Universidade de Aveiro
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FILIPA VITAL MORAIS

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IN VIVO AND *IN VITRO* ECOTOXICITY ASSESSMENT OF PHARMACEUTICALS AND NANOPLASTICS FOR EARLY LIFE STAGES OF AMPHIBIANS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Eco-toxicologia e Análise de Risco, realizada sob a orientação científica da Doutora Isabel Maria Cunha Antunes Lopes, Investigadora Principal do CESAM (Centro de Estudos do Ambiente e Mar) e do Departamento de Biologia da Universidade de Aveiro e Doutor Marcelino Miguel Guedes de Jesus Oliveira, Investigador Auxiliar do CESAM e do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Ecotoxicidade, *Xenopus laevis*, *Pelophylax perezi,* linhas celulares de anfíbios, nanoplásticos de poliestireno.

resumo

A expansão tecnológica permitiu o desenvolvimento de substâncias ativas que promoveram uma melhoria de qualidade da vida humana e um aumento populacional. No entanto, o aumento do consumo de substâncias farmacêuticas, em termos de quantidade e variedade, promoveu a sua presença ambiental, nomeadamente nos sistemas aquáticos. Considerando a natureza bioativa destas substâncias, podem esperar-se efeitos indesejáveis na biota presente nos ecossistemas que recebem estas substâncias químicas. Entre esses organismos encontram-se os anfíbios, organismos que demonstram uma elevada sensibilidade às mudanças ambientais. O presente estudo visou avaliar os efeitos individuais de dois fármacos (propranolol e haloperidol), isoladamente e em exposições combinadas com nanoplásticos (partículas de poliestireno de 50 nm), para diferentes fases da vida aquática (embriões e girinos) de *Xenopus laevis* e *Pelophylax perezi*. Os parâmetros avaliados incluíram mortalidade, malformações e efeitos no crescimento. Adicionalmente, foram avaliados os efeitos *in vitro* dos xenobióticos selecionados, em duas linhas celulares (A6, uma linha epitelial derivada do rim de um macho adulto; e XTC-2, uma linha fibroblástica derivada de um girino), avaliando a viabilidade celular, de forma a validar a sua relevância como uma alternativa não animal para avaliar os riscos potenciais para os anfíbios. Assim, embriões e girinos de *X. laevis* e *P. perezi* e as duas linhas celulares (A6 e XTC-2) foram expostos aos fármacos e nanoplásticos, sozinhos ou em misturas binárias. Globalmente, os resultados mostraram que girinos de *X. laevis* são mais sensíveis aos fármacos testados do que os respetivos embriões. A comparação das duas espécies revela que os girinos de *X. laevis* tendem a ser ligeiramente mais sensíveis do que *P. perezi*. A exposição individual a nanoplásticos de 50 nm de poliestireno não causou efeitos nos parâmetros avaliados (letais ou não letais), embora estes tenham sido ingeridos pelos girinos. A comparação das duas abordagens selecionadas, *in vivo* versus *in vitro*, revelou que os ensaios *in vitro* foram menos sensíveis aos fármacos. *In vitro*, a exposição apenas a nanoplásticos de poliestireno não causou efeitos significativos sobre a viabilidade celular das linhas celulares testadas. Contudo, nas misturas, observou-se um efeito significativo da sua presença, com as linhas celulares A6 a revelarem-se mais sensíveis do que a linha celular XTC-2. *In vivo*, a mistura causou um aumento da mortalidade induzida pelos fármacos particularmente para a menor concentração de nanoplásticos, demonstrando a relevância de testar concentrações de xenobióticos relevantes do ponto de vista ambiental e a necessidade de caracterizar completamente os nanoplásticos nos meios de ensaio (e.g., concentração, tamanho, capacidade de formar agregados).

keywords

Ecotoxicity, *Xenopus laevis*, *Pelophylax perezi,* amphibian cell line, polystyrene nanoplastics.

abstract

The technological expansion allowed the development of active substances that promoted an improvement of human life quality and a population increase. However, the increase in the consumption of pharmaceutical substances, in terms of quantity and variety, has promoted its environmental presence, namely in aquatic systems. Considering the bioactive nature of these substances, undesirable effects may be expected in the biota inhabiting ecosystems that receive these chemicals. Among this biota are the amphibians, which constitute a group of organisms that shows a high sensitivity to environmental changes. The present study aimed to assess the individual and combined effects of two pharmaceutical drugs (propranolol and haloperidol), alone and in combined exposures with nanoplastics (50 nm polystyrene particles), to aquatic life stages (embryos and tadpoles) of *Xenopus laevis* and *Pelophylax perezi*. Assessed endpoints included mortality, malformations and growth (as length and weight). Furthermore, effects of the selected xenobiotics on two amphibian cell lines (A6, an epithelial line derived from the kidney of an adult male; and XTC-2, a fibroblast-like line derived from a tadpole) were assessed using *in vitro* assays, by monitoring cell viability, to validate its relevance as a non-animal alternative to assess potential risks to amphibians. Thus, embryos and tadpoles of *X. laevis* and *P. perezi* and the two cell lines (A6 and XTC-2) were exposed to pharmaceuticals and nanoplastics, alone or in binary mixtures. Overall, the results showed that *X. laevis* tadpoles were more sensitive than the respective embryos to the tested pharmaceuticals. When comparing the two species, the tadpoles of *X. laevis* tended to be slightly more sensitive than those of *P. perezi*. Single exposure to 50 nm polystyrene nanoplastics caused no effects on the selected endpoints (lethal or non-lethal), despite their ingestion by the tadpoles. The comparison of the two selected approaches, *in vivo* versus *in vitro*, revealed that the latter were less sensitive to the pharmaceuticals. *In vitro*, individual exposure to polystyrene nanoplastics caused no significant effects on the cell viability of the tested cell lines. However, in mixtures, significant effect of its presence was observed for A6 cell lines, which revealed to be more sensitive than XTC-2 cell line. *In vivo*, for the pharmaceuticals drugs, the mixture caused a higher mortality at a lower concentration of nanoplastics, demonstrating the relevance of testing environmentally relevant concentrations of xenobiotics and the need to fully characterize nanoplastics in the test media (e.g., concentration, size, ability to form aggregates).

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Index of Acronyms

CTR – Control

CTR SOL – Solvent control

DMSO – Dimethyl sulfoxide

FBS – Fetal bovine serum

FETAX – Frog embryo teratogenesis

G – Gosner developmental stage

HAL – Haloperidol

IU – International Units

IUCN – International Union for Conservation of Nature

L15 – Leibovitz 15

MTT – 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide

NF – Nieuwkoop and Faber

NP – Nanoplastics

OECD – Organisation for Economic Co-operation and Development

PBS – Phosphate-buffered saline

PE – Polyethylene

PMMA – Polymethylmethacrylate

PP – Polypropylene

PROP – Propranolol

PS – Polystyrene

SVL – Snout-to-vent length

TL – Tail length

TBL – Total body length

UK – United Kingdom

USA – United States of America

UV – Ultraviolet

WWF – World Wildlife Fund

WWTP – Wastewater Treatment Plant

Introduction

1.1 Pharmaceuticals in the aquatic environment

Pharmaceuticals have become intrinsic to society, helping to improve life quality and expectancy. Pharmaceuticals are thus being increasingly used throughout the years, for medical and veterinary purposes, making them ubiquitous environmental contaminants. In a recent analysis made by the IQVIA institute it is estimated that the global pharmaceutical market will exceed $1.5 trillion by 2023 (IQVIA, 2022). Consumption of various pharmaceuticals categories have increased in OECD countries between 2000 and 2017: anti-diabetic and anti-depressant drugs usage doubled; anti-hypertensive drugs consumption grew by 70%; and cholesterol-lowering agents by a factor of 3 (OECD, 2019).

These substances can enter the environment by point sources (sources of easy identification; U.S. Environmental Protection Agency, 2020), such as hospital and industrial waste and nonpoint sources (harder to identify and resolve), such as agriculture, urban runoffs and leakages from waste treatment systems (Li, 2014). Overall, various sources of pharmaceuticals contamination for the aquatic compartment have been identified (Figure 1). Wastewater is considered an important source of pharmaceuticals release to the environment as wastewater treatment plants are unable to efficiently remove pharmaceuticals and their metabolites (Branchet et al., 2021), resulting from body excretion (Courtier et al., 2019; Sui et al., 2015). The improper disposal of untreated wastewater (Courtier et al., 2019; Sammut Bartolo et al., 2021), industrial pharmaceutical wastewater and livestock activities are other important sources of contamination (Courtier et al., 2019). Overall, several factors contribute to the levels of pharmaceuticals found in the aquatic environment but the rising consumption levels of pharmaceuticals and low efficiency removal of pharmaceuticals on the wastewater treatment plants are the main factors.

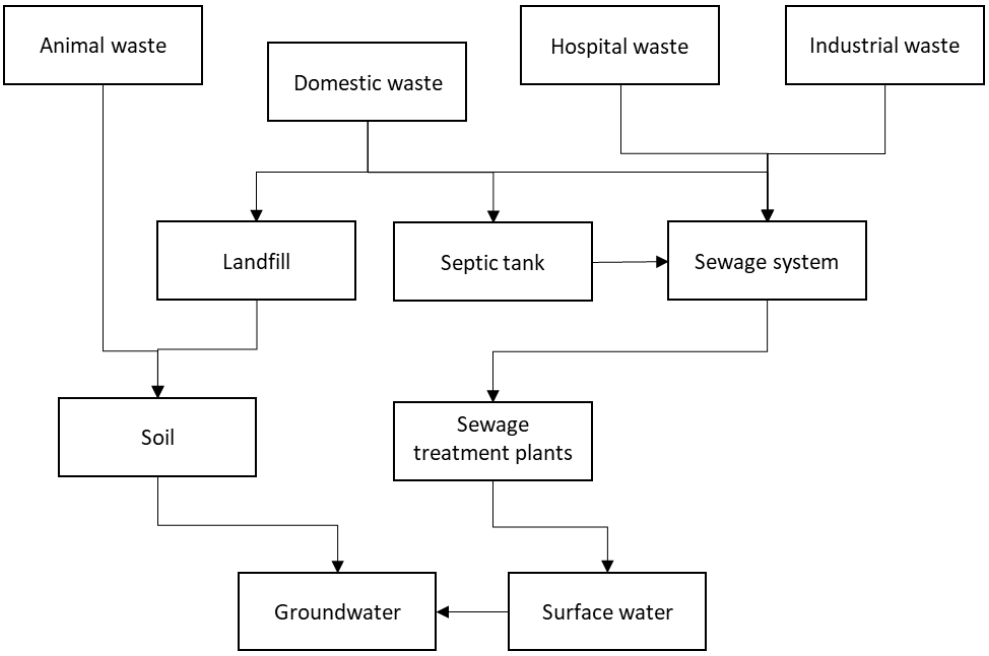


Figure 1. Sources of pharmaceutical contamination (Adapted: Li, 2014).

Although higher concentrations of pharmaceuticals have been reported in surface and wastewaters, pharmaceuticals have also been found in groundwater in a range between ng L^{-1} and µg L^{-1} (Sui et al., 2015). It is thus clear that, nowadays, pharmaceuticals can be found in all aquatic compartments. This environmental presence raises concern as pharmaceuticals are designed to be bioactive at low concentrations (Chavoshani et al., 2020) and may induce adverse effects on non-target organisms. Effects such as feeding impairment, growth inhibition, altered reproduction and locomotion have already been reported for several aquatic organisms, such as *Daphnia magna, Synechococcus leopolensis, Gambusia holbrooki* and *Brachydanio rerio* (Branchet et al., 2021; Fent et al., 2006; Mezzelani et al., 2018).

