Humane acute testing with tadpoles for risk assessment of chemicals: Avoidance instead of lethality

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HIGHLIGHTS

• Fish toxicity data are not a secure surrogate to predict the effects on amphibians.
• Avoidance indicates the threshold at which organisms will flee from contamination.
• A reduction in the population density due to emigration is analogous to mortality.
• Test with amphibians based on spatial avoidance is a more humane method.
• Avoidance tests provide an ethical advantage to ecotoxicity tests (Refinement - 3 Rs).

ABSTRACT

In spite of the sensitivity of amphibians to contamination, data from fish have been commonly used to predict the effects of chemicals on aquatic life stages. However, recent studies have highlighted that toxicity data derived from fish species may not protect all the aquatic life stages of amphibians. For pesticide toxicity assessment (PTA), EFSA has highlighted that more information on lethal toxicity for the aquatic life stages of amphibians is still needed to reduce uncertainties. The current review aims to propose a test with amphibians based on spatial avoidance, as a more humane alternative method to the lethality tests for chemicals. A review of lethal toxicity tests carried out with amphibians in the period between 2018 and 2021 is presented, then we discuss the suitability of using fish toxicity data as a surrogate to predict the effects on more sensitive amphibian groups. The possible differences in sensitivity to chemicals may justify the need to develop further tests with amphibian embryos and larvae in order to reduce uncertainties. A new test is proposed focused on the avoidance behaviour of organisms fleeing from contamination to replace lethal tests. As avoidance indicates the threshold at which organisms will flee from contamination, a reduction in the population density, or its disappearance, at the local scale due to emigration is expected, with ecological consequences analogous to mortality. Avoidance tests provide an ethical advantage over lethal tests as they respect the concepts of the 3 Rs (mainly Refinement), reducing the suffering of the organisms.

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1. Introduction

Historically, the use of animals in experimentation comes from ancient Greece (500 BCE) as a surrogate for humans (Guerrini, 2003). From that period on, scientific thought about animal experimentation has evolved considerably, so that the lack of intelligence and notions of justice and injustice were initially indicated as reasons for animals not being protected against suffering (Guerrini, 2003). From the 19th century onwards, animal experimentation started to be criticised, which led to the creation of the Society for the Protection of Animals Liable to Vivisection (1875) and the consolidation of the Law of Cruelty to Animals of Britain in 1876 (Rogers, 2007). During the following years, many initiatives that focused on welfare in animal experimentation were launched, such as: the British Cruelty to Animal Act, in the United Kingdom (Pankevich et al., 2012; DGOTA, 2014), the Animal Welfare Act, in the USA (AWA, 1966), the Act on Animal Experimentation, in the Netherlands (Bordes, 2005), and the Universal Declaration of Animal Rights by the UNESCO (UNESCO, 1978). In 1986, the Council of the European Communities adopted the Directive 86/609/EEC to guarantee identical legislative, regulatory and administrative provisions in all member states related to the use of animals in experimentation (EEC, 1986). Nowadays, the Directive 2010/63/EU is the legal framework that regulates animal experimentation procedures in the European Union, establishing more humane procedures for animals proven to feel pain: living non-human vertebrate animals (including larval forms with autonomous feeding or reproduction and foetal forms of mammals in the last third of their normal development) and cephalopods (EU, 2010).

The use of animals in experimentation is a practice that deserves special environmental and ethical concern due to the high quantity of organisms used and their suffering. In this sense, alternative methods for animal experimentation have been sought to replace the animals used in laboratory tests (Garthoff, 2005), for instance, computer models, cells and tissue cultures and alternative organisms (the use of invertebrates or microorganisms instead of vertebrates) (Doke and Dahwale, 2015). Similarly, Rai and Kaushik (2018) also proposed in-silico simulation, informatics, 3D cell culture models, and organ-on-chips as innovative and alternative methods to animal experimentation. The use of these alternative methods has some disadvantages, which makes it difficult to fully replace animals (Van Norman, 2019). It is important to consider that the extrapolation of the effects from the sub-organismal level to the population and community levels, or even to the individual level, presents many uncertainties, since, from the functional (e.g., genetic and physiological mechanisms) and behavioural (e.g., ecological niche) points of view, organisms are much more than merely a group of cells, just as populations and communities are much more complex than groups of individuals. In the case of ecotoxicity studies, the replacement is very difficult because the aim of these studies is to assess the effects that chemicals cause on biota, considering from the sub-organismal to the community and ecosystem levels. In this cascade of effects, the various genetic, biochemical, physiological, and behavioural disruptions might vary according to the surrounding environment. Thus, it is important to know the chemical processes of the contaminants, like toxicokinetics and toxicodynamics, and how organisms deal with the absorption of contaminants (detoxification: from complexion to the elimination of chemicals). The use of alternative methods can lack relevance if they do not simulate the organismal complexity of the individuals, their arrangement or their functionality accurately.

