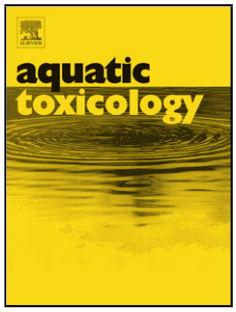

Title: Copper-Driven Avoidance And Mortality In Temperate And Tropical Tadpoles

Author: Cristiano V.M. Araújo Cândida Shinn Matilde Moreira-Santos Isabel Lopes Evaldo L.G. Espíndola Rui Ribeiro

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COPPER-DRIVEN AVOIDANCE AND MORTALITY IN TEMPERATE AND TROPICAL TADPOLES

Cristiano VM Araújo[*a], Cândida Shinn[a], Matilde Moreira-Santos[a], Isabel Lopes[b], Evaldo LG Espíndola[c], Rui Ribeiro[a]

[a]IMAR-Instituto do Mar, Department of Life Sciences, University of Coimbra, Apartado 3046, 3001-401 Coimbra, Portugal

[b]Department of Biology & CESAM, University of Aveiro, Campus de Santiago, 3810-193, Aveiro, Portugal

[c]NEEA-Núcleo de Estudos em Ecossistemas Aquáticos, CRHEA, USP, Universidade de São Paulo, Brazil

Abstract

Amphibians have experienced an accentuated population decline in the whole world due to many factors, one of them being anthropogenic contamination. The present study aimed to assess the potential effect of copper, as a worldwide and reference contaminant, on the immediate decline of exposed population due to avoidance and mortality responses in tadpoles of three species of amphibians across climatic zones: a South American species, *Leptodactylus latrans*, a North American species, *Lithobates catesbeianus*, and a European species, *Pelophylax perezi*. A non-forced exposure system with a copper gradient along seven compartments through which organisms could freely move was used to assess the ability of tadpoles to detect and avoid copper contamination. All species were able to avoid copper at a concentration as low as 100 µg L^{-1}. At the lowest (sublethal) concentrations (up to 200 µg L^{-1}) avoidance played an exclusive role for the population decline, whereas at the highest concentrations (>450

[*]Corresponding author. Tel: + 351-239855781; fax: + 351-239855789.

E-mail address: cristiano.araujo@icman.csic.es (CVM Araújo).

μg L^{-1}) mortality was the response determining population decline. The median concentrations causing exposed population immediate decline were 93, 106 and 180 μg L^{-1} for *Le. latrans*, *Li. catesbeianus* and *P. perezi*, respectively. Contaminants might, therefore, act as environmental disruptors both by generating low-quality habitats and by triggering avoidance of tadpoles, which could be an important response contributing to dispersion patterns, susceptibility to future stressors and decline of amphibian populations (together with mortality).

Keywords: amphibian population decline; anuran larvae; avoidance; contamination; environmental disruption.

1. Introduction

Global amphibian decline has been recognized as a phenomenon of major concern as amphibians are one of the groups most threatened of extinction (Stuart et al., 2004; McCallum, 2007). The Global Amphibian Assessment worldwide project (by the International Union for Conservation of Nature) has recently reported that almost one-third (32%) of the world amphibian species are threatened (1,896 species) and that at least 43% of all species are declining (IUCN, 2012), thus anticipating that amphibians will continue to be threatened, at least in the near future. Among the several causes for such decline (e.g. habitat loss and destruction, UV-B radiation, invasive species, increased disease susceptibility, over-exploitation as food resource, climate change) chemical contamination is considered a highly threatening factor (Beebee and Griffiths, 2005; Nyström et al., 2007; Blaustein et al., 2010; Hayes et al., 2010). The threat of contamination acting as an environmental disruptor is linked to the reduction and/or fragmentation of habitat and its quality loss, causing a decrease in the density and viability of populations, an increase in the susceptibility to future stressors, and changes in dispersion patterns between neighboring habitats (Ribeiro and Lopes, 2013;Wilson and Hopkins, 2013).