1.2 Propranolol

Propranolol, a β-adrenergic receptor antagonist agent, is used as a cardioprotective drug in treatment of cardiovascular diseases such as hypertension (Brunton et al., 2018), coronary artery disease (Peixoto et al., 2020) and congestive heart failure (Brunton et al., 2018). Additionally, it has also been used in the prophylaxis treatment of migraine (Brunton et al., 2018), treatment of essential tremor (Al-Majed et al., 2017), treatment of anxiety related to public performing (Dowd et al., 2007) and treatment of post-traumatic stress disorder (Dowd et al., 2007). It has recently been suggested that propranolol can be used in the treatment of cancer, preventing disease progression and metastases, as well as changing the tumour

microenvironment (Fumagalli et al., 2020). Its discovery in the early 1960s, by James Black and his team, awarded them with the 1988 Nobel Prize for Physiology or Medicine (Nobel Prize Outreach AB, 2021).

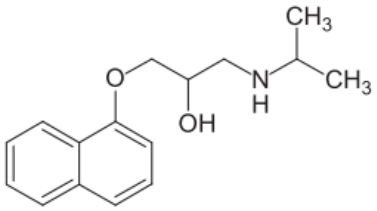


Figure 2. Chemical structure of propranolol (Source: Al-Majed et al., 2017).

Propranolol is highly lipophilic (Ågesen et al., 2019; Fumagalli et al., 2020) and is practically all absorbed in the gastrointestinal tract, being intensively metabolized in the liver (Brunton et al., 2018), by O-dealkylation, side chain oxidation, glucuronic acid conjugation and ring oxidation (Routledge & Shand, 1977). Around 25% of the non-metabolized drug reaches the systemic circulation (Brunton et al., 2018). The elimination of propranolol in urine in its non-metabolized form is small, only around 1 to 4% (Al-Majed et al., 2017; Routledge & Shand, 1977). 4-hydropropranolol, that also has beta adrenergic antagonist activity, is one of the metabolites of propranolol, and shows a higher elimination rate (Al-Majed et al., 2017; Brunton et al., 2018).

Beta-blockers can be separated into two groups: nonselective and selective. The former group shows a similar affinity for both β-1 (mainly present in the heart) and β-2 (expressed in various types of smooth muscle cells located in vessels) receptors (Fumagalli et al., 2020; Peixoto et al., 2020).

Propranolol inhibits β-1 and β-2 receptors but does not affect β-3 receptors (present in brown adipose tissue) unless in higher concentrations. This beta blocker competes with agonists such as catecholamines, epinephrine and norepinephrine, for beta receptor sites (Dowd et al., 2007), therefore inhibiting sympathetic effects. Propranolol can cross the blood-brain barrier (as well as placenta) allowing it to act on central nervous system (Steenen et al., 2016). The efficiency of propranolol treatments can be influenced by variables like gender, age, ethnicity (Ågesen et al., 2019).

β-receptors are present not only on human tissues but also in other animals, including in *Xenopus laevis*, an amphibian model species commonly used in the field of physiology, where these receptors are identified even in early life stages (Devic et al., 1997). This presence is an indication that beta blockers may affect a wide range of species once released in the environment.

The reported maximal removal efficiency of propranolol in a sewage treatment plant is 96% (Fent et al., 2006). This high efficiency and the low elimination of its unchanged form would allow the assumption that propranolol should not be found in the aquatic environment. However, propranolol is among the most commonly detected pharmaceuticals found in aquatic systems (Chavoshani et al., 2020). The levels of propranolol found in different waterbodies worldwide is presented in Table 1. In Portugal, specifically in the Douro River estuary, propranolol was found in 38% of the samples analysed by Madureira et al. (2010) with a maximum reported concentration of 3.18 ng L^{-1} (Madureira et al., 2010). In another report, samples were collected at polluted areas of the Douro River in which this blocker appeared in a range between the concentrations 22.0 and 54.0 ng L^{-1} (Madureira et al., 2010). Although propranolol was found in the groundwaters analysed in Spain at a maximum concentration of 9.38 ng L^{-1}, the metabolite 4-hydropropranolol was also present at a 100% frequency of the samples at a maximum concentration of 21.4 ug L^{-1} (López-Serna et al., 2013).

Table 1. Reported concentrations of propranolol in the aquatic environment.

Location	Maximum concentration	Reference
Belgium coast, North Sea	1 ng L^{-1}	Wille et al., 2010
Barcelona groundwater, Spain	9.38 ng L^{-1}	López-Serna et al., 2013
Tyne estuary, UK	107 ng L^{-1}	Roberts et al., 2006
Gulf of Cadiz, Spain	5.9 ng L^{-1}	Biel-Maeso et al., 2018
Yangtze estuary, China	142 ng L^{-1}	Yang et al., 2011
Douro River estuary, Portugal	3.18 ng L^{-1}	Madureira et al., 2010

The potential effects of propranolol have been studied in various aquatic organisms. In zebrafish larvae, a 96h LC_{50} of 2.48 mg L^{-1} was determined. Propranolol also caused a significant increase of mortality of the zebrafish embryos (at concentrations of 8 and 16 mg L^{-1}), decreased heart rate (starting in the lowest concentration tested, 1 mg L^{-1}) and hatching rate (at propranolol concentrations of 8 mg L^{-1} and above; Sun et al., 2014).

1.3 Haloperidol

Haloperidol is a butyrophenone-derivative antipsychotic drug, first synthesized in 1958 by Janssen Laboratories. It was initially approved by the U.S. Food and Drug Administration in

1976 (Lin et al., 2019), to prevent surgical shock (Adams et al., 2013), and has since then been applied in the treatment of schizophrenia (Kudo & Ishizaki, 1999) as well as in cases of Tourette disorder (Lin et al., 2019). It has also been used in the treatment of symptoms of autism (Posey et al., 2008). This drug is often used in hospital emergency settings in cases of nausea, vomiting and abdominal pain (Shahsavari et al., 2021) and has shown potential for the treatment of headaches in adults (Honkaniemi et al., 2006). However, it has also shown severe side effects, such as tremors, muscle stiffness and uncontrollable shaking, leading to the increase of the usage of second-generation antipsychotic drugs to replace this drug. Nonetheless, haloperidol is still one of the most commonly prescribed antipsychotic drugs (Adams et al., 2013).

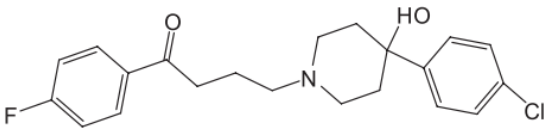


Figure 3. Chemical structure of haloperidol (Source: Silveira et al., 2013).

Haloperidol acts as an antagonist on the dopamine (D_2) receptor in the central nervous system (Fox et al., 1994). Haloperidol can be administered orally, intravenously as well as intramuscular. Haloperidol is highly lipophilic and is distributed through the body (Kudo & Ishizaki, 1999) specially in the brain (Dowd et al., 2007). It has a bioavailability between 60 and 75% for oral administration (Dowd et al., 2007). It is metabolized in the first pass metabolism in the liver into various metabolites, with the main metabolite being reduced haloperidol (Dowd et al., 2007). Excreted mainly in the form of metabolites (Dowd et al., 2007), it can also be excreted in the unmetabolized form, though at a low percentage (of around 1%; Kudo & Ishizaki, 1999). The average removal of haloperidol in municipal WWTP is 53% (UNESCO and HELCOM, 2017).

The presence of haloperidol in aquatic systems has not been extensively reported. The available data regarding aquatic environmental levels is shown in Table 2. In a sewage treatment plant (Gothenburg, Sweden), the drug was found at a concentration of 374 ng L^{-1} (Fick et al., 2010), although it has not been reported in the other studied treatment plants. The same study also reported the presence of haloperidol in the blood plasma of *Oncorhynchus mykiss* present in the sewage effluent, at a concentration of 1.2 ng mL^{-1}. In the Pacific Ocean, close to San Francisco (USA), it was detected at a maximum concentration of 56 ng L^{-1} (Nödler et al., 2014), although not detected in other 3 locations tested in the same report (San Francisco Bay, USA; Mediterranean Sea, Israel; Balearic Sea, Spain). In Baltic Sea countries (Denmark, Estonia,

Finland, Germany, Poland, Russia and Sweden), haloperidol was detected in all samples from municipal wastewater treatment plants influent as well as effluent (UNESCO and HELCOM, 2017), with the maximum concentration detected being below 0.1 µg L^{-1}. In the Bezdrevský stream (located in Czech Republic), haloperidol was detected in 3 out of 10 plasma samples of *Squalius cephalus* fish, presenting a mean concentration of 0.14 ng mL^{-1}, showing a moderate risk of inducing pharmacological effect as the mean concentration in the fish plasma is below the human therapeutic plasma concentrations (HTPC) but it exceeds 10% of HTPC (Cerveny et al., 2021).

Table 2. Reported concentrations of haloperidol in the aquatic environment.

Location	Maximum concentration	Reference
Pacific Ocean, USA	56 ng L^{-1}	Nödler et al., 2014
Zivny stream, Czech Republic	Not detected	Grabicova et al., 2017
Rio Grande, Brazil	0.1 µg L^{-1}	Silveira et al., 2013

The dopamine receptor in which haloperidol acts is evolutionary conserved in various species (Gunnarsson et al., 2008), including in *Xenopus laevis* (Martens et al., 1993), revealing the possibility that various species can be sensitive to the drug. Zebrafish larvae exposure to 3.4 mg L^{-1} of haloperidol was reported to alter swimming behaviour (lower swimming speed as well as erratic movements; Giacomini et al., 2006). The 72h LC_{50} of haloperidol for zebrafish embryo was determined to be 9.7 x 10^{-6} µg L^{-1} (Lin et al., 2019). The same study reported that haloperidol concentrations as low as 10^{-7} µg L^{-1} can cause the formation of edema on the pericardium in the embryonic zebrafish at 48 hours (Lin et al., 2019).

1.4 Nanoplastics in the aquatic environment

Plastic is used in everyday life scenarios, having contributed to the improvement of human life quality. This material shows various advantages such as being lightweight, versatile and low cost (Shim et al., 2018). In 2019, 368 million tonnes of plastic were produced worldwide with considerable percentage of plastics in Europe being directed for packaging of products (39.6%), followed by building and construction (20.4%), automotives (9.6%), electrical and electronic (6.2%), household, leisure and sports (4.1%) and agriculture (3.4%) too (PlasticsEurope, 2020). According to World Wildlife Fund (WWF), 95% of the waste in the

Mediterranean is plastic. In the Portuguese beaches, 72% of the marine litter is microplastics (Jornal de Negócios, 2018). Of the diverse polymer commercially available, polystyrene is among the most common in the aquatic environment (de Sá et al., 2018).

In the environment, plastic materials tend to degrade, by biotic and abiotic factors, originating smaller plastic particles. Small plastic particles may also be directly released from different products and industrial activities. Thus, based on their size of entrance in the environment, small plastic particles may be ranked as primary particles, when reaching the environment in small sizes as a result of products such as cosmetics, cleaning products (Pinto et al., 2016) and textiles such as clothes. When resulting from large plastic items that are degraded over time, they are considered secondary particles. This fragmentation can happen before entering the aquatic environment, as for example the frequent washing of clothes that can lead to separation of synthetic fibres (Pinto et al., 2016), and/or after entering the aquatic systems due to exposure to elements, such as UV, biological activities as well as mechanical processes (El Hadri et al., 2020). The size range of the particles can also allow their classification as microplastics, particles smaller than 5 mm and bigger than 100 nm for some authors or 1000 nm for others. This not consensual value is the upper size for the nanoplastics range (El Hadri et al., 2020).