Probably, one of the most accepted replacement procedures in ecotoxicology occurs among ecological groups, where fish are commonly used as a surrogate for the aquatic life stages of amphibians, although for some chemicals fish are not an accurate surrogate (Ortiz-Santaliestra et al., 2018; Glaberman et al., 2019). The European Food Safety Authority (EFSA) has identified that, for some chemical compounds (namely pyrethroids), fish may not be good alternatives to assess the risk to the aquatic stages of amphibians, as the latter tends to be more sensitive, therefore more data is needed to be able to suggest assessment factors to apply to existing fish data (EFSA, 2007). Although the recommendation of using lethality tests is based on this important ecological reasoning, two inconvenient aspects for animal welfare can be highlighted: i) the concentrations used to detect mortality tend to be very high and might suppose the maximal suffering that an animal can experience and ii) the experimental procedure using a mandatory intensity of exposure for a given period (forced-exposure) supposes a continuous suffering during the experiments. Therefore, the use of mortality as an ecotoxicological response essentially does not meet the recommendation of the Directive 2010/63/EU (EU, 2010) to end the procedures where an animal experiences pain equivalent to or higher than that caused by the introduction of a needle (the humane endpoint). From a regulatory perspective, the endpoints considered of relevance in ecotoxicology are those that may predict a population decline (Agerstrand et al., 2020); however, the application of more humane experimental procedures that meet this requisite without losing biological relevance should be encouraged. In this regard, the avoidance response, which focuses on the spatial displacement of organisms escaping from contamination (Lopes et al., 2004 and reviews by Araújo et al., 2016; Moreira-Santos et al., 2019), could be considered a more humane alternative to replace lethality tests. This idea is premised on the fact that when the organisms move away from a contaminated area the effect, at the local population level, is identical to mortality (Lopes et al., 2004; Hellou, 2011), even though no long-lasting direct toxic effect occurs regarding the individuals. Therefore, the aim of the present critical review is to propose spatial avoidance tests, using a non-forced multi-compartmented approach, as an alternative to lethality tests. This alternative considerably reduces the animal’s suffering as it prevents the continuous exposure to chemicals, given that the animals are not mandatorily exposed to the test concentrations and can move freely among different concentrations (gradient or patchy scenarios), choosing the most favourable one. Initially, we reviewed (from 2018 to July of 2021) how frequently mortality was used as an endpoint to assess the toxicity of chemicals on the larval stages of amphibians, at development stages considered as animal experimentation (Gosner stage ≥25, Nieuwkoop and Faber stage ≥45 and Harrison stage ≥42). This time period was established to give continuity to the review by Ortiz-Santaliestra et al. (2018). Later, the alternative of using fish ecotoxicity data as surrogates for the aquatic stage (tadpoles) of amphibians is discussed. To support the hypothesis that tadpoles avoid contamination, experimental evidence that tadpoles, of many species, are able to detect and escape chemical contamination is provided. Furthermore, the sensitivity of this avoidance response is compared with that of lethality. Finally, we discuss the ecological importance of spatial avoidance when the non-forced exposure approach is used and how humane this method is when considering one of the principles of the 3 Rs: Refinement (reducing suffering and improving the living conditions of animals) (NC3Rs, 2009).

2. Lethality as an endpoint in amphibian toxicity tests

The amount of data available relating to amphibians in the aquatic system has been considered too limited to make comparisons concerning toxicity with alternative animal groups accurately (EFSA PPR Panel, 2018). Currently, among vertebrates, only fish, birds and mammals are recommended for use in ecological risk assessments (EEC, 1986). However, the EFSA Panel on Plant Protection Products (PPP) and their Residues recognized the need for further data on the acute and chronic effects of active substances or PPPs on amphibians in simple laboratory tests (EFSA PPR Panel, 2018). However, although test data concerning amphibians is not required for the submission of new chemicals (only terrestrial phases are required), all previously available information must be provided to the authorities.

Based on Ortiz-Santaliestra et al. (2017, 2018), Table 1 presents the latest studies using lethality as an endpoint with amphibian larval stages. Briefly, a total of 62 studies were found, in which 36 species
Table 1
Studies on toxicity using amphibian tadpoles from 2018 to 2020, in which mortality was used as the endpoint.