Many amphibian species have been shown to be susceptible to toxic effects of different contaminant classes (e.g. agrochemicals, metals, nitrogenous compounds and industrial effluents), which can act on enzymatic activity, morphological and histological development, behavior, growth, reproduction, and survival (James and Little, 2003; Ferrari et al., 2005; Shinn et al., 2008, 2013; Relyea and Jones, 2009; Ossana et al., 2010; Gürkan and Hayretdağ, 2012; Marques et al., 2013). The ability to avoid contaminants has been another endpoint studied in amphibians. However, such studies have mainly focused on swimming ability, such as traveled distance, speed and frequency of swimming (Bridges, 1997; Chen et al., 2007; Shinn et al., 2008; Denoël et al., 2013), and on oviposition (Takahashi, 2007; Vonesh and Buck, 2007). In addition, all these studies have been performed under forced exposure conditions, with no alternative towards which organisms could present avoidance or preference responses. Although the ability of tadpoles to detect and avoid contamination moving towards less contaminated zones has been scarcely investigated (but see study by Steele et al., 1989), this response is highly relevant because it indicates possible changes regarding the pattern of the organisms' displacement dynamics and, thus, potential implications for a population immediate decline (PID in Rosa et al., 2012; see also Gutierrez et al., 2012).

The present study has therefore focused on two key objectives: (i) to investigate the role of a metal (copper, Cu) as habitat disruptor by triggering avoidance response in tadpoles of three species of amphibians from different geographic regions, namely *Leptodactylus latrans* (Steffen, 1815), *Lithobates catesbeianus* (Shaw, 1802) and *Pelophylax perezi* (López-Seoane, 1885) (hereafter *Le. latrans*, *Li. catesbeianus*, and *P. perezi*); (ii) to estimate the exposed population immediate decline (PID) due to combined avoidance and mortality responses. To achieve these two goals, a non-forced exposure system (simulating a large water body with heterogeneously distributed contamination) with a Cu gradient through which organisms could freely move was employed. The use of the three species allows for a more global approach regarding the consequences of contamination for amphibian populations. Although Cu is an essential metal for animals and plants and a substantial input of Cu into aquatic compartments comes from natural sources, it was selected given that residual levels from domestic, industrial and agricultural activities have increased in many aquatic ecosystems worldwide (Ossana et

al. 2010; Aronzon et al., 2011). Lastly, Cu can be highly toxic to amphibians at levels often measured at contaminated sites (Redick and La Point, 2004; Aronzon et al., 2011; Lance et al., 2012; Xia et al., 2012).

2. Materials and methods

2.1. Species: characteristics, origin and culture conditions

Three amphibian species were selected to carry out this study. *Leptodactylus latrans* (sapo-ranallanero, butter frog or common thin-toed frog, also known as *Le. ocellatus* and *Rana latrans* - Lavilla et al., 2010) is a species widely present in South America (Araújo et al., 2009; Coelho et al., 2012; Heitor et al., 2012), generally found in open grasslands near temporary or permanent ponds, streams or marshes (Heyer et al., 1990). *Lithobates catesbeianus*, known as the bullfrog, is originally from North America, currently occurring as invasive species in lentic ecosystems across different regions, such as Europe, Asia and South America (Giovanelli et al., 2008). The Perez' frog, *P. perezi*, is a native species in Europe (found in southern France and across the Iberian Peninsula), inhabiting a wide variety of temporary and permanent water bodies, such as streams, ditches and irrigation canals (Loureiro et al., 2010). At present, the IUCN has listed the populations of *Le. latrans* and *P. perezi* as stable and of least concern, while *Li. catesbeianus* is listed as increasing and of least concern (http://www.iucnredlist.org/initiatives/amphibians, last visited May 2013).

Fresh feral *Le. latrans* egg masses were collected from an outdoor tank containing natural water (pH = 8.4; dissolved oxygen = 6.9 mg L^{-1}; conductivity = 47 µS cm^{-1}; salinity = 0), located at CRHEA (Centro de Recursos Hídricos e Ecologia Aplicada, São Carlos, São Paulo, SE Brazil). *Lithobates catesbeianus* tadpoles were obtained from a frog farm located near São Carlos city and transported to the laboratory in tap water (pH = 8.1; dissolved oxygen = 7.0 mg L^{-1}; conductivity = 27 µS cm^{-1}; salinity = 0). Both species were maintained in plastic aquaria containing tap water (pH = 7.4; conductivity = 30 µS cm^{-1}) with continuous and gentle aeration (dissolved oxygen above 7 mg L^{-1}), at 25 ºC and under a photoperiod of 12:12 h light/darkness. A few individuals of the