The formation of secondary nanoplastics has been observed in various types of plastic such as polyethylene (PE) and polypropylene (PP) microplastic fragments under UV irradiation (El Hadri et al., 2020).

Nanoplastics due to their small size show a higher surface area in relation to the volume ratio which leads to being more reactive (Almeida et al., 2019), increasing the probability of the adsorption of various chemicals to its surface. This has been studied with various contaminants such as metals and organic pollutants (El Hadri et al., 2020).

Some studies have already addressed the potential effects of nanoplastics on *X. laevis*. Tusselino et al. (2015) reported toxic effects of 50 nm uncoated polystyrene nanoparticles, in the concentrations 4.5, 9.0 and 18.0 mg L^{-1} on the *X. laevis* development, leading to increased mortality in embryos, anomalous distribution of pigmentation; malformations of the head, gut, and tail; edema in the anterior ventral zone; and a shorter body length compared with the wild type (Tussellino et al., 2015). In another study, *X. laevis* tadpoles at developmental stage NF 36 exposed to polystyrene microplastics in a concentration range between 0.125 and 12.5 µg mL^{-1}, displayed no significant alterations in terms of development and in the swimming behaviour, although the microparticles were ingested by the tadpoles (De Felice et al., 2018). When early life stages of *X. laevis* were exposed to 40 nm polymethylmethacrylate (PMMA) nanoplastics, no

significant effects occurred on mortality and malformations in the embryos. However, for the highest concentration tested (1000 µg L^{-1}) a significant reduction of the body length was found (Venâncio et al., 2022). The same study reported that, in tadpoles no mortality was found but body weight and body length showed significant effects. Additionally, tadpoles exposed to 1000 µg L^{-1} showed an increase of externalization of the gut.

1.5 Environmental contamination and amphibians

According to the International Union for Conservation of Nature (IUCN), amphibians constitute the group of vertebrates with a higher percentage of species considered endangered, with 41% classified as danger of extinction (IUCN, 2021). Several factors have been identified as responsible for the worldwide decline of populations and species of amphibians, namely the degradation and fragmentation of their habitats, infectious diseases, introduced species, commercialization (as pets and for human food) and chemical contamination, among many others (Collins, 2010). The impacts of chemical contamination on aquatic life stages of amphibians have gained increasing concern in the last two decades, and many studies have been published since the 2000s addressing the ecotoxicity of chemicals on these life stages of amphibians. There are some characteristics of these organisms that may make them more sensitive to chemical contamination, comparatively to other aquatic species, namely with other aquatic vertebrates like fish. The fertilization and embryonic development of the majority of amphibians species (namely anurans) is external, exposing directly the gametes and all the aquatic life stages to the chemical contamination; the eggs of amphibians have no protective shells (though in most species the egg masses may be covered by a jelly-coat that may constitute a physical barrier); their skin is highly irrigated and permeable to gases and chemicals present in the environmental matrices, among other characteristics (Mitchell et al., 2005). In addition, many species undergo through metamorphosis, a process where amphibians are very vulnerable as drastic physiological (e.g. metabolism starts to produce urea instead of ammonia) and morphological (e.g. formation of lungs) changes occur in their body, and thus, may turn them more sensitive to chemical contamination during this life stage (Gilbert, 2000). These peculiarities make difficult the ecotoxicity assessment of chemicals to aquatic life stages of amphibians based on data generated for other aquatic taxa. It is then perceivable the relevance to generating ecotoxicity data specifically to this group of vertebrates aiming at their accurate protection. To date, the ecotoxicity of many chemicals has already been characterized for embryos and tadpoles of amphibian's species, however, these studies were focused on a narrow

number of chemicals (mainly pesticides and inorganic compounds; Bridges, 2010; Ortiz-Santaliestra et al., 2018; Weltje et al., 2013). Documented effects of chemical contamination on different life stages of amphibians range from impaired survival to sublethal responses like decreased growth rates, feeding inhibition, presence of developmental abnormalities, alterations in behaviour, among other responses (Costa et al., 2020; Egea-Serrano et al., 2012; Venâncio et al., 2022). Given the large diversity of chemicals being produced and released into the environment and as well their different modes of action, it is important to generate new ecotoxicity data for the chemicals that have not been evaluated to date.

Xenopus laevis is a standard anuran species commonly used to estimate the effects of chemical contamination in amphibians, and most of the available guidelines for ecotoxicity testing with amphibians (e.g. OECD, 2009, 2015) were designed specifically for this species, though they have been used with other anuran species. However, this species has been suggested to be less sensitive to some groups of chemicals than other amphibian species, thus being necessary to evaluate as well the ecotoxicity of chemicals to other amphibian species to establish safety assessment factors to be applied to the ecotoxicity data generated for *X. laevis*. In a review, Yu et al. (2013), compared the sensitivity of *X. laevis* with that of other amphibian species for a set of chemicals, reporting that *X. laevis* larvae were less sensitive to malathion than 4 of the 7 analysed anuran species; and for endosulfan, *X. laevis* larvae were less sensitive than 8 of the 11 analysed species. Taking in consideration these results, it is important to generate more ecotoxicity data for other species of amphibians, namely from different geographic regions (e.g., *X. laevis* originates from tropical and sub-tropical regions, species from temperate regions should also be studied) and belonging to different orders (Anura, Caudata and Gymnophiona).

Adding to the differential sensitivity to chemicals among species, different developmental stages must also be considered within the context of ecotoxicity to amphibians, as they may be exposed through diverse pathways, as different physiologies and morphologies present may translate in different sensitivities. For example, Santos et al. (2013) showed that *P. perezi* embryos and tadpoles had different responses to Cu and NaCl. Copper induced a higher mortality in tadpoles than in embryos whereas the sensitivity to NaCl was the opposite.

1.5.1 Amphibian model and standard species: *Xenopus laevis*

As mentioned above, *X. laevis*, the South African clawed frog, is considered a standard model species in amphibian ecotoxicology, being commonly used for laboratory toxicity experiments (Bernardini et al., 1999). The life cycle of the species is fully aquatic and, thus, are

exposed to waterborne contaminants through their life cycle. In the laboratory, reproduction can be induced by gonadotropic hormones injection in both male and female individuals. Afterwards the male latches for various hours on top of the female, forming amplexus; while the female releases the oocytes, the male releases the spermatozoa on top of them, promoting their fertilization (Bernardini et al., 1999). This species presents several characteristics that makes it a good model for research such as the entire aquatic life cycle, the easy laboratory maintenance (Bernardini et al., 1999), easy to reproduce under captivity conditions, releases a large number of embryos per clutch and the development stages of *X. laevis* are well defined by Nieuwkoop and Faber in the Normal Table of *Xenopus laevis* (Nieuwkoop & Faber, 1994). This data is essential in the usage of *X. laevis* as a proper organism model in scientific studies.

In the beginning of the twentieth century, *X. laevis* was frequently used in South Africa for educational purposes (Gurdon & Hopwood, 2000). However, this species is mainly known for its use in pregnancy tests. In 1930, Hogben reported that hypophysectomised female *Xenopus* suffered ovarian involution, though both implantation of glands and injection of anterior pituitary extracts induced ovulation. His finding was the principle for the creation of the *Xenopus* pregnancy test (Gurdon & Hopwood, 2000). The process involved a female individual being injected with urine concentrate from a human female suspected of being pregnant. The release of eggs by the female frog between eight to twelve hours following the injection indicates a positive result for the female human whose urine it belonged to (Weisman et al., 1942). This test was mainly used between 1940 and 1950s causing the species to be introduced in various laboratories in Europe as well as North America (Gurdon & Hopwood, 2000). Only in the 1960s, the use of the Hogben test started to decline, being surpassed by immunological methods. While the *Xenopus* pregnancy test was still mainstream, this species started to be used in various areas, such as in molecular biology (Cannatella & Sa, 1993), biomedical research (Tokarz et al., 2021) and developmental biology (Gurdon & Hopwood, 2000) causing it to become a staple in scientific studies.

1.5.2 Amphibian model species: *Pelophylax perezi*

This species, endemic to the Iberian Peninsula as well as the south of France, has been introduced in Azores and Madeira, in Baleares and Canary Islands as well as United Kingdom. (Rebelo et al., 2019). It can inhabit both permanently as well as temporarily aquatic sites such as lakes and ponds, as well as rivers and lagoons (Masó et al., 2011). This species also can appear in the terrestrial environment (in its adult developmental stage), although it is rare its appearance far from waterbodies (Rebelo et al., 2019).

The reproduction occurs in the aquatic environment and, depending on the geographic site, can happen between March and October (Rebelo et al., 2019) with the female laying up to the thousands of eggs. The tadpoles mainly feed on detritus, algae and bacteria that grow on underwater surfaces (Rebelo et al., 2019). As this species is very common in Portugal and has a high ecological plasticity, it is an interesting species in ecotoxicity studies as well as in studies addressing climate change scenarios.

1.6 Alternatives to animal experimentation in amphibian ecotoxicology

The use of animals in scientific research has raised a negative opinion in the general public, due to ethical concerns. Legislators began implementing directives to address this concern focusing on the protection of the animal's welfare as these can sense pain and suffering (e.g., directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes). The use of the "three Rs policy" became a staple of animal experimentation. The principles, replacement, reduction and refinement, were first proposed by W.M.S. Russell and R.L. Burch in 1959's in "The Principles of Humane Experimental Technique".

Methods for testing have been improved to follow the "Three R's" whilst still being able to determine the toxicity of various compounds. An alternative method to the testing relies on the usage of embryos and larval stages, of amphibians as well as fish, with no independent feeding as these stages are not included in the European Directive, as that is limited to "independently feeding larval forms and foetal forms of mammals as from the last third of their normal development". Although the use of early life stages is allowed by the European Directive, it still uses organisms in the studies.

Another increasingly used method in biomedical research involves the usage of cell lines, for example to assess the effects that chemicals may pose to biological entities. Currently, around 150 amphibian cell lines have been established, 40 of these had as source the African clawed frog (e.g. cell lines A6 and XTC-2; Vo, 2021).

The cell line model assays show three main advantages: (1) allows for the reduction of animals used in an assay (Langlois, 2021; Scott et al., 2020); (2) allows the identification of mode of action of various chemicals (Rehberger et al., 2018) and (3) allow for the testing of a bigger number of chemicals when compared to assays with tadpoles and embryos.

Despite the importance of the *in vitro* methodologies there are some concerns: (1) as these assays only evaluate the effects at a cellular level, when the data is extrapolated to the level of organisms (which are much more complex and with other emergent properties) can

include a high degree of uncertainty and (2) for compounds that have to be metabolized, the metabolic competence of a cell may be limited requiring a more complex system (e.g., organs), making these models not the most suitable. As a result, these *in vitro* assays are mainly used for initial toxicity screening and to verify that a chemical compound may be toxic for the specific species, in this case, amphibians, having to be followed by *in vivo* assays to assure true results.

In any case, the application of cell lines for screening has already greatly reduced the need of animals, as it prevents the need to use embryos or tadpoles for preliminary assessments of the toxicity of compounds. However, these methods must be carefully validated and assessed.