<table>
<thead>
<tr>
<th>Year</th>
<th>Species (Family; Order)</th>
<th>Contaminants</th>
<th>Exposure time (h)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2019</td>
<td><em>Bufo pulchella</em> (Hyliidae: Anura)</td>
<td>Growth, development, body mass, and morphological effects</td>
<td>96</td>
<td>Pérez-Iglesias et al. (2018a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Imaethapipy-based herbicide formulation</td>
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<td>Pivot H</td>
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<tr>
<td></td>
<td></td>
<td>Sediment with various contaminants</td>
<td>240</td>
<td>Sasanine et al. (2018)</td>
</tr>
<tr>
<td></td>
<td><em>Ceratophrys ornata</em> (Ceratophryidae: Anura)</td>
<td>Behavioural changes, growth inhibition and morphological abnormalities</td>
<td>48/96</td>
<td>Natale et al. (2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pirimicarb-based commercial formulation</td>
<td></td>
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<td></td>
<td></td>
<td>Chlorpyrifos</td>
<td></td>
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<tr>
<td></td>
<td><em>Holobatrachus rugulosus</em> (Dicroglossidae: Anura)</td>
<td>Avoidance of predators, changes in swimming behaviour and interactive effects of copper and imidacloprid</td>
<td>24</td>
<td>Sievers et al. (2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Atrazine (herbicide), cypermethrin (insecticide) and tebuconazole (fungicide)</td>
<td></td>
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<tr>
<td></td>
<td><em>Physalaemus cuvieri</em> (Leptodactylidae: Anura)</td>
<td>Motility and malformations</td>
<td>96</td>
<td>Rutkoski et al. (2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herbicide atrazine</td>
<td></td>
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<tr>
<td></td>
<td><em>Physalaemus gracilis</em> (Leptodactylidae: Anura)</td>
<td>Growth inhibition, development retardation, thyroid gland histology, and axial malformations</td>
<td>96</td>
<td>Zhang et al. (2018a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rac-Cyproconazole, 1-enantiomers, 2-enantiomers, 3-enantiomers and 4-enantiomers</td>
<td></td>
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<tr>
<td></td>
<td><em>Rana nigromaculata</em> ( Ranidae: Anura)</td>
<td>Weight, body length and development stages</td>
<td>96</td>
<td>Zhang et al. (2018b)</td>
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<td></td>
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<td>Triadimefon and triadimenol</td>
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<td>Colloidal silicon dioxide nanoparticles</td>
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<td></td>
<td><em>Rhinella schneideri</em> (Biofoniidae: Anura)</td>
<td>–</td>
<td>48/96</td>
<td>Pérez-Iglesias et al. (2018b)</td>
</tr>
<tr>
<td></td>
<td><em>Silurana tropicalis</em> (Pipidae: Anura)</td>
<td>Growth inhibition, development retardation, thyroid gland histology, and axial malformations</td>
<td>96</td>
<td>Saka et al. (2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Seven 1,3,5-triazine (s-triazine) herbicides (ametryn, prometryn, dimethametryn, simazine, atrazine, propazine, cyanazine)</td>
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<td></td>
<td><em>Ambystoma talpoideum</em> (Ambystomatidae: Caudata)</td>
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<td>1152</td>
<td>Weir et al. (2019)</td>
</tr>
<tr>
<td></td>
<td><em>Hypнопhassa pardinis</em> (Hyliidae: Anura)</td>
<td>–</td>
<td>96</td>
<td>Daam et al. (2019)</td>
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<tr>
<td></td>
<td><em>Leptodactylus fuscus</em> (Leptodactylidae: Anura)</td>
<td>–</td>
<td>2,4-dichlorophenoxyacetic acid herbicide (DMA 806)</td>
<td>Freitas et al. (2019)</td>
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<td>2,4-dichlorophenoxyacetic acid herbicide (DMA 806)</td>
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<td></td>
<td><em>Lithobates clamitans</em> ( Ranidae: Anura)</td>
<td>Energy storage, development, respiration rates, swimming performance and avoidance behaviour</td>
<td>96</td>
<td>Moreira et al. (2019)</td>
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<td>Diuron Nortox 500 SC pesticide</td>
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<td></td>
<td><em>Rhinella arenarum</em> (Biofoniidae: Anura)</td>
<td>Growth inhibition, development retardation, thyroid gland histology, and axial malformations</td>
<td>96</td>
<td>Zhang et al. (2018a)</td>
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<td>Zhang et al. (2018b)</td>
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<td><em>Ambystoma opacum</em> (Ambystomatidae: Caudata)</td>
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<td>Weir et al. (2019)</td>
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<td><em>Hypнопhassa pardinis</em> (Hyliidae: Anura)</td>
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<td>Daam et al. (2019)</td>
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<td>Freitas et al. (2019)</td>
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<td></td>
<td><em>Lithobates catesbeianus</em> ( Ranidae: Anura)</td>
<td>Energy storage, development, respiration rates, swimming performance and avoidance behaviour</td>
<td>96</td>
<td>Freitas et al. (2019)</td>
</tr>
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<td><em>Lithobates clamitans</em> ( Ranidae: Anura)</td>
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<td>Moreira et al. (2019)</td>
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<td>Zhang et al. (2018b)</td>
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<td><em>Ambystoma opacum</em> (Ambystomatidae: Caudata)</td>
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<td><em>Hypнопhassa pardinis</em> (Hyliidae: Anura)</td>
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<td>Daam et al. (2019)</td>
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<td><em>Lithobates catesbeianus</em> ( Ranidae: Anura)</td>
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<td>Energy storage, development, respiration rates, swimming performance and avoidance behaviour</td>
<td>96</td>
<td>Moreira et al. (2019)</td>
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Table 1 (continued)