floating macrophyte species *Pistia stratiotes* were placed in the aquaria during acclimatization. *Pelophylax perezi* egg masses were collected in a lentic area of a freshwater brook (40°23'10.9"N, 8°14'5.3"W) within the hydrological basin of the Mondego River (Central Portugal) (pH = 7.5; dissolved oxygen = 6.5 mg L^{-1}; conductivity = 126 µS cm^{-1}; salinity = 0.1). Egg masses were transported to the laboratory immediately after collection and placed in an aquarium with FETAX medium (Dawson and Bantle, 1987). After hatching, larvae were transferred to 500 mL glass vessels also containing FETAX, and maintained at 20 ºC on a 16:8 h light/darkness cycle. Organisms of the three species were maintained under the outlined culture conditions until reaching Gosner stage 25 (Gosner, 1960), at which point they were used in the tests. Culture conditions were considered acceptable as until the tests were performed no mortality was recorded for *Li. catesbeianus*, whereas for *Le. latrans* and *P. perezi* mortality was below 10%. Organisms used in the tests were actively swimming and presented mean ± standard deviation (n = 10) total body length (tip of the head to the tip of the tail) of 0.9 ± 0.1 cm (*Le. latrans*), 1.0 ± 0.1 cm (*Li. catesbeianus*) and 1.0 ± 0.1 cm (*P. perezi*). For testing, it was preferred to use tadpoles rather than adults due to their higher sensitivity to contaminants and because it is a life stage confined to the aquatic environment (Bridges, 1997).

2.3. System for avoidance tests

A multi-compartmented non-forced static system was used in the tests (Fig. 1). Each system comprised of seven compartments, with a total length of 105 cm and total volume of 980 mL (system #1) for tests with *Le. latrans* and *Li. catesbeianus*, and total length of 98 cm and total volume of 350 mL (system #2) for tests with *P. perezi*. Each compartment was constructed from two plastic flasks glued at the cut-out bases using white glue (Sikaflex-11FC^{+}, Baar, Switzerland). The compartments were then connected with glue at the mouth of the glued bottles in order to obtain a 7-compartment system. The total capacity of each compartment was 140 mL (system #1) and 50 mL (system #2), but only 125 and 45 mL, respectively, of test solution were used during each test.

A calibration of the avoidance system was performed in order to verify the stability of the contamination gradient. A sodium chloride (NaCl) solution was used for this purpose as an accurate relationship with conductivity values could be easily obtained. Five NaCl concentrations (17, 33, 50, 66, and 83 mg L^{-1}) were prepared using a stock solution of 100 mg L^{-1} (considered 100%) plus a control (0%) of tap water used in the dilutions. The parameters of the NaCl concentration-conductivity calibration curves for system #1 were, $y=2082x + 27.06$, $r^2=0.9998$, $p<0.0001$, $n=7$, and for system #2, $y=2215x + 572.4$, $r^2=0.9999$, $p<0.0001$, $n=7$. For calibration, the individual compartments were isolated from each other with plasticine plugs wrapped in parafilm while each of the seven NaCl solutions was carefully disposed in its respective compartment. The plugs were then removed to form a linear concentration gradient. The calibration procedure lasted 12 h, as this was the maximum exposure time in the avoidance tests with organisms. Conductivity values were recorded at 0 (initial values before introducing the dilutions into the compartments) and 12 h (final values measured directly inside each compartment). The system calibration was performed in triplicate without organisms. Data of the initial and final NaCl concentrations of the calibration procedure are presented in Table 1. The variation observed between the initial and final NaCl concentration was of 0 to 7% for system #1 and of 1 to 22% for system #2.

2.4. Avoidance tests

Using culture water in all compartments, controls were carried out to verify the existence of no mortality and the random distribution of the tadpoles in the absence of contamination, i.e., no preference/avoidance for any compartment of the test system. Each control experiment was performed once and the number of replicate systems, number of organisms introduced into each compartment and the total number of organisms per replicate system and per experiment were, respectively: 3, 4, 28, and 84 for *Le. latrans*; 4, 5, 35, and 140 for *Li. catesbeianus* and *P. perezi*.