1.7 Aim of Study

The main objective of the present work was to assess the individual and combined effects of two pharmaceuticals (propranolol and haloperidol) and a nanoplastic (50 nm polystyrene) to aquatic life stages of *Xenopus laevis* and *Pelophylax perezi.* Furthermore, the suitability of *in vitro* assays as non-animal alternatives, of *in vivo* assays, to be used at early and screening phases of chemical risk for amphibians was evaluated. To attain these objectives, embryos and tadpoles of *X. laevis* and *P. perezi* and two cell lines of *X. laevis* were exposed to single and binary mixtures of each pharmaceutical and the nanoplastic.

Material and Methods

2.1 Chemicals

Propranolol hydrochloride (CAS RN 318-98-9; purity >99.0%), haloperidol (CAS RN 52-86-8; purity >98.0%) and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium Bromide (MTT) (CAS RN 298-93-1; purity > 98,0%) were purchased from Tokyo Chemical Industry (TCI; Belgium). All other chemicals were analytical grade and acquired from Sigma-Aldrich (Spain).

Preparation of pharmaceuticals solutions

For *in vivo* assays, propranolol stock solution (95 mg L^{-1}) was prepared in FETAX media and haloperidol stock solution (37.6 mg L^{-1}) was prepared with FETAX medium supplemented with 0.01% DMSO to improve solubilization. Test concentrations of each pharmaceutical were prepared by dilution of the stock in FETAX.

For *in vitro* assays, stock solutions of propranolol (250 g L^{-1}) and haloperidol (55 g L^{-1}) were prepared in DMSO. Stock was further diluted in complete cell culture media to a working stock of 1 g L^{-1} and 200 mg L^{-1} respectively. Test concentrations of each pharmaceutical were prepared by further dilution of working stock in complete cell culture media.

Nanoplastics synthesis and characterization

The plastic particles used in this thesis, polystyrene (PS-50) nanoplastics were synthesized by mini-emulsion polymerization using sodium dodecyl sulphate (SDS) as a stabilizer (Almeida et al., 2019). The nanoplastics were prepared and characterized by the research team. After the polymerization step, nanoplastics were subjected to dialysis in ultrapure water, for the duration of 7 days with daily water renewal.

The characterization of the nanoplastics were performed both in ultrapure water and in the different tested media, evaluating the hydrodynamic size using dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern). Morphological characterization was performed by transmission electron microscopy (Hitachi, H9000 NAR). To avoid aggregation/agglomeration events, nanoplastics were subjected to a 10 minute treatment in an ultrasonic bath (Sonorex Digitec DT100 H, Bandelin), prior to dilution in the different tested media.

The tested concentrations, 10 mg L^{-1} and 10 µg L^{-1}, were obtained by dilution of the stock suspension, 40 g L^{-1}, in the appropriate medium. The lowest concentration, 10 µg L^{-1}, was determined as an ecological relevant concentration meanwhile the highest concentration was based on previous studies made with fish cell lines (Almeida et al., 2019).

2.2 Biological models

Anuran species

The ecotoxicity of propranolol, haloperidol and of each of these two chemicals combined with 50 nm polysterene nanoplastics (PS-NP) was assessed in two anuran species and two cell lines: (i) *Xenopus laevis*, is an anuran species autochthonous from sub-tropical and tropical regions. It is considered a standard model species to assess the impacts of chemicals in amphibians. In fact, most available guidelines have been developed specifically for this species; (ii) *Pelophylax perezi*, is also an anuran species from temperate regions (restricted to the Iberian Peninsula and South France, though recently has been introduced in other places like the Azores islands and the UK). It is ubiquitous in several aquatic habitats holding no protection classification by IUCN.

To obtain aquatic life stages of *X. laevis* to perform the *in vivo* assays, adults of this species were maintained the Department of Biology, University of Aveiro, in glass aquaria filled with demineralized and dechlorinated water, to which artificial sea salt was added (OceanFish, Prodac, Italy) to reach a conductivity of 500 µS cm^{-1}, under controlled conditions of photoperiod (14:10h light:dark) and temperature (23 ± 1°C). The water in the aquaria was changed completely twice a week and partially (~80%) once. Each animal was fed three times a week with *Tenebrio molitor* larvae combined with pellets of vitamins and minerals appropriate for this species (XE40, Mucedola, Italy). Embryos were obtained after injecting a female and male with human chorionic gonadotropin, 500 IU and 100 IU respectively, in the dorsal lymph sac. The two animals were then placed in a tank, filled with the same water used in the culture aquaria, overnight. The next day, eggs were gently collected and screened (using a Zeiss Stemi 508 stereomicroscope) to discard the non-viable ones. A portion of viable embryos were immediately used in the embryo teratogenicity assays. The remaining embryos were allowed to grow, under the photoperiod and temperature conditions similar to those described for the adults, in plastic vessels containing FETAX medium, with constant aeration until reaching Nieuwkoop and Faber (NF) stages 45-46 (Nieuwkoop and Faber, 1994). During this period, the medium was changed every two days. As they reach NF 45-46, the tadpoles were used to run the tadpoles toxicity assay.

To obtain embryos of *P. perezi*, egg masses of this species were collected in a freshwater pond located at Gafanha de Áquem (Aveiro (40°35'48.8"N 8°41'43.4"W), Portugal). In the laboratory, embryos collected between Gosner stages (G) 8 and 10 (Gosner, 1960) were checked under a stereomicroscope (Zeiss Stemi 508), and non-viable eggs were discarded. As described for *X. laevis*, a part of the viable embryos was used to perform the embryos teratogenicity assay,

and the remaining was allowed to develop in FETAX medium, until reaching Gosner stage 25, under the same laboratory conditions as those mentioned above to rear *X. laevis* tadpoles.

Cell lines

The cell lines used in the thesis were A6 (ECACC 89072613) (Rafferty, 1969), an epithelial line derived from the kidney of an adult male *X. laevis,* and XTC-2 (Pudney et al., 1973), a fibroblast-like line derived from a Xenopus tadpole. Cells were cultured with diluted Leibovitz 15 (L-15) media supplemented with 2 mM L-glutamine (Biowest, France); A6 cells with 55% of L-15 and XTC-2 with 65% of L-15 to maintain an optimal osmolarity for amphibian cells. Media was supplemented with 10% Fetal Bovine Serum (FBS) (Biowest, France), 100 units mL^{-1} of Penicillin G, 100 µg mL^{-1} of Streptomycin and 25 µg mL^{-1} of Amphotericin B (Gibco, US). Cells were maintained in an incubator, at 25ºC under atmospheric air. Cells were routinely cultured in cellular flasks and TrypLE™ Express (ThermoFisher Scientifics) was used as dissociation agent. All assays were performed in flat-bottom clear 96-well plates (Orange Scientific).

2.3 Embryo teratogenicity assay

The toxicity of the studied chemicals in embryos of *X. laevis* and *P. perezi* was assessed by performing the Frog Embryo Teratogenesis Assay procedure (ASTM, 2012) with some minor adjustments. Embryos at the developmental stage NF 8-11 or G 8-10, respectively, were exposed to 7 conditions, a negative control (FETAX medium only) and 6 concentrations of each pharmaceutical. In this assay, *X. laevis* embryos were exposed to 7.63, 9.16, 11.0, 13.2, 15.8 and 19 mg L^{-1}. These concentrations were based on preliminary assays.

The control consisted of 5 replicates and each pharmaceutical tested concentration had 3 replicates performed. Tests were performed in plastic Petri dishes (one per replicate) with 20 embryos and 10 mL of test solution. Thus, 100 organisms were used per control and 60 per pharmaceutical test concentration. Organisms were exposed for 96 hours with test media renewal at 48 hours. Assays were performed under similar condition of laboratory culture: 23±1°C with a photoperiod of 14h:10h light:dark. Test media characteristics (e.g., pH and conductivity) were measured (WTW Multi 3410) at the beginning and in the end of each assay to ensure that the conditions did not change during the test. Mortality and hatching were checked daily, at the same time of day. Any dead organism was removed to avoid contamination of the media during the rest of the assay and the possible growth of microorganisms. Mortality, hatching, development stage, malformations and body length were the endpoints observed in the assay.

2.4 Tadpoles toxicity assays

Tadpoles of *X. laevis* (between NF stages 45-46) and *P. perezi* (between G stages 24-25) were randomly selected for this assay. Tadpoles were exposed to a negative (Fetax medium) and six different concentrations of the selected pharmaceuticals. For the test with haloperidol an extra control, a solvent control consisting of 0.01% of DMSO was performed. *X. laevis* and *P. perezi* were exposed to 0.40, 0.61, 0.91, 1.36, 2.05 and 3.07 mg L^{-1} of haloperidol. Propranolol concentrations tested with *P. perezi* were: 2.71, 3.26, 3.91, 4.69, 5.63, 6.75 mg L^{-1}, for *X. laevis* the following concentrations were tested: 0.99, 1.39, 1.94, 2.72, 3.80 and 5.32 mg L^{-1}. These concentrations are based on previous tests.

To evaluate the influence of PS-50 nm in the toxicity of the two pharmaceuticals, two concentrations of PS-50 nm were tested, individually and in mixture with *P. perezi* tadpoles – 10 mg L^{-1} and 10 µg L^{-1}. The mixtures consisted of the LC_{50} of one of the pharmaceuticals, haloperidol or propranolol, with a nanoplastic concentration, 10 mg $L^{-1,}$ and 10 µg L^{-1}. The mixture was sonicated for 10 minutes before the beginning of the assay.

Each replicate was performed with 150 mL of test media. Five replicates per condition were tested, each with 3 tadpoles per replica. Before the beginning of the assay, a sample of 15 tadpoles were weighed and photographed to obtain the initial lengths. The assay was carried in with constant aeration, at 23±1°C with a photoperiod of 14h:10h light: dark. The tadpoles were fed with 1.8 mg L^{-1} of TetraMin™ at the beginning of the assay and at the 48 hours exposure period. At the end of the assay, the parameters analysed included mortality, development stage, malformations, body length, and weight. Every 24 hours, the mortality was checked, and dead organism removed to avoid contamination. Conductivity (µS cm^{-1}) and pH were measured at the beginning and end of each assay using a WTW Multi 3410.

2.5 Cytotoxicity assays

Assays were performed in clear bottom 96 well plates. The number of cells per well was determined by a preliminary assay, assessing optimal cell growth rate and the time in which the absorbance/fluorescence reading is ideal. The estimated optimal cell density was 1×10^4 cells per well. Three independent experiments were performed, in quadruplicates for each concentration. Cell viability was assessed after 24, 48 and 72h and lethal concentrations (LC_{50}, LC_{20} and LC_{10}) were calculated based on thiazolyl blue tetrazolium bromide (MTT absorbance measured at 570 nm and 690 nm; Thermo Scientific Multiskan Spectrum) assay.

Cells were transferred to the test plates and allowed to adhere overnight. To test the effects of the selected pharmaceuticals single exposure in A6 and XTC-2 cell lines, a 250 g L^{-1} stock solution of propranolol and a 55 g L^{-1} stock solution of haloperidol were prepared in DMSO and working solutions were prepared in complete cell culture media. The concentrations tested were 0.025, 0.076, 0.229, 0.686, 2.06, 6.17, 18.5, 55.6, 167 and 500 mg L^{-1} for propranolol; and 0.195, 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25.0, 50.0 and 100 mg L^{-1} for haloperidol. For the assessment of the *in vitro* effects of nanoplastics, the stock suspension of polystyrene nanoplastics was diluted in cell culture media to obtain the desired concentrations: 0.005, 0.015, 0.046, 0.137, 0.412, 1.24, 3.70, 11.1, 33.3 and 100 mg L^{-1}. A control with cell culture media only and a solvent control with cell culture media and the maximum concentration of DMSO tested was included in the experiments. For the combined exposures, the cells were exposed to 10 mg L^{-1} or 10 µg L^{-1} of PS nanoplastics in the presence of the pharmaceutical (propranolol or haloperidol) in the same concentrations tested for the single exposure.