<table>
<thead>
<tr>
<th>Year</th>
<th>Species (Family; Order)</th>
<th>Stage</th>
<th>Number of organisms without control</th>
<th>Other endpoints</th>
<th>Contaminants</th>
<th>Exposure time (h)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2020</td>
<td>Microhyla fusipes (Microhyla: Anura)</td>
<td>GS 26-28</td>
<td>180</td>
<td>Enzymatic activities, growth, and locomotion</td>
<td>Cadmium</td>
<td>48</td>
<td>Hu et al. (2019)</td>
</tr>
<tr>
<td></td>
<td>Odontophrynus americanus (Ceratophryidae: Anura)</td>
<td>GS 26-30</td>
<td>120</td>
<td>Enzyme activities, hormone levels, cardiac rates, behaviour performance, development rate and growth</td>
<td>Pyriproxyfen insecticide</td>
<td>48</td>
<td>Lajmanovich et al. (2019a)</td>
</tr>
<tr>
<td></td>
<td>Pelophylax perezi (Ranidae: Anura)</td>
<td>GS 25</td>
<td>420</td>
<td>Growth and somatic growth</td>
<td>Natural sea water (SW), sodium chloride (NaCl) and sodium chloride before and after short-term exposure to low levels of salinity (NaCl 375)</td>
<td>96</td>
<td>Venancio et al. (2019)</td>
</tr>
<tr>
<td></td>
<td>Physalaemus albonotatus (Leptodactylidae: Anura)</td>
<td>GS 23-27</td>
<td>120</td>
<td>Morphology, development, body, visceral abnormalities and liver histology</td>
<td>Commercial herbicide formulated with 2,4-D</td>
<td>96</td>
<td>Curi et al. (2019)</td>
</tr>
<tr>
<td></td>
<td>Physalaemus cuvieri (Leptodactylidae: Anura)</td>
<td>GS 25</td>
<td>800</td>
<td>–</td>
<td>Herbicides: acetochlor, ametryn, glyphosate, and metribuzin</td>
<td>96</td>
<td>Daam et al. (2019)</td>
</tr>
<tr>
<td></td>
<td>Physalaemus nattereri (Leptodactylidae: Anura)</td>
<td>GS 25</td>
<td>120</td>
<td>Energy storage, development, respiration rates, swimming performance and avoidance behaviour</td>
<td>2,4-dichlorophenoxyacetic acid herbicide (DMA 806)</td>
<td>96</td>
<td>Freitas et al. (2019)</td>
</tr>
<tr>
<td></td>
<td>Rana catesbeiana (Ranidae: Anura)</td>
<td>GS 25</td>
<td>220</td>
<td>Body mass and development stage</td>
<td>Perfluorooctanesulfonic acid (PFOS), perfluorooctanoic (PFOA) acid and their mixtures</td>
<td>96</td>
<td>Flynn et al. (2019)</td>
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<tr>
<td></td>
<td>Rana phaeoccephala (Ranidae: Anura)</td>
<td>GS 26</td>
<td>280</td>
<td>–</td>
<td>Neonicotinoid insecticide clothianidin</td>
<td>96</td>
<td>Holsworth et al. (2019)</td>
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<td></td>
<td>Rhinella arenarum (Bufonidae: Anura)</td>
<td>GS 26-30</td>
<td>210</td>
<td>Enzymatic activities, hormone levels, miotic index, DNA damage, development stage, and growth</td>
<td>Glyphosate and arsenic mixture</td>
<td>48</td>
<td>Lajmanovich et al. (2019b)</td>
</tr>
<tr>
<td></td>
<td>Boana pardalis (Hyliidae: Anura)</td>
<td>GS 25</td>
<td>72</td>
<td>Locomotor swimming performance and thermal tolerance limits</td>
<td>Chloryprifos</td>
<td>96</td>
<td>Quiroga et al. (2019)</td>
</tr>
<tr>
<td></td>
<td>Bombeinus pulchellus (Micrathenidae: Anura)</td>
<td>GS 29-42</td>
<td>100</td>
<td>Motility</td>
<td>Five pesticides (cypermethrin, chlorpyrifos, endosulfan, glyphosate and 2,4-dichlorophenoxyacetic acid)</td>
<td>144/192/216</td>
<td>Agostini et al. (2020)</td>
</tr>
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<td></td>
<td>Euthysticus cyanophytyctis (Dicroglossidae: Anura)</td>
<td>GS 25-30</td>
<td>900</td>
<td>–</td>
<td>Arsenic, chromium and herbicide almix 20WP</td>
<td>24/48/72/96</td>
<td>Samanta et al. (2020)</td>
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<td>Leptodactylus latinasus (Leptodactylidae: Anura)</td>
<td>GS 25 and GS 36</td>
<td>100</td>
<td>Swimming activity, morphological abnormalities, and enzymatic activities</td>
<td>Imaezathapy-based herbicide formulation</td>
<td>96</td>
<td>Pérez-Iglesias et al. (2020)</td>
</tr>
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<td></td>
<td>Leptodactylus latrans (Leptodactylidae: Anura)</td>
<td>GS 29-42</td>
<td>720</td>
<td>Motility</td>
<td>Five pesticides: (cypermethrin, chlorpyrifos, endosulfan, glyphosate and 2,4-dichlorophenoxyacetic acid)</td>
<td>144/192/216</td>
<td>Agostini et al. (2020)</td>
</tr>
<tr>
<td></td>
<td>Lithobates catesbeianus (Ranidae: Anura)</td>
<td>GS 25</td>
<td>120</td>
<td>Swimming activity; morphological analysis; respirometry and avoidance DNA damage</td>
<td>Mining tailing</td>
<td>96</td>
<td>Girotto et al. (2020)</td>
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<tr>
<td></td>
<td>Lithobates sphenocephalus (Ranidae: Anura)</td>
<td>GS 25</td>
<td>144</td>
<td>–</td>
<td>Bacillus thuringiensis kustaki biopesticide</td>
<td>96</td>
<td>Motta et al. (2020)</td>
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<td></td>
<td>Lithobates sylvaticus (Ranidae: Anura)</td>
<td>GS 26</td>
<td>348</td>
<td>Malformations in metamorphosis</td>
<td>NaCl</td>
<td>96</td>
<td>Green and Salice (2020)</td>
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<tr>
<td></td>
<td>Physalaemus cuvieri (Leptodactylidae: Anura)</td>
<td>GS 25-26</td>
<td>210</td>
<td>Length, mass and malformations</td>
<td>Glyphosate-based herbicide Roundup</td>
<td>96</td>
<td>Herak et al. (2020)</td>
</tr>
<tr>
<td></td>
<td>Physalaemus gracilis (Leptodactylidae: Anura)</td>
<td>GS 25-26</td>
<td>210</td>
<td>Length, mass and malformations</td>
<td>Glyphosate-based herbicide Roundup</td>
<td>96</td>
<td>Herak et al. (2020)</td>
</tr>
<tr>
<td></td>
<td>Physalaemus gracilis (Leptodactylidae: Anura)</td>
<td>GS 25</td>
<td>300</td>
<td>Swimming activity and biochemical traits</td>
<td>Chlorpyrifos insecticide</td>
<td>96</td>
<td>Rutkoski et al. (2020a)</td>
</tr>
<tr>
<td></td>
<td>Physalaemus gracilis (Leptodactylidae: Anura)</td>
<td>GS 25</td>
<td>600</td>
<td>–</td>
<td>–</td>
<td>168</td>
<td>(continued on next page)</td>
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</table>
(especially belonging to the order Anura and with a preference for the Gosner stages between 25 and 36) and about 70 contaminants (pesticides, herbicides, fungicides and heavy metals, among others) have been studied. Data in Table 1 and the review by Ortiz-Santaliestra et al. (2018) may facilitate future comparisons of the sensitivity for each specific toxicant between amphibians and other animal groups. Furthermore, it may also facilitate comparisons among toxicity endpoints, specially the one here proposed: avoidance assays.