For avoidance tests with Cu, seven concentrations (nominal concentrations: 0, 110, 220, 330, 430, 540, and 650 µg L^{-1}) were prepared and disposed in each compartment. Five

organisms per compartment were then introduced and only after the plasticine plugs were removed, to form a Cu gradient. Tests were performed in quadruplicate for *Le. latrans* and *Li. catesbeianus* and in triplicate for *P. perezi*, totalling 20 and 15 organisms per tested Cu concentration, respectively. All tests were performed in the dark at 26 (*Le. latrans* and *Li. catesbeianus*) and 20 ºC (*P. perezi*). After 12 h exposure, the distribution of alive and dead organisms along the compartments was checked. Samples of each compartment were taken to determine, by atomic absorption gas chromatography (method 3113 B – APHA, 1995), the final actual Cu concentration: 35, 115, 180, 210, 445, 500, and 610 μg L^{-1} for the test with *Le. latrans*; 35, 115, 210, 300, 455, 510, and 580 μg L^{-1} for the test with *Li. catesbeianus*; and 24, 160, 220, 320, 390, 490, and 580 μg L^{-1} for the test with *P. perezi*.

2.5. Statistical analysis

The randomness of the distribution of organisms among compartments within each avoidance system, when exposed exclusively to control water for 12 h, was checked with the Kruskal-Wallis test. In the avoidance test with Cu, the number of avoiders was computed as the number of expected organisms minus the number of observed organisms. The expected number of organisms was determined from the exposed organisms (those introduced in a given compartment) plus immigrants (expected organisms in the compartment adjacent of higher concentration minus the organisms observed in that compartment). The avoidance percentage in each compartment was determined as the number of avoiders divided by the expected ones. For the highest concentration, immigrant organisms were not expected, so the number of expected organisms was equal to the number of organisms initially introduced in that concentration. Mortality percentages were determined from the number of dead organisms out of all observed organisms. The exposed population immediate decline (PID, in %) induced by Cu was calculated for each concentration used in the avoidance test via the integration of avoidance and mortality results. Copper concentrations that caused avoidance, mortality and PID of 50% of the population (AC_{50}, LC_{50} and PID_{50}, respectively) and corresponding 95% confidence intervals (CI) were calculated using

PriProbit 1.63 software (Sakuma, 1998). Calculations were performed taking into account the real copper concentrations measured at the end of the tests.

3. Results

Results of the control distribution showed that organisms distributed randomly in the systems in the absence of contamination, with no statistically significant difference (Kruskal-Wallis Statistic - H) among the number of organisms observed in each compartment: p=0.6761, H=4.005 for *Le. latrans*; p=0.6265, H=4.372 for *Li. catesbeianus*; p=0.0899, H=10.953 for *P. perezi*.

Percentages of avoidance, mortality and PID for each tested Cu concentration are shown in Fig. 2. All species were able to avoid sublethal Cu concentrations. At concentrations around 200 μg L^{-1} the avoidance was ca. 80%, while mortality was lower than 5%. At concentrations higher than 200 μg L^{-1} the percentage of avoidance declined and increases in mortality began to be recorded. The PID curve followed the same trend as the avoidance response at the lowest concentrations (until ca. 200 μg L^{-1}), whereas at the highest concentrations (>450 μg L^{-1}) mortality was the response leading to the population immediate decline.

Avoidance was a response three to six times more sensitive than mortality. On the other hand, the effective Cu concentrations for avoidance and PID that affected 50% of the population were very similar (Table 2). Regarding the AC_{50} values, the three species responded similarly to Cu exposure, with a difference of less than double, although avoidance by *P. perezi* showed to be relatively less sensitive (highest AC_{50} value) (Table 2) than that of the other species.

4. Discussion

Avoidance from Cu contamination by tadpoles of three species of amphibians, *Le. latrans*, *Li. catesbeianus* and *P. perezi*, was studied in a multi-compartmented non-forced system. All species showed to be able to detect and avoid sublethal Cu concentrations and the sensitivity of this response for the three species was very similar. Avoidance, being more sensitive than mortality, played a more relevant role for the exposed population immediate decline, but only at the lowest concentrations as at the highest concentrations it was mortality that played an evident role in declining the amphibian population (see below). Taking as a reference the concentration of 100 μg L^{-1} Cu, at which the avoidance response was considerable, other studies using a forced exposure revealed different sublethal effects at that same concentration, such as longer time to metarmophosis in *Li. sphenocephalus* (Lance et al., 2012) and decreased swimming performance and time to metamorphosis in *Rana pipiens* (Chen et al., 2007). Avoidance showed to be, therefore, a sensitive, obvious and reliable sublethal response that could have important repercussions for amphibian population migration dynamics: although dispersal occurs mainly in adults, the avoidance of tadpoles is expected to increase with each decrease in the gradient of contamination. Thus, for amphibians inhabiting large water bodies, particularly those with a heterogeneously distributed contamination, the present results reinforce the hypothesis of underestimating the risk of population decline and possible extinction if only forced exposure tests are used (Rosa et al., 2012).