A MTT stock solution was prepared in ultra-pure water as to obtain a final concentration of 5 mg/mL. Following the removal of the media from the 96-well culture plates, cells were washed with phosphate-buffered saline (PBS) and exposed to 10% MTT solution (diluted in PBS) and incubated for 4h. Afterwards, the MTT solution was removed and 100% DMSO was added to lyse the cells and solubilize the purple coloured formazan crystals. These crystals are a result of MTT being converted to formazan by viable cells with an active metabolism. The absorbance was measured at 570 nm and 690 nm in a microplate reader (Thermo Scientific Multiskan Spectrum). Conversion of MTT into formazan is proportional to the number of viable cells present in each well and was calculated using the following equation:

$$\%\ \text{Cell viability} = \frac{\text{sample absorbance} - \text{cell free sample blank}}{\text{mean media control absorvance}} \times 100$$

2.6 Data analysis

To identify differences in the monitored parameters for *in vivo* assays, one-way analysis of variance was performed followed by the Dunnett's post-hoc test. For the *in vitro c*ytotoxicity curves, the statistical analysis was assessed using a nonlinear regression fitting curve with variable slopes (4P) and lethal concentrations were interpolated from the cytotoxicity curves. Statistical differences between single and combined treatments were verified with a paired t-test. Normality of data was checked through the Shapiro-Wilk's test and the homogeneity of

variance through the Bartlett's test. Significant differences were set at a p value of 0.05. These analyses were carried out in Graphpad Prism 6 software.

Results

3.1 Embryo teratogenicity assays with propranolol

The survival rate of *X. laevis* embryos are presented in Figure 4. All organisms exposed to the control were alive and less than 5% presented malformations after the 96h exposure period, thus, filling the validity criteria for this assay. On the contrary, all tested concentrations of propranolol significantly impaired the survival of embryos (which was always ≤65%; F= 42.6; p<0.0001). Concentrations equal or higher than 11 mg/L induced 100% of mortality. In the two lowest propranolol concentrations, larvae were mostly unreactive after a physical stimulus, with the heartbeat being the parameter considered to assess the survival of the organisms. The estimated LC_{20} and LC_{50} (and confidence limits at 95%) after the 96h exposure period for propranolol were 7.23 (6.73-7.56) and 8.10 (7.79-8.36) mg/L, respectively.

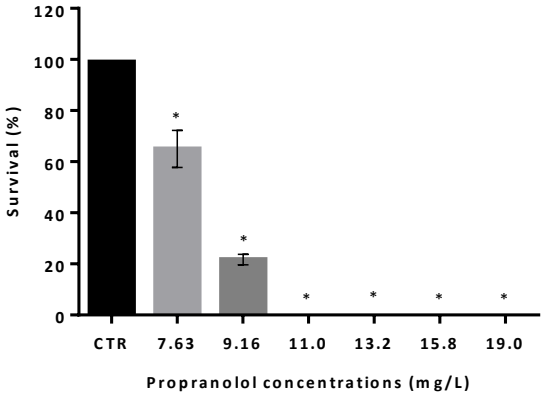


Figure 4. Average survival percentage of *Xenopus laevis* embryos after being exposed, for 96h, to a control and various propranolol concentrations. Error bars represent standard deviation. * indicates significant differences relatively to the control (p<0.0001).

Exposure to propranolol induced severe malformations in the embryos of *X. laevis* (Figure 5); 84.6% and 92.3% of the organisms exposed to 7.63 mg/L and 9.16 mg/L, respectively, showed malformations, the majority being edema, either in the heart or in proctodaeum (some examples can be seen in Figure 6).

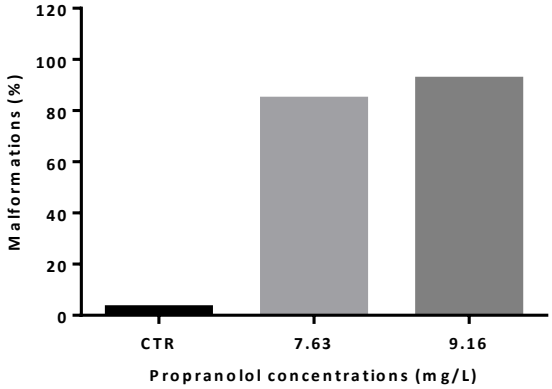


Figure 5. Proportion of larvae of *Xenopus laevis* with malformations after an exposure, for 96h, to propranolol.

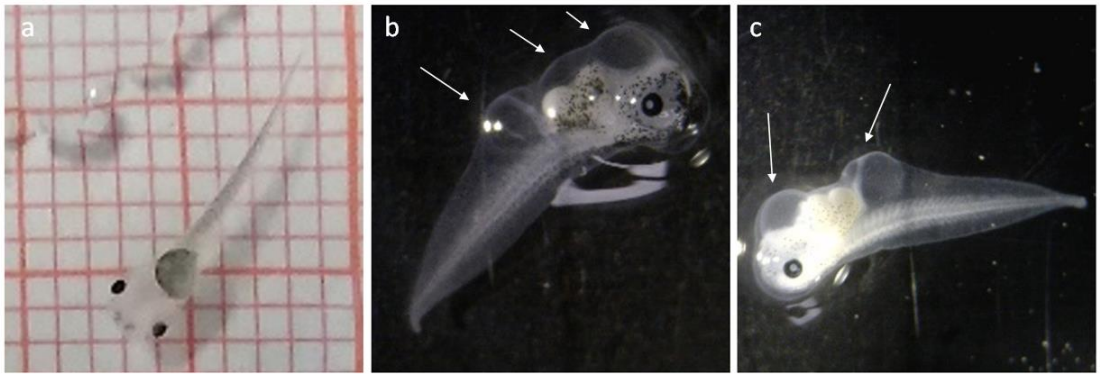


Figure 6. Photographs illustrating larvae of *Xenopus laevis* at the end of the embryo teratogenicity assay. (a) larvae exposed to the control without malformations; (b) larvae exposed to 7.63 mg/L of propranolol with severe malformations including a heart edema and edema in proctodaeu; (c) larvae exposed to 9.16 mg/L of propranolol with an heart edema, edema in proctodaeu and hypopigmentation. (Photos taken with DinoCapture 2.0)

The tested concentrations of propranolol also affected the developmental stages of the embryos. A significant delay in the developmental stages was observed for organisms exposed to 7.63 and 9.16 mg/L when compared to control organisms ($F=335.7$; $p<0.0001$; Figure 7). After the 96h exposure, more than 90% of the embryos in the control were in NF 46, while at 7.63 mg/L of propranolol most larvae (46%) were either in the stage NF 43 and NF 44 and at 9.16 mg/L, 77% of larvae were at NF 43.

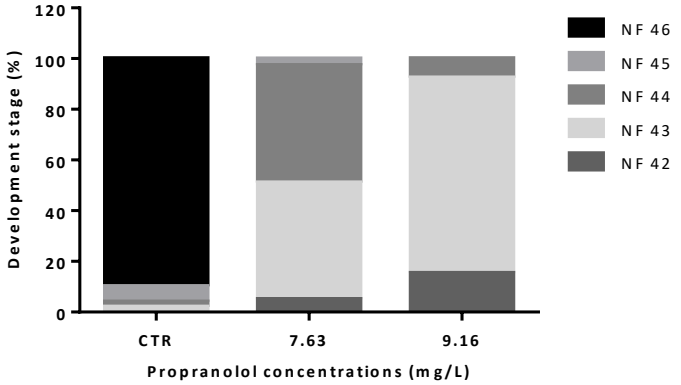


Figure 7. Percentage of *Xenopus laevis* larvae at developmental stages NF 42, NF 43, NF 44, NF 45 and NF 46, after 96h of exposure to propranolol.

Propranolol exposure also caused a significant decrease in total body length (TBL), snout-to-vent length (SVL) and tail length (TL) of the organisms exposed to 7.63 and 9.16 mg/L ($F \leq 174.0$, $p < 0.0001$; Figure 8).

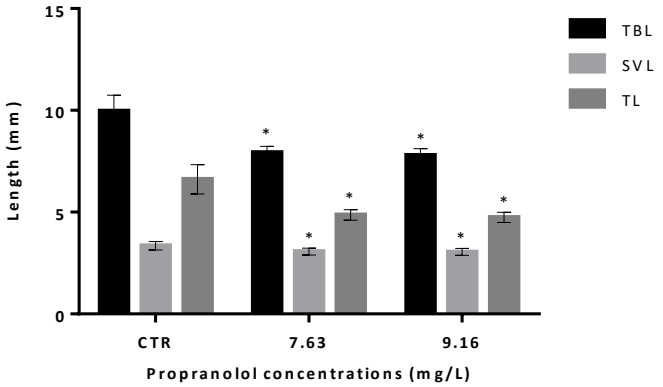


Figure 8. Average length of total body (TBL), snout-to-vent (SVL) and tail (TL) of *Xenopus laevis* larvae, after being exposed for 96h to propranolol. Error bars represent standard deviation. * indicates significant differences relatively to the respective control ($p < 0.0001$).

3.2 Tadpoles toxicity assay with propranolol

Figure 9 shows the survival rate of *X. laevis* and *P. perezi* after being exposed, for 96h, to six concentrations of propranolol. Mortality and malformations observed in the control were

lower than 10% after the 96h exposure period, thus, filling the validity criteria for this assay. With the *X. laevis* tadpoles, the survival of the tadpoles in the highest two concentrations tested, 3.80 and 5.32 mg/L, was significantly affected by the exposure to propranolol, with a mortality rate of 53.3% and 100%, respectively ($F=16.20$, $p<0.0001$). The LC_{20} and LC_{50} (and 95% CL) determined for this species were: 2.51 (2.02-2.85) and 3.27 (2.87-3.77) mg/L respectively. Exposure to propranolol impaired the movement of tadpoles, with effects increasing with the concentration increase. The survival of *P. perezi* tadpoles was significantly affected by concentrations of propranolol higher than 3.26 mg/L ($F=60.61$, $p<0.0001$), with 3.91 mg/L eliciting a mortality rate of 66.7% and the remaining concentrations 100% mortality. The LC_{20} and LC_{50} (and 95% CL) determined for propranolol were: 3.12 (2.78-3.34) and 3.58 (3.34-3.85) mg/L, respectively. Furthermore, all tadpoles exhibited difficulties in moving after a physical stimuli, similar to the effect observed with the *X. laevis* tadpoles.

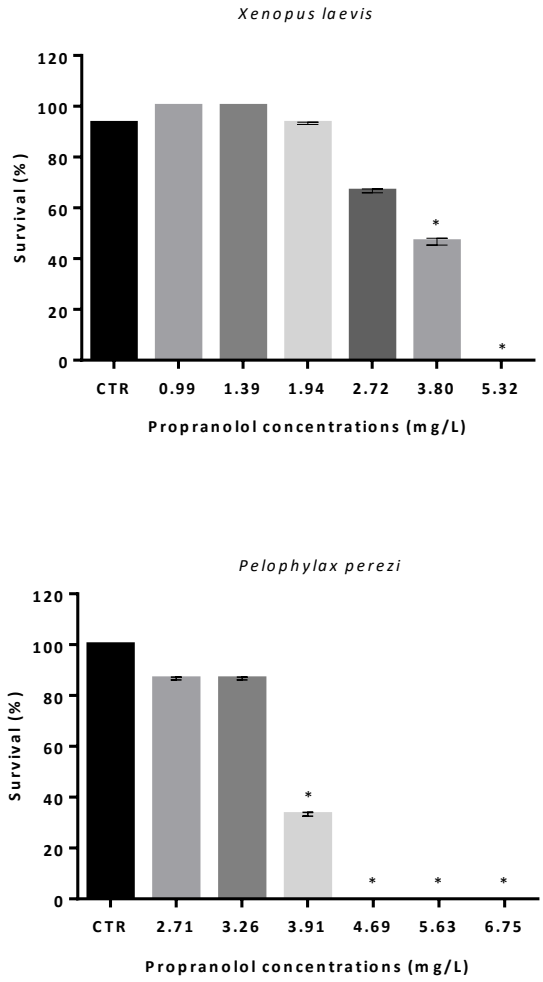


Figure 9. Survival of *Xenopus laevis* (up) and *Pelophylax perezi* (down) tadpoles after being exposed for 96h to various propranolol concentrations. Error bars represent standard deviation. * indicates significant differences relatively to the control ($p<0.0001$).