3. Fish as surrogates for amphibians

The problem about the decline of amphibian populations and species started to be of major concern since the 90s (Wake, 1991). Several factors were pointed out as drivers for this loss of biodiversity: habitat change, UV radiation, diseases, introduction of invasive species, and environmental contamination (Alford and Richards, 1999; Stuart et al., 2004; McCallum, 2007; IUCN: https://www.iucnredlist.org). One of the first studies that reviewed ecotoxicological data concerning amphibians was published by Power et al. (1989), which was later completed by Pauli et al. (2000). At first, the comparison of the data on ecotoxicity concerning fish and amphibians justified the use of the former as a substitute for the latter. However, even then, there were already suspicions that fish might not be good amphibian surrogates. However, Bridges et al. (2002) found a good efficiency in the use of fish as

| Year | Species (Family; Order) | Stage
g | Number of organisms without control | Other endpoints | Contaminants | Exposure time (h) | References |
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<tr>
<td></td>
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<td></td>
<td>Enzymatic activities, biochemical responses</td>
<td>Cypermethrin- (Cyprin 250 CE) and fipronil- (TerraFort®) based insecticides</td>
<td>144/192/ 216</td>
<td>Rutkoski et al. (2020b)</td>
</tr>
<tr>
<td></td>
<td>Rhinella arenarum</td>
<td>GS 29-</td>
<td>100</td>
<td>Motility</td>
<td>Five pesticides (cypermethrin, chlorpyrifos, endosulfan, glyphosate and 2,4-dichlorophenoxyacetic acid)</td>
<td>96</td>
<td>Agostini et al. (2020)</td>
</tr>
<tr>
<td></td>
<td>(Bifonidae: Anura)</td>
<td>42</td>
<td></td>
<td>Mancoseb fungicide</td>
<td>Herbicide with glyphosate(GLY)-dicamba (DIC) and glyphosate(GLY)-flurochloridone (FLC)</td>
<td>96</td>
<td>Asparch et al. (2020)</td>
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<td></td>
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<td></td>
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<td></td>
<td>Soluble and emulsifiable concentrations of dimethoate</td>
<td>48</td>
<td>Martinuzzi et al. (2020)</td>
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<td>AI203, NiO/AI203 and Ni/AI203</td>
<td>96</td>
<td>Svarz et al. (2020)</td>
</tr>
<tr>
<td></td>
<td>Rhinella fernandaeae</td>
<td>GS 29-</td>
<td>100</td>
<td>Motility</td>
<td>Five pesticides (cypermethrin, chlorpyrifos, endosulfan, glyphosate and 2,4-dichlorophenoxyacetic acid)</td>
<td>144/192/ 216</td>
<td>Agostini et al. (2020)</td>
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<td></td>
<td>(Bifonidae: Anura)</td>
<td>42</td>
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<td>Chloropyrifos</td>
<td>96</td>
<td>Turhan et al. (2020)</td>
</tr>
<tr>
<td>2021</td>
<td>Xenopus laevis</td>
<td>NF 46</td>
<td>390</td>
<td>–</td>
<td>Glyphosate pure (GLY) and glyphosate-based product (Roundup)</td>
<td>96</td>
<td>Costa et al. (2021a)</td>
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<td></td>
<td>(Pipidae: Anura)</td>
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<td>Chlorpyrifos</td>
<td>96</td>
<td>COSTA ET AL. (2021)</td>
</tr>
<tr>
<td></td>
<td>Ceratophrys ornata</td>
<td>GS 31</td>
<td>590</td>
<td>Swimming alterations, presence of morphological abnormalities, and prey consumption</td>
<td>–</td>
<td>72</td>
<td>Luz et al. (2020)</td>
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<td></td>
<td>(Ceratophryidae: Anura)</td>
<td></td>
<td></td>
<td></td>
<td>Hydroxychloroquine and azithromycin</td>
<td>72</td>
<td>Luz et al. (2020)</td>
</tr>
<tr>
<td></td>
<td>Elachitoctis bicolor</td>
<td>GS 30-</td>
<td>330</td>
<td>Biochemical responses</td>
<td>Herbicide DIC Cowboy Elite SURCOS®</td>
<td>48</td>
<td>Attademo et al. (2021)</td>
</tr>
<tr>
<td></td>
<td>(Microhydridae: Anura)</td>
<td>34</td>
<td></td>
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<td>herbicide</td>
<td>96</td>
<td>Viriato et al. (2021)</td>
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<tr>
<td></td>
<td>Lithobates caeruleusius</td>
<td>GS 31-</td>
<td>168</td>
<td>Healthiness, haematological profile, and histopathological analysis</td>
<td>2,4, D (DMA ® 806)</td>
<td>144/192/ 216</td>
<td>Arcaute et al. (2020)</td>
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<td></td>
<td>(Ranidae: Anura)</td>
<td>36</td>
<td></td>
<td></td>
<td>Polyeethylene glycol</td>
<td>72</td>