Only at concentrations higher than 200 μg L^{-1} of Cu - when tadpoles were possibly not able to move towards less contaminated zones - did the importance of the mortality for the population decline increase. This decrease or even loss of the ability to avoid contamination, which can be due to moribundity, was similarly recorded in stream macroinvertebrates of the genus *Anomalocosmoecus* exposed to crude oil (Araújo et al., submitted) and in cladocerans and copepods exposed to metals and to the insecticide endosulfan (Gutierrez et al., 2012). Many other contaminants have shown to weaken the swimming ability of tadpoles, thus possibly impairing avoidance ability (Wojtaszek et al., 2004; Chen et al., 2007; Shinn et al., 2008; Denoël et al., 2013). A possible effect of Cu on the neuromuscular function of tadpoles has been hypothesized as the cause of decreased swimming ability and, consequently, ability to escape (Chen et al., 2007).

When exposed to high concentrations at which avoidance response is impaired (therefore they cannot escape from the toxic habitat), organisms would be more susceptible to suffer the lethal toxic effects of the contaminant.

Copper has been extensively studied regarding its toxicity to many amphibian species, showing to be a toxic substance that can cause effects on morphological and histological development, behavior, swimming activity, growth, reproduction, and survival (Ferreira et al., 2004; Chen et al., 2007; Ossana et al., 2010, Gürkan and Hayretdağ, 2012). The results of the present study indicate that Cu can also effectively trigger an avoidance response in tadpoles of different amphibian species even at non-lethal concentrations. A similar response has been described by Lopes et al. (2004) and Gutierrez et al. (2012) for cladocerans and copepods. Given that avoidance can lead to a population immediate decline by the displacement of organisms to more favorable zones (Moreira-Santos et al., 2008; Rosa et al., 2012), its consequences are more enhanced at the ecosystem level than at the individual level. Contaminants can, thus, act as lethal toxicants as well as habitat disruptors. The former role can be differentiated by directly measuring acute or chronic responses on organisms, while a role as habitat disruptor can be observed by generating habitats with low quality and triggering avoidance before toxic effects are detected. The latter effect is particularly important given that concentrations at which it might occur could be considered non-risky as no toxic effect at the individual level would be usually observed. This has been shown for the cladoceran *Daphnia magna* exposed to atrazine (Rosa et al., 2012) and the copepod *Boeckella occidentalis intermedia* exposed to crude oil (Araújo et al., submitted) in avoidance experiments under laboratory conditions. Habitat disruption caused by contaminants has also been hypothesized based on historical and experimental evidence: elevated nitrate and phosphate concentrations and resulting decline of *Litoria aurea* populations (Harmer et al., 2004), habitat degradation due to increasing levels of fertilizers and pesticides (Beebee and Griffiths, 2005), unsuccessful reproduction of amphibians linked to eutrophication (Nyström et al., 2007), and selective oviposition of the gray tree frog triggered by the presence of a pesticide (Vonesh and Buck, 2007). Accordingly, even at concentrations considered safe at the individual level, if avoidance is induced by the presence of a contaminant, modifying and reducing the quality of the environment, the

stability of populations could be seriously affected (Vonesh and Buck, 2007; Vonesh and Kraus, 2009; Ribeiro and Lopes, 2013).

The disrupting effect of contaminants on ecosystems can be comparable to the loss and fragmentation of habitats (Green, 2003; Beebee and Griffiths, 2005; Ribeiro and Lopes, 2013; Wilson and Hopkins, 2013). Habitats with low quality due to contamination are less probable to effectively support an amphibian population or even to serve as sink habitats for surrounding populations, as distance between high-quality habitats is increased and dispersion rates between neighboring habitats is reduced (Wilson and Hopkins, 2013). Heard et al. (2012) suggested that the rapid decline of the Australian frog *Litoria raniformis* may have been due to metapopulation collapse, driven by habitat loss, degradation and fragmentation, and stochastic perturbations. This scenario deserves special attention given that amphibian populations are structured in small subpopulations with permanent dispersion of individuals from one subpopulation to another, being prone to stochastic events and, therefore, dependent on good-quality surrounding habitats (Beebee and Griffiths, 2005; Wilson and Hopkins, 2013).