Propranolol induced the occurrence of malformations in tadpoles of *P. perezi*, with more than 80% of the tadpoles exposed to 3.91 mg/L exhibiting at least one type of malformation. The malformation that appeared most frequently were haemorrhage, with 23.1% of tadpoles in the 2.71 mg/L showing this type of malformation, 46.2% in 3.26 mg/L and 80% in 3.91 mg/L (Figure 10 and 11). In the case of *X. laevis*, the occurrence of malformations was low, with the highest percentage being 13.3% in both 0.99 and 1.39 mg/L of propranolol. All malformations detected were haemorrhage close to the heart.

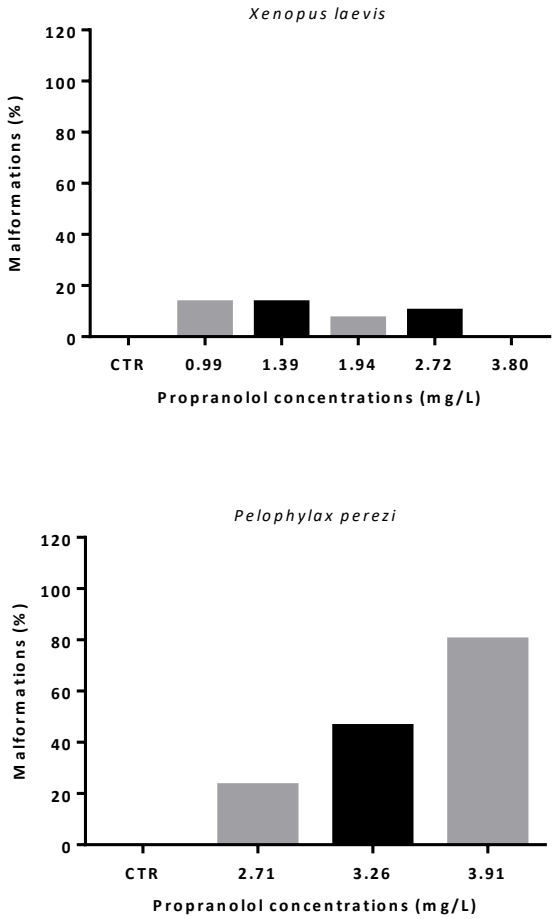


Figure 10. Proportion of *Xenopus laevis* (up) and *Pelophylax perezi* (down) tadpoles with malformations after exposure, for 96h, to various propranolol concentrations.

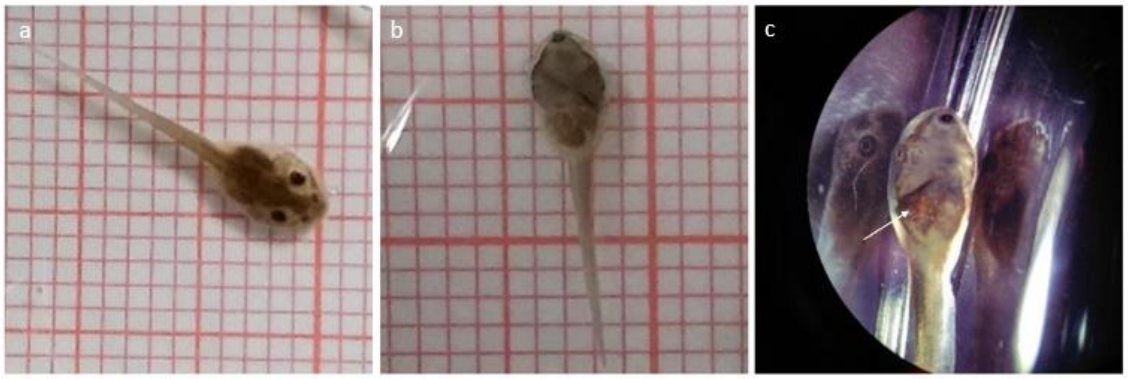


Figure 11. Photographs illustrating tadpoles of *Pelophylax perezi* at the end of the tadpole toxicity assay. (a) control tadpole without malformations; (b) tadpole exposed to 2.71 mg/L of

propranolol without malformations; (c) tadpole exposed to 3.26 mg/L of propranolol with a hemorrhage, the most commom malformation in this assay.

Significant differences were detected in the developmental stages of *X. laevis* tadpoles exposed to the following concentrations of propranolol: 1.39, 2.72 and 3.80 mg/L ($F = 10.76$, $p<0.0001$; Figure 12). No significant differences were observed in the developmental stages of *P. perezi* tadpoles exposed to propranolol when compared to the control ($F = 1.45$, $p>0.05$; Figure 12): 86% and 100% of the tadpoles were in G 25 stage in the control and propranolol concentrations, respectively.

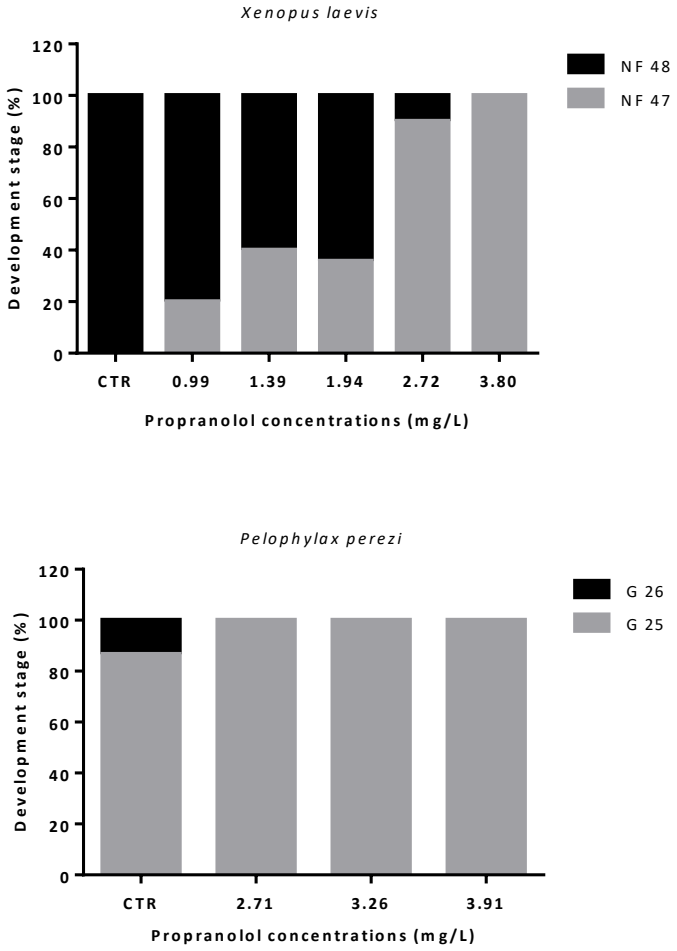


Figure 12. Percentage of *Xenopus laevis* (up) and *Pelophylax perezi* (down) tadpoles at developmental stages NF 47 and NF 48 and G 25 and G 26 respectively, after 96h of exposure to a range of concentrations of propranolol.

Exposure of *X. laevis* to propranolol, caused significant decrease in the weight of the tadpoles, when compared to the control group ($F=27.21$; $p<0.0001$; Figure 13), in all tested concentrations of propranolol. Likewise, all lengths analysed were significantly different from the control ($F\leq22.59$; $p<0.0001$; Figure 14). For *P. perezi* tadpoles, the results indicate that exposure to propranolol did not affect body weight of the tadpoles (Figure 13). On the contrary, lengths (total body, snout-to-vent and tail) were significantly reduced in tadpoles exposed to 2.71, 3.26 and 3.91 mg/L comparatively to the control group ($F\leq10.4$; $p<0.0001$; Figure 14).

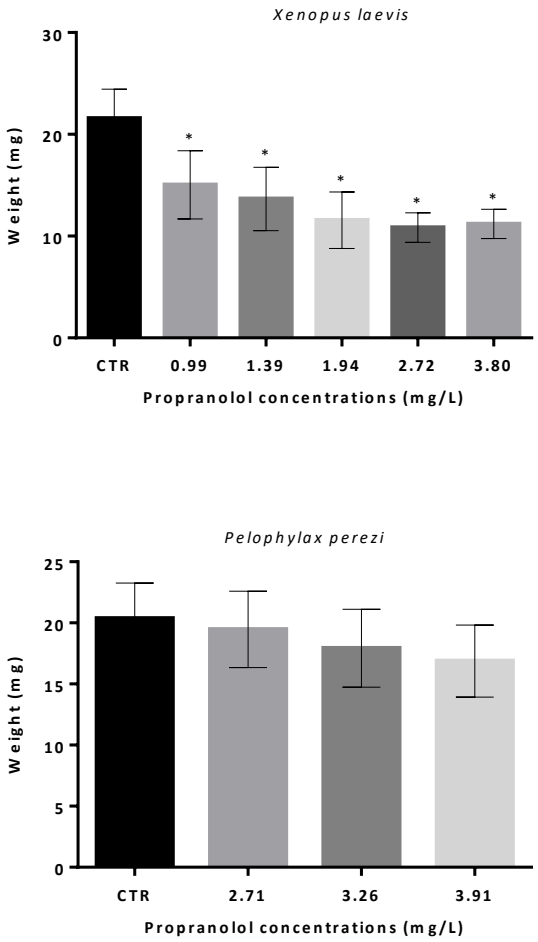


Figure 13. Average weight of the tadpoles of *Xenopus laevis* (up) and of *Pelophylax perezi* (bottom), after being exposed for 96h to a range of propranolol concentrations. Error bars represent standard deviation. * indicates significant differences relatively to the control ($p<0.0001$).