<td>Nascimento et al. (2021)</td>
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<td></td>
<td>Physalaemus cavierti</td>
<td>GS 27</td>
<td>360</td>
<td>Toxicity biomarker and polyethylene glicol accumulation</td>
<td>–</td>
<td>72</td>
<td>Nascimento et al. (2021)</td>
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<td></td>
<td>(Leptodactylidae: Anura)</td>
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<td>Hydroxychloroquine and azithromycin</td>
<td>72</td>
<td>Luz et al. (2021)</td>
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<td>Polypedates maculatus</td>
<td>GS 19-</td>
<td>300</td>
<td>Analysis of RNA-seq data, differential transcript expression analysis</td>
<td>Cadmium chloride</td>
<td>24/48</td>
<td>Ojha et al. (2021)</td>
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<td></td>
<td>(Rhacophoridae Anura)</td>
<td>20</td>
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<td>2,4,6-trinitrotoluene (TNT), the new insensitive munition formulation (IMX-101), the insensitive munition constituents nitrogeniusidine (NQ), and 1-methyl-3-nitroguanidine (MeNQ)</td>
<td>96</td>
<td>Gust et al. (2021)</td>
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<td>Rana pipiens</td>
<td>GS 25</td>
<td>640</td>
<td>–</td>
<td>Chlorothalonil fungicide</td>
<td>504</td>
<td>Aguaroni et al. (2021)</td>
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<td>(Ranidae: Anura)</td>
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<td>Herbicide DIC Cowboy Elite SURCOS®</td>
<td>48</td>
<td>Attademo et al. (2021)</td>
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<td>Morphological and behavioural alterations</td>
<td>Herbicide DIC Cowboy Elite SURCOS®</td>
<td>48</td>
<td>Attademo et al. (2021)</td>
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<td>Biochemical responses</td>
<td>Herbicide DIC Cowboy Elite SURCOS®</td>
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<td>Attademo et al. (2021)</td>
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<td>Morphometric and gravimetric data</td>
<td>Four neonicotinoids: (acetamiprid, clothianidin, dinofuran, and imidacloprid) and fipronil</td>
<td>96</td>
<td>Saka and Tada (2021)</td>
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<td>Feding, growth and weight gain rates, biochemical responses, avoidance</td>
<td>Gold nanorods</td>
<td>72/12</td>
<td>Costa et al. (2021b)</td>
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<td>Changes in growth, behavioural endpoints, neurotransmitters, antidepressant system and thyroid development</td>
<td>Metamilip</td>
<td>96</td>
<td>Liu et al. (2021)</td>
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</tbody>
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* Gosner stage – GS (Gosner, 1960); Harrison stage – HS (Harrison, 1969); Nieuwkoop and Faber stage – NF (Nieuwkoop and Faber, 1994).
surrogates for amphibians in their aquatic phases. In that study, the authors compared data on lethality for the southern leopard frog and banded toad tadpoles relating to the contaminants: copper, carbaryl, pentachlorophenol (PCP), permethrin, and 4-nonylphenol. Similarly, at least in the short term, fish were found to be good surrogates for tadpoles when testing pesticides by Aldrich (2009). A study by Fryday and Thompson (2012) provided EFSA with information on the risk assessment of pesticides concerning amphibians. The first part of that study presented recommendations to use fish as surrogates for Anura and Caudata amphibians in toxicity tests. More recently, according to Welte et al. (2013), the extrapolation of fish sensitivity to amphibians should be done with caution because some contaminants, such as the corticosteroid hormone dexamethasone, are not detected in fish. Therefore, using fish as surrogates for amphibians for these types of chemicals may not be appropriate. This is due to specific interference with the biochemical pathways involved in the metamorphosis of amphibians. Also, some compounds like pyrethroids have been shown to affect fish and amphibians differently (Ortiz-Santalestra et al., 2018; Glaberman et al., 2019). The herbicide atrazine also caused different effects on the non-sensory system of fish is generally made up of: (i) inner ears, mechanical, and noreceptor and electroreceptor cells, forming the lateral acoustic system (touch, balance and hearing), (ii) the olfactory system (smell), (iii) the taste buds (taste) and (iv), the optical or visual system (vision) (Moor, 2001).