5. Conclusion

Tadpoles of *Le. latrans*, *Li. catesbeianus* and *P. perezi* showed to be able to avoid Cu contamination. Avoidance was a more reliable and sensitive response than mortality. Therefore, at lower concentrations the avoidance response plays a more important role than mortality in the population decline. Although further studies are needed to gauge the ecological implications of the present results (e.g. other contaminants, avoidance response in space and over time), avoidance is suggested to be an important response contributing to the local decline of amphibian populations across climatic zones. On the other hand, contaminants, by triggering avoidance, might exert an important role as environmental disruptors generating low quality habitats that can affect the dispersion pattern of amphibians.

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Figure captions

Figure 1. Schematic diagram of the multi-compartmented non-confined static avoidance assay system featuring one of the seven compartments. For systems #1 and #2, respectively: A, 8 x 2.5 and 7 x 1.5 cm; B, 140 and 50 mL; C, 4 and 3 cm; D, 1 and 0.9 cm; E, 15 and 14 cm.

Figure 2. Concentration-response curves for avoidance and mortality responses, and of the estimated PID (exposed population immediate decline) of tadpoles of three species of amphibians exposed to copper.

561 **Tables**

562 Table 1. NaCl concentrations (± standard deviation; mg L^{-1}) of the dilutions used in the
563 calibration of the avoidance test systems #1 and #2 at 0 h (initial; before introducing the
564 dilutions in the compartments; $n = 1$) and 12 h (final; inside each compartment; $n = 3$).

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566 Table 2. Copper concentrations (and respective 95% confidence intervals; µg L^{-1}) that
567 cause avoidance (AC_{50}), mortality (LC_{50}) and exposed population immediate decline
568 (PID_{50}) of 50% of the tested amphibian species.

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570 calibration of the avoidance test systems #1 and #2 at 0 h (initial; before introducing the
571 dilutions in the compartments; n = 1) and 12 h (final; inside each compartment; n = 3).

NaCl (0 h)	System #1	System #2
	12 h	12 h
0	1.9 (±0.5)	0.03 (±2.0)
17	18.2 (±0.5)	20.7 (±0.9)
33	35.5 (±3.0)	36.2 (±2.9)
50	49.3 (±3.1)	50.7 (±3.6)
66	65.8 (±1.7)	63.2 (±2.3)
82	81.6 (±2.5)	77.6 (±3.8)
100	99.2 (±2.6)	97.8 (±4.3)

572

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574 cause avoidance (AC_{50}), mortality (LC_{50}) and exposed population immediate decline
575 (PID_{50}) of 50% of the tested amphibian species.

Species	AC_{50}	LC_{50}	PID_{50}
Le. latrans	102 (73 - 122)	606 (525 - 768)	93 (34 - 186)
Li. catesbeianus	101 (nc)	372 (196 - 1442)	106 (0.7 – 231)
P. perezi	178 (176 - 181)	487 (467 - 512)	180 (63 – 247)

576 nc: not calculated.

577

Highlights:

- Three species of amphibian were studied for avoidance response to copper.
- A seven-compartment non-forced system with a copper contamination gradient was used.
- Avoidance and mortality were integrated to predict the exposed population decline.
- Avoidance was a reliable and more sensitive response than mortality.
- Cooper showed to be an environmental disruptor even at sublethal concentrations.