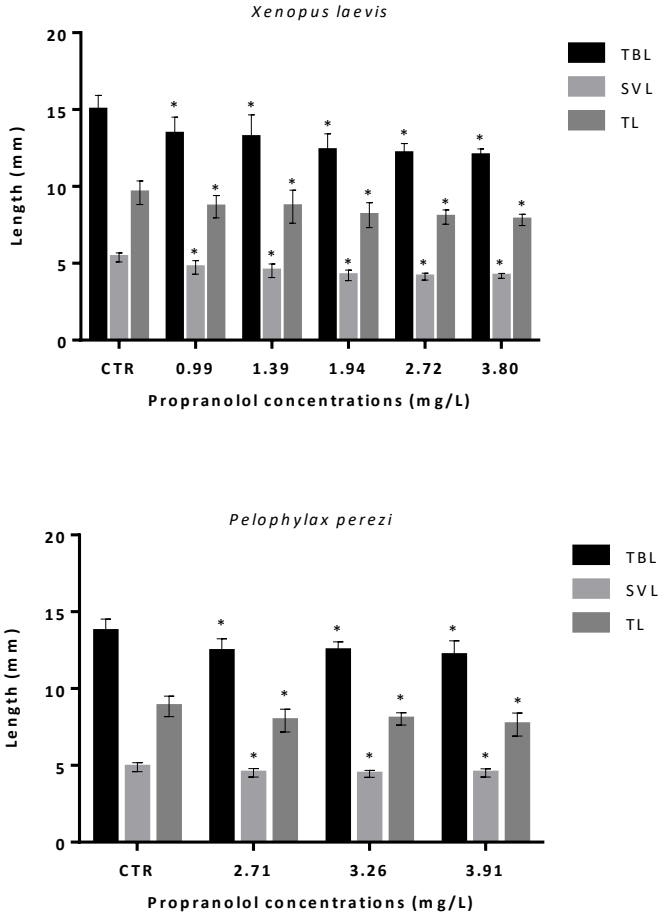


Figure 14. Average length of total body (TBL), snout-to-vent (SVL) and tail (TL) of tadpoles of *Xenopus laevis* (up) and of *Pelophylax perezi* (bottom), after being exposed for 96h to a range of propranolol concentrations. Error bars represent standard deviation. * indicates significant differences relatively to the control ($p<0.0001$).

3.3 *In vitro* cytotoxicity assays with propranolol

Cytotoxicity curves of XTC-2 and A6 cells when exposed to a range of concentrations of propranolol for each time point are represented in Figure 15. In the XTC-2 cell line assay, the cytotoxicity curve at 24h was statistically different from both, 48h and 72h time points ($p<0.05$). Meanwhile there was no statistical difference between the effects at 48h and 72h ($p=0.660$). For the A6 cell line, no statistical differences between 24h with 48h and 72h effects were observed ($p\geq0.0759$). However, 48h and 72h time points were significantly different ($p<0.05$). The estimated lethal concentrations of propranolol to both cell lines tested are presented in Table 3.

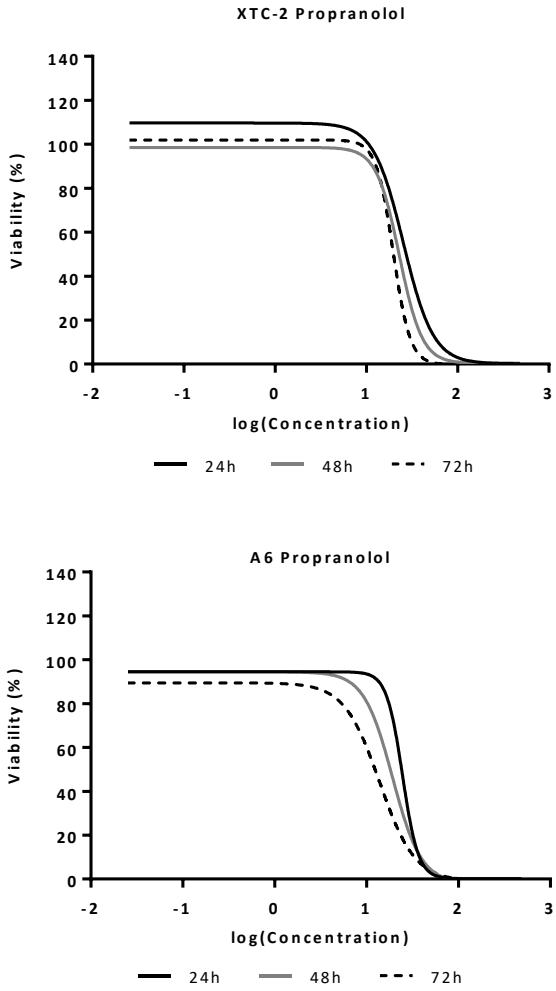


Figure 15. Cytotoxicity curves of XTC-2 (up) and A6 (down) cells when exposed to a range of concentrations of propranolol at the three time points (24, 48 and 72h).

Table 3. Estimated lethal doses (LC_{10}, LC_{20} and LC_{50}) to A6 and XTC-2 cell line after 24h, 48h and 72h exposure to propranolol. LCs were calculated through interpolation of a nonlinear regression with a four-parameter dose-response curve. Values missing indicate that LCs were out of the curve range.

		XTC-2	A6
24h	LC_{10}	14.4 (10.7 – 18.5)	13.6 (7.13 – 17.6)
	LC_{20}	17.6 (13.7 – 21.9)	17.6 (13.2 – 32.6)
	LC_{50}	27.3 (22.4 – 35.8)	24.1 (20.4 – 38.7)
48h	LC_{10}	11.7 (6.85 – 16.4)	6.46 (4.83 – 8.75)
	LC_{20}	15.1 (10.4 – 18.8)	10.3 (8.57 – 12.0)
	LC_{50}	22.8 (19.1 – 35.2)	18.2 (16.9 – 19.4)
72h	LC_{10}	12.8 (6.69 – 17.5)	-
	LC_{20}	14.9 (8.02 – 18.0)	5.37 (4.56 – 6.33)
	LC_{50}	19.8 (10.6 – 38.6)	12.4 (11.4 – 13.3)

3.4 Tadpoles toxicity assay with haloperidol

The survival rate of *X. laevis* and *P. perezi* tadpoles in various haloperidol concentrations are presented in Figure 16. Only the two highest concentrations of haloperidol significantly reduced the survival of the tadpoles of the two species ($F \leq 58.91$; $p < 0.0001$). The estimated LC_{20} and LC_{50} for *P. perezi* were 1.70 (1.32-1.95) mg/L and 2.20 (1.90-2.58) mg/L, respectively. For the *X. laevis*, the software was not able to calculate the confidence intervals for the lethal concentrations indicating that the intervals were "very wide". However, the lethal concentrations LC_{20} and LC_{50} obtained were 0.931 and 1.45 mg/L.

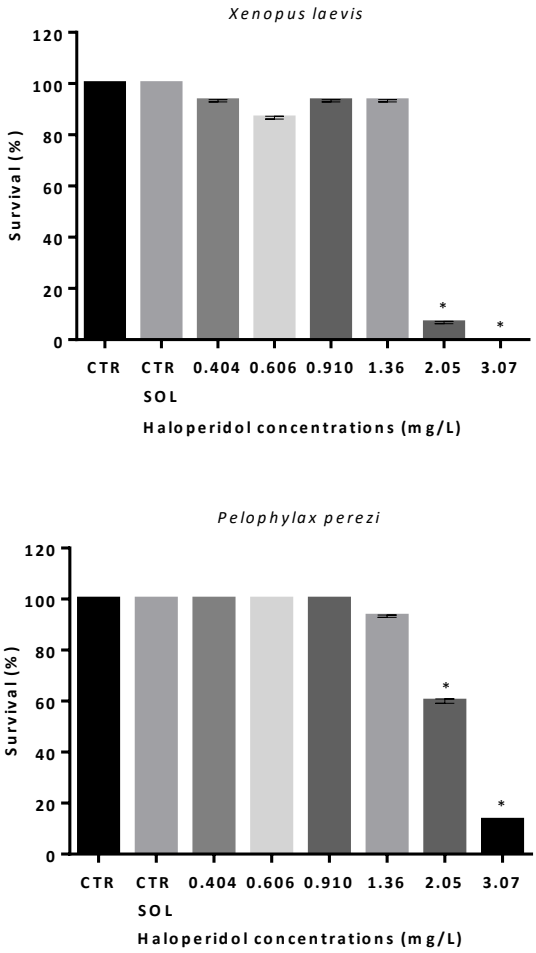


Figure 16. Average survival of *Xenopus laevis* (up) and *Pelophylax perezi* (down) tadpoles after being exposed for 96h to various haloperidol concentrations. Error bars represent standard deviation. * indicates significant differences relatively to the control ($p<0.0001$).

Malformations were observed in tadpoles of *X. laevis* exposed to haloperidol (Figure 17): 35% and 39% of the tadpoles exposed to 0.606 and 0.910 mg/L respectively, exhibited malformations, being the most common haemorrhage. In *P. perezi*, 21.4% and 66.7% of tadpoles presented malformations when exposed to, respectively, 1.36 and 2.05 mg/L. However, no malformations were observed in organisms exposed to the other tested concentrations (Figure 17). All the malformations for both species were haemorrhages.

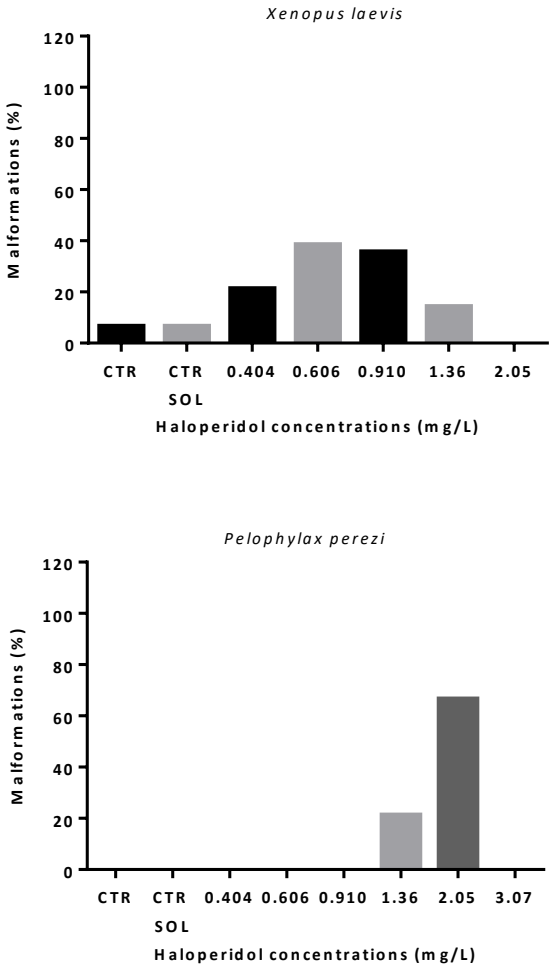


Figure 17. Proportion of tadpoles of *Xenopus laevis* and *Pelophylax perezi* with malformations after being exposed for 96h to various haloperidol concentrations.

The tadpoles of *X. laevis* exposed to 0.910 and 1.36 mg/L were at earlier developmental stages relatively to those from the control group ($F=13.38$; $p<0.0001$; Figure 18). The results shown for *X. laevis* at 2.05 mg/L represents a single tadpole and, for this reason, were not included in the statistical analysis. Similarly, to the results for malformations, haloperidol induced no significant effects in the development of tadpoles of *P. perezi*, with more than 80% of the tadpoles, in each treatment, being at G 25 ($F=1.489$; $p=0.1810$; Figure 18).