Even if, from the perspective of physiological functionality, tadpoles and fish could suffer from contamination in a very similar way, from the perspective of detection and avoidance of contamination, the different sensory mechanisms used by these groups might produce very different responses (Coombs and van Netten, 2005; Saccomanno et al., 2020). The sensory system of fish is generally made up of: (i) inner ears, mechanoreceptor and electroreceptor cells, forming the lateral acoustic system (touch, balance and hearing), (ii) the olfactory system (smell), (iii) the taste buds (taste) and (iv), the optical or visual system (vision) (Moorman, 2001). However, for the detection of contamination, the most important ones to be considered are smell and taste (Tierney, 2016). In their aquatic stages, amphibians depend heavily on their sensory capacity for the chemical detection of the environment (Troyer and Turner, 2015), smell is the most recurrent mechanism (mainly in view of the life stage at which amphibians are considered) (Duellman and Trueb, 1985). Due to the differences in the mechanisms used to perceive the environment, fish should not be considered a safe surrogate to predict the effects of contaminants on amphibians, especially if the avoidance response is to be assessed.

4. Defining avoidance: contamination-driven displacement of organisms

Avoidance response is considered here as the ability of organisms to detect contamination and flee towards a more favourable area. Therefore, an important step to measure this response (and which has contributed to increase its ecological relevance) is the use of a non-forced exposure scenarios, which is the main difference regarding traditional ecotoxicity assays. In these exposure scenarios organisms are able to move among the compartments with different levels of contamination, instead of being exposed exclusively to one concentration. The non-forced exposure approach has existed since the 1940s; when a bi-compartmental system was proposed by Jones (1947). Based on the same goal, many different systems have been proposed to test the ability of organisms to avoid contamination (see review by Lutterschmidt and McCoy, 2010).

Even if, from the perspective of physiological functionality, tadpoles and fish could suffer from contamination in a very similar way, from the perspective of detection and avoidance of contamination, the different sensory mechanisms used by these groups might produce very different responses (Coombs and van Netten, 2005; Saccomanno et al., 2020). The sensory system of fish is generally made up of: (i) inner ears, mechanoreceptor and electroreceptor cells, forming the lateral acoustic system (touch, balance and hearing), (ii) the olfactory system (smell), (iii) the taste buds (taste) and (iv), the optical or visual system (vision) (Moorman, 2001). However, for the detection of contamination, the most important ones to be considered are smell and taste (Tierney, 2016). In their aquatic stages, amphibians depend heavily on their sensory capacity for the chemical detection of the environment (Troyer and Turner, 2015), smell is the most recurrent mechanism (mainly in view of the life stage at which amphibians are considered) (Duellman and Trueb, 1985). Due to the differences in the mechanisms used to perceive the environment, fish should not be considered a safe surrogate to predict the effects of contaminants on amphibians, especially if the avoidance response is to be assessed.