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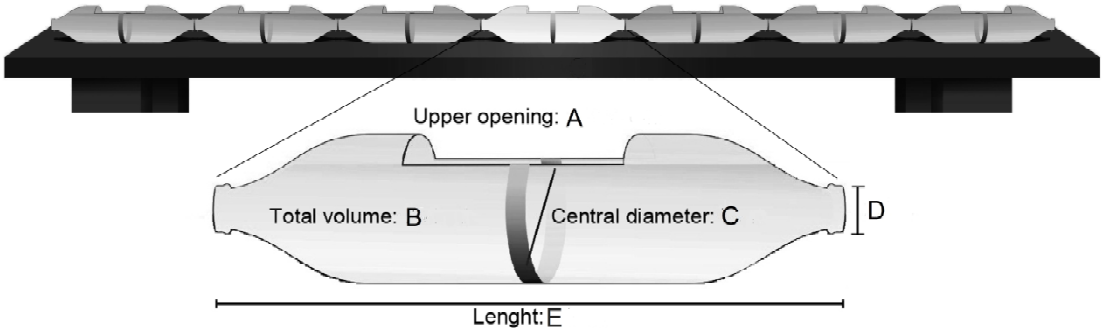

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587 assay system featuring one of the seven compartments. For systems #1 and #2,
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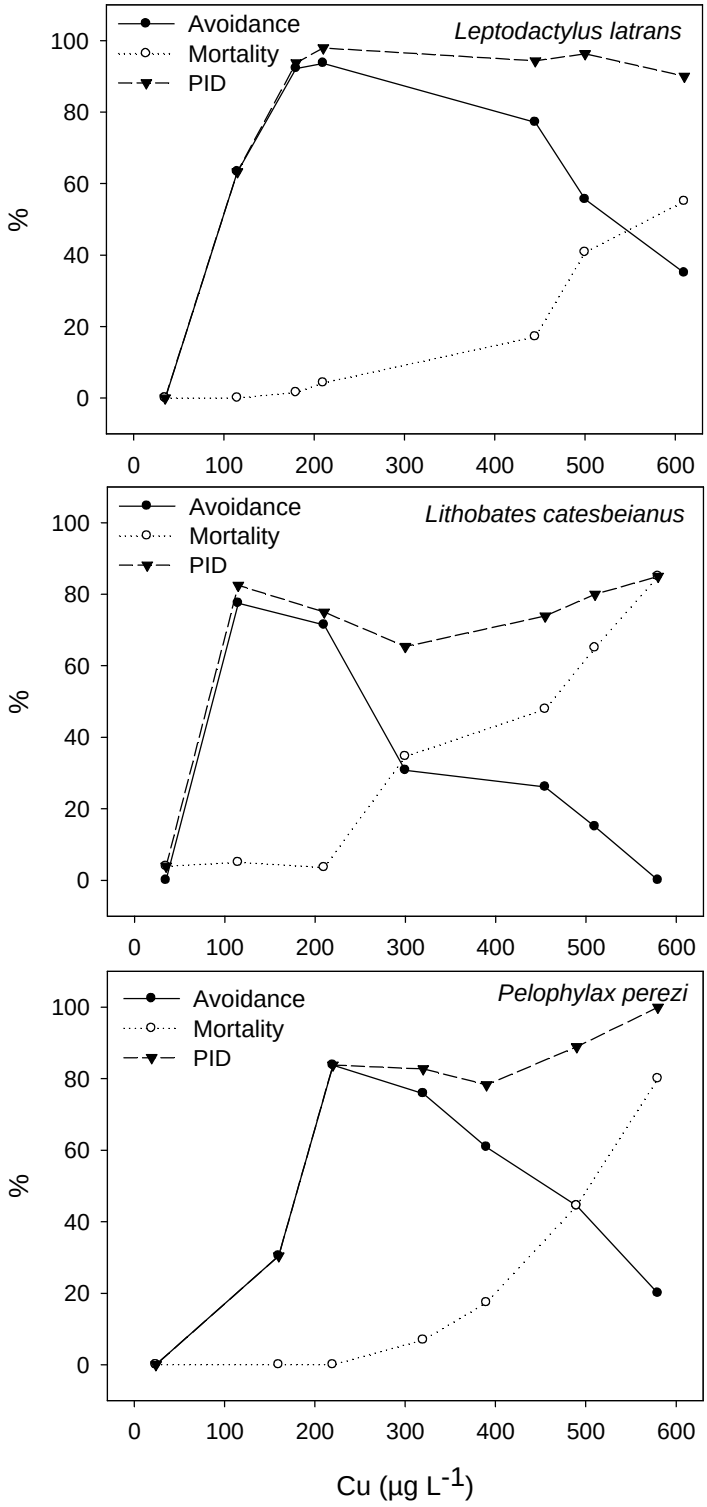

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