017; PPDB, 2017) to control the development of mosquitoes such as *Aedes aegypti*, the vector of Dengue, Chikungunya and Zika diseases. Due to specificity of pyriproxyfen, low impact on the environment and toxicity to mammals are expected (Invest and Lucas, 2008), but toxicity on *Apis melifera* bees (Chen et al., 2016) and non-target species such as crab, shrimp and *Daphnia spp* have been described (McKenney, 2005; 4th WHO Pesticide Evaluation Scheme (WHOPES), 2001).

Few data are available on the toxicity of pyriproxyfen to vertebrates such as fish and fish ELS. Erratic swimming of *Xiphophorus maculatus* (Caixeta et al., 2016) and embryos deformities at extremely high concentration of pyriproxyfen (Truong et al., 2016) have been reported. Foremost, we did not find studies on toxicological interaction of pyriproxyfen and other chemicals such as microcystin in fish.

Rhamdia quelen (Silver catfish) is an omnivorous fish from Central and South America (Fishbase.org, 2017). In Southern Brazil, *R. quelen* is widely consumed by human population so that there is extensive aquaculture activity with well-known artificial reproduction. On this way, some ecotoxicological studies with larvae and adult fish are available (Kreutz et al., 2008; Pimpão et al., 2013; Brito et al., 2017).

The present study investigated the effects of environmentally realistic concentrations of microcystin-LR algal extract, pyriproxyfen and their association (co-exposure) on early life stage of *R. quelen* through analyses of survival and larvae deformities. In addition, a mathematical model (Brito et al., 2017) was applied to evaluate the effects of these chemicals on the population size of *R. quelen* along further generations.

2. Materials and methods

2.1. Microcystin

The microcystins (MC) used in the current study was obtained from the algal extract of *Microcystis aeruginosa* – strain: BB05 – Barra Bonita, provided by UFSCar – Federal University of São Carlos, Brazil (WDCM: CCMA-UFSCar 835) and cultivated in ASM-1 modified environment (Jacinavicius et al., 2017) in Technology Institute of Parana State, Brazil. The growth of cultures was interrupted when 10^6 cells mL^{-1} was reached. Then, the cultures were centrifuged (3000*g* at 4 °C for 15 min), and lyophilized for 3 days. Lyophilized cells were suspended in pure water (0.01 g mL^{-1}), sonicated for 3 min, and centrifuged (3000*g*, 4 °C for 5 min). The pellet was re-extracted according to the same procedure 3 times. The supernatants were combined and stored at − 20 °C until use (Silva-Stenico et al., 2009). Microcystins in supernatant were analyzed by mass spectrometer after filtration by PVDF 0.45 µm filter, according with Dahlmann et al. (2003) with modifications. A total of 10,500 µg L^{-1} of microcystin-LR and traces of microcystin-LW and microcystin-LF, below the instrument detection limit, were observed. Microcystin-RR and YR were not detected in the supernatant.

Before experiments, the supernatant solution was diluted in reconstituted water (0.0065 g L^{-1} $CaCl_2$, 0.1335 g L^{-1} $MgSO_4$, 0.0004 g L^{-1} KCl, 0.0105 g L^{-1} $NaHCO_3$) previously filtered in 0.45 µm filter, to obtain 200 µg L^{-1} MC-LR (stock solution). From the stock solution the test concentrations of 1 µg L^{-1} (M1), 10 µg L^{-1} (M2) and 100 µg L^{-1} (M3) were prepared. According to Brazilian law, 1 µg L^{-1} (= 1 ppb, M1) is the maximum allowed microcystins concentration for drinking-water (Brasil, Ministério da Saúde, 2011), whereas 10 µg L^{-1} (M2) and 100 µg L^{-1} (M3) was described in algae blooms, but MC can reach 25,000 µg L^{-1} in eutrophicated waters (World Health Organization et al., 1999).

2.2. Pyriproxyfen

Sumilarv™ product from Sumimoto Chemical (Chemical Abstract Service [CAS] Registry Number 95737-68-1), composed by 0.5% of pyriproxyfen in volcanic sand of slow releasing, was obtained from Health Secretary of Parana State, Brazil. The stock solutions (20 and 200 µg L^{-1}) were prepared with reconstituted water after 15 min of shaking and filtered in 80 g filter paper to remove the volcanic sand. From the stocks, test concentrations (10 µg L^{-1}) were prepared based on that recommended to mosquito control in drinking-water (World Health Organization (WHO), 2008), and 100 µg L^{-1} which may be found in residential water tanks with low volume capacity (BRASIL, 2014).

2.3. Test solutions of microcystin, pyriproxyfen and their association

A total of 12 experimental groups were established: control, microcystin (M1, M2, M3), pyriproxyfen (P1, P2) and the association of both (P1M1, P1M2, P1M3, P2M1, P2M2, P2M3). The concentrations of microcystin and pyriproxyfen were further confirmed in these test solutions. *Microcystin*: LC-ESI-MS/MS (Accela UHPLC, Thermo Fisher Scientific) coupled to a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific) with positive mode electrospray ionization - ESI (Dahlmann et al., 2003, with modifications), using 5 mg L^{-1} standard microcystin RR, YR and LR (Fluka™). *Pyriproxyfen*: liquid chromatography/tandem mass spectrometer (Applied Biosystems – API 4000) adapted from EPA Method 538 (U.S. EPA, 2009), using 100 mg L^{-1} standard pyriproxyfen (Accustandard™). For microcystin, the real concentrations (in MC-LR) were similar to those calculated: M1 = 0.95 µg L^{-1}, M2 = 9.40 µg L^{-1} and M3 = 97.8 µg L^{-1}. For pyriproxyfen, the real concentrations were lower than calculated: P1 = 0.17 µg L^{-1} and P2 = 1.85 µg L^{-1}. These values were due to the slow release of pyriproxyfen into the water from the commercial product.

In the mixtures, the concentrations of microcystin (in MC-LR) were close to those of the respective isolated solutions: M1 = 0.92 (P1M1) and 1.06 µg L^{-1} (P2M1); M2 = 9.01 (P1M2) and 10.0 µg L^{-1} (P2M2) and M3 = 94.7 (P1M3) and 90.1 µg L^{-1} (P2M3). For pyriproxyfen, P1 levels ranged from 0.10 (P1M1) to 0.17 µg L^{-1} (P1M2) and P2 levels ranged from 0.84 in (P2M1) to 2.44 µg L^{-1} (P2M3). However, these low concentrations are more realistic, being closer to the environmentally observed levels of pyriproxyfen (Belenger et al., 2014; Ccanccapa et al., 2016).

2.4. Procedures for eggs fertilization and exposure

2.4.1. Parental spawning

one male and one female of adult *Ramdia quelen* were used to obtain the fertilized eggs, according to procedures established from Panama fish-farming (Santa Catarina State, Brazil, www.pisciculturapanama.com.br) and described by Graeff et al. (2008), with modifications. The female fish received two intramuscular injections of pituitary extract of carp fish diluted in saline solution (0.5 mg kg^{-1} and 4.5 mg kg^{-1}), respectively, with intervals of 8 h between injections. The moment of spawning was calculated considering the sum of hour and temperature (hour-degree) after injection, which vary between 210 and 240 h-degrees according to fish species. The spawning was performed in a plastic