4.1. Avoidance vs lethality in tadpoles

In ecotoxicological studies where mortality and avoidance were simultaneously assessed, it was noticeable that the avoidance response was shown to be more sensitive than mortality.

In the study by Araújo et al. (2014), the avoidance and lethality responses of the tadpoles of three anuran species (Leptodactylus latrans, Lithobates catesbeianus and Pelophylax perezi) exposed to copper were compared. The results showed that avoidance was verified at concentrations lower than those causing mortality: 50% of avoidance occurred at concentrations lower than those causing mortality: 50% of avoidance occurred at 100 μg.L⁻¹, while 20% mortality was only observed at 200 μg.L⁻¹. This result indicates that extinctions of local populations may occur in certain habitats, where such an event is not expected, owing to mortality.

In several studies, avoidance was shown to occur faster and at lower concentrations than mortality, as demonstrated by Vasconcelos et al. (2016) with L. catesbeianus GS 21 tadpoles exposed to the pesticide abamectin and by Moreira et al. (2019) with tadpoles of the same anuran species exposed to the herbicide diuron. Vasconcelos et al. (2016) reported a median lethal concentration (96-h LC₅₀) of 55 ± 4 μg.L⁻¹ and the median avoidance concentration (12-h AC₅₀) of 36 μg.L⁻¹ while Moreira et al. (2019) described a 96-h LC₅₀ of 31 ± 3.7 mg.L⁻¹ and about 90% of individuals fleeing concentrations of 2.5–5 mg.L⁻¹, during the same exposure period (96 h). An even greater sensitivity of the avoidance response was found in L. fuscus, L. catesbeianaus and Physalaemus nattereri tadpoles exposed to the herbicide 2,4-dichlorophenoxy-acetic acid (Freitas et al., 2019). In view of the results of these lethality tests, the most sensitive species was L. fuscus, whose 96-h LC₅₀ value was 28.01 ± 4.18 mg.L⁻¹. In the avoidance tests, tadpoles showed a similar behavior regardless of the species, with an avoidance of 50% of the population at concentrations of 242.5 μg.L⁻¹ (in a 21-d exposure); two orders of magnitude more sensitive than lethality. More recently, Girotto et al. (2020) exposed the amphibian species L. catesbeianaus to mining tailings, using lethality and avoidance tests. Although no mortality was recorded in the lethality tests, for a 96-h exposure (even at 100% of a stock solution containing 50 g.L⁻¹ of mining tailings), avoidance occurred at the lowest concentrations (10, 25 and 50%);
decreasing with the increasing concentrations) for 16-d and 20-d exposures times.

Although the number of studies with tadpoles in which mortality (under forced exposure) and avoidance (non-forced exposure) have been simultaneously assessed is relatively small, all the evidence points to the higher sensitivity of avoidance when compared to mortality, also for other organisms (reviewed by Moreira-Santos et al., 2019).

5. Conclusions

(1) If test organisms are mobile and able to detect contamination, then avoidance tests, in a non-forced exposure approach, involve a much lower sensation of anxiety and pain than mortality tests, and therefore represent an alternative in accordance with the evolution of animal experimentation.

(2) The use of avoidance as an ecotoxicity endpoint is also important from an ecological perspective. Even at concentrations that do not cause the death of organisms, if they flee to adjacent habitats, the loss of amphibian diversity can be critical for the location avoided. This dynamic of contamination-driven migration allows us not only to study the environmental conditions that trigger avoidance, but also to include new concepts related to habitat colonisation. Thus, the role of contamination may now be seen from a broader spatial perspective, in which the connectivity among habitats should be considered.

(3) The avoidance/recolonisation approach makes it possible to integrate landscape ecology with ecotoxicology and study habitats not simply as isolated environmental compartments, but, instead, as part of a whole. This approach draws attention to a new perspective to the risk of contaminants related to the disturbance of spatial dynamics, which goes beyond merely considering classical toxic effects, at the physiological level.

(4) Another important point to be highlighted about avoidance testing is its feasibility and rapidity of results. Avoidance experiments usually take very short times of exposure, from 3 to 24 h, although more extended time periods may also be used.

(5) The present study proposes an alternative methodology for toxicity tests with tadpoles, making animal experimentation with amphibians more humane. Taking into account an evident need to perform toxicity tests on amphibians, for the purpose of pesticide toxicity assessment, the avoidance methodology proposed here potentially contributes to performing them in an ethically more effective way, reducing suffering and distress, as required by current legislation.

Author statement

All authors conceived the ideas, contributed in data collection and analysis of data, and participated in the development, drafting and editing of the manuscript.

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All authors conceived the ideas (conceptualization), contributed in data collection and analysis of data, and participated in the development, drafting and editing of the manuscript.

Declaration of competing interest

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

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on identifying and testing-out for use.


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