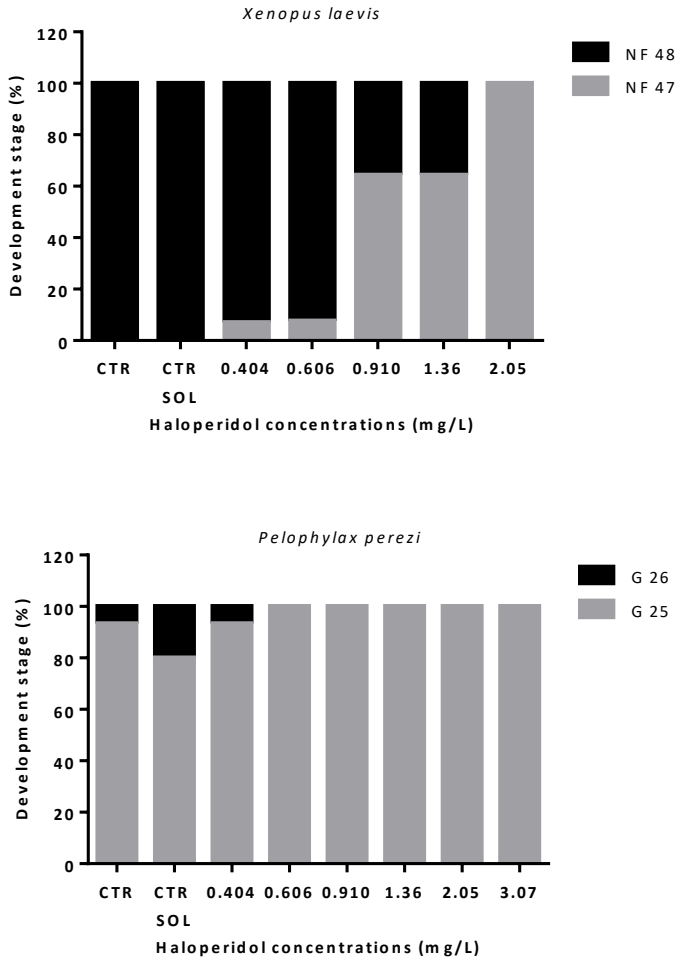


Figure 18. Percentage of *Xenopus laevis* (up) and *Pelophylax perezi* (down) tadpoles at the NF 47 or NF 48 and G 25 or G 26, respectively, after 96h of exposure to a range of concentrations of haloperidol.

Exposure to haloperidol caused a significant reduction in the weight of tadpoles of *X. laevis* for all tested concentrations (please note that concentration 2.05 mg/L was not included in statistical analyses as it refers only to one organism) ($F=35.2$; $p<0.0001$; Figure 19). As for *P. perezi*, only tadpoles exposed to concentrations of haloperidol equal or above 1.36 mg/L showed a significantly lower body weight relatively to those from the control ($F=10.40$; $p<0.0001$).

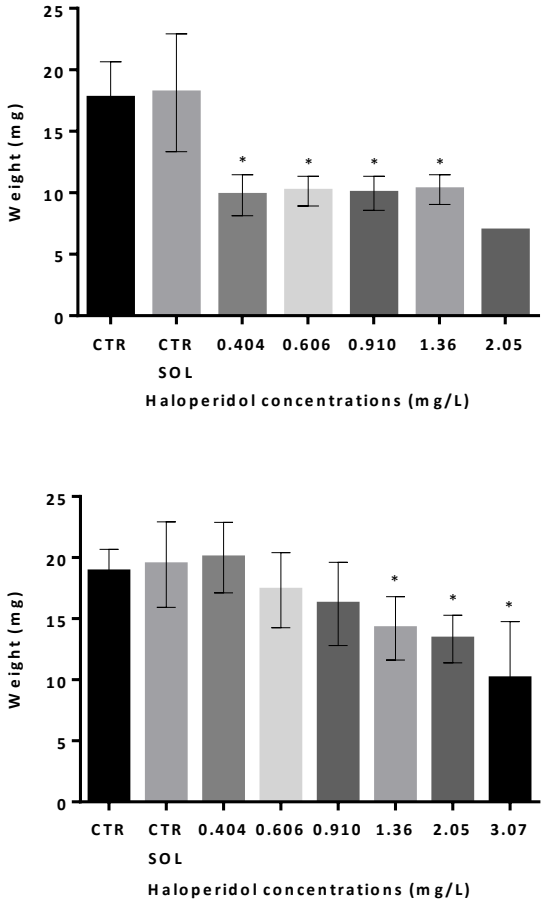


Figure 19. Average weight of tadpoles of *Xenopus laevis* (up) and *Pelophylax perezi* (bottom), after being exposed for 96h to a range of haloperidol concentrations. Error bars represent standard deviation. * indicates significant differences relatively to the control ($p<0.0001$).

The body lengths of tadpoles of the two species were affected by exposure to haloperidol (Figure 20). The total body and snout-to-vent lengths were reduced in tadpoles of *X. laevis* exposed to all tested concentrations ($F\leq26.83$; $p<0.0001$); however, tail length was only significantly smaller than controls in tadpoles exposed to 0.404 and 0.606 mg/L ($F=3.004$; $p\leq0.0156$; Figure 20). For *P. perezi* tadpoles, total body and tail lengths were only significantly reduced at the highest tested concentration (3.07 mg/L), while snout-to-vent was significantly smaller in tadpoles exposed to haloperidol concentrations equal or higher than 1.36 mg/L ($F\leq7.840$; $p<0.0001$).

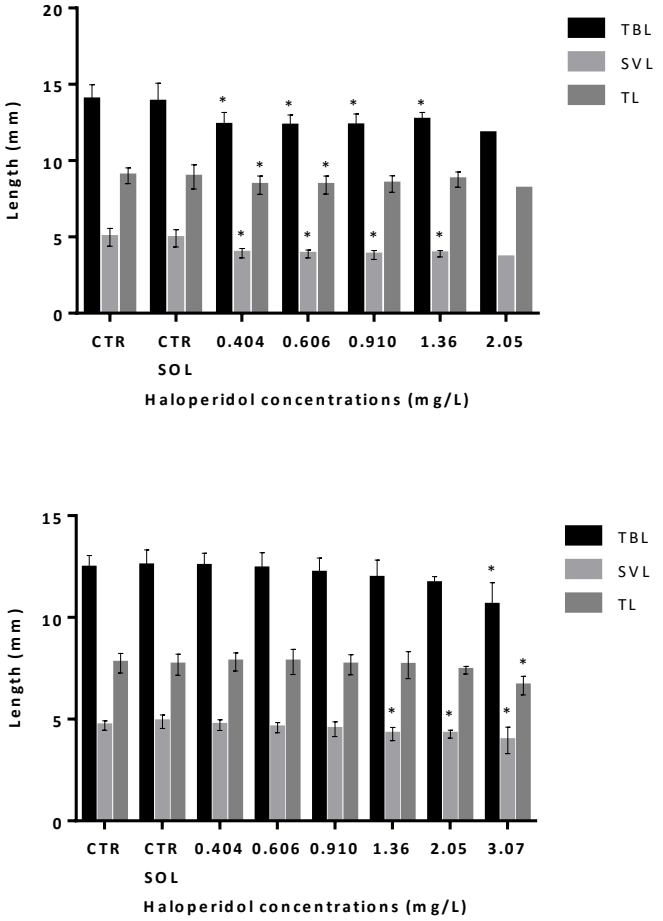


Figure 20. Average length of total body (TBL), snout-to-vent (SVL) and tail (TL) of tadpoles of *Xenopus laevis* (up) and of *Pelophylax perezi* (bottom), after being exposed for 96h to a range of haloperidol concentrations. Error bars represent standard deviation. * indicates significant differences relatively to the control ($p<0.0001$).

3.5 *In vitro* cytotoxicity assays with haloperidol

The cytotoxicity curves for the single exposure to haloperidol in both amphibian cell lines are represented below Figure 21. In the XTC-2 cells, the cell viability after 24h and 48h exposure to haloperidol was not significantly different ($p=0.2524$). However, significant differences were found between 24h and 72h as well as 48h with 72h ($p=0.0216$; $p=0.0427$, respectively). In A6 cell, significant differences in cytotoxic curves of haloperidol were only found between 48h and 72h ($p=0.0253$). The estimated lethal concentrations of haloperidol to both cell lines tested are presented in Table 4.

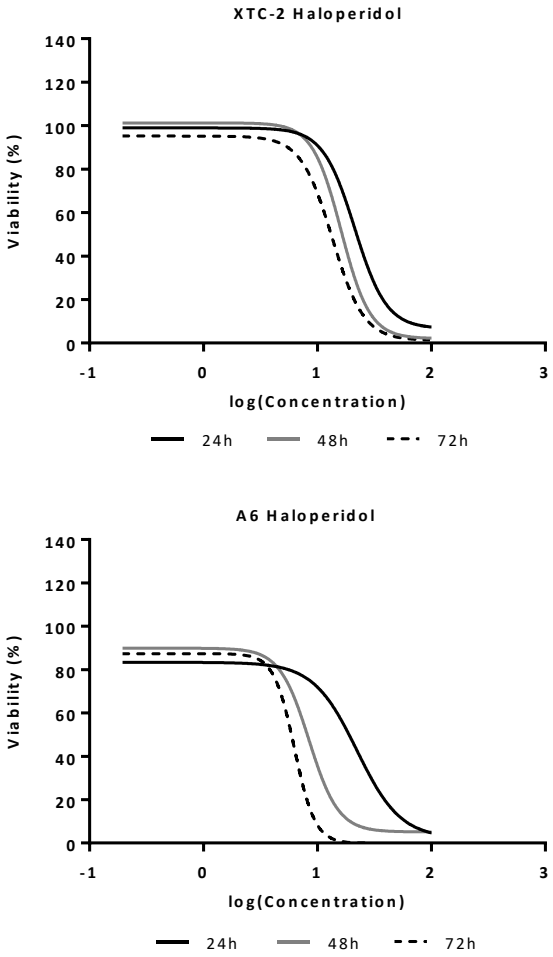


Figure 21. Cytotoxicity curves of XTC-2 (up) and A6 (down) cells when exposed to a range of concentrations of haloperidol alone at the three time points (24, 48 and 72h).

Table 4. Estimated lethal doses (LC_{10}, LC_{20} and LC_{50}) to A6 and XTC-2 cell line after 24h, 48h and 72h exposure to haloperidol. LCs were calculated through interpolation of a nonlinear regression with a four-parameter dose-response curve. Values missing indicate that LCs were out of the curve range.

		XTC-2	A6
24h	LC_{10}	10.4 (7.34 – 14.3)	-
	LC_{20}	13.8 (10.6 – 17.0)	5.56 (0.0 - 13.6)
	LC_{50}	22.1 (18.9 – 26.0)	19.0 (12.3 – 27.9)
48h	LC_{10}	8.90 (6.87 – 11.2)	-
	LC_{20}	11.1 (9.12 – 13.1)	4.63 (3.62 – 5.81)
	LC_{50}	16.5 (14.4 – 19.2)	8.15 (7.12 – 9.35)
72h	LC_{10}	5.49 (3.92 – 7.28)	-
	LC_{20}	8.02 (6.69 – 9.39)	3.83 (2.49 – 5.27)
	LC_{50}	13.2 (12.0 – 14.6)	5.92 (4.63 – 6.89)

3.6 Toxicity assay with tadpoles with combined exposures of pharmaceuticals and nanoplastics

The average survival of *P. perezi* tadpoles when exposed to single and combined exposures of propranolol and PS-50 nm are represented in Figure 22 ($F=50.66$; $p<0.0001$). The mixture of propranolol and 10 mg/L PS-50 nm showed a significantly lower mortality (33.3%) than the registered for propranolol single exposure. Meanwhile, the mortality rate of propranolol mixture with 10 µg/L PS-50 nm was not significantly different from the single exposure to propranolol.

The effects of *X. laevis* tadpoles exposure to PS-50 nm nanoplastics, alone and combined with the haloperidol (at a concentration corresponding to the $LC_{50,96h}$) is showed in Figure 22 ($F=3.598$; $p<0.05$). Mortality in the control was lower than 10% thus validating the assay. Individual exposure to nanoplastics, at both concentrations, elicited mortality effects within the same range of control (6.67%). In the treatments, haloperidol $LC_{50,}$ and the mixture of haloperidol LC_{50} with PS-50 nm 10 mg/L, the mortality reached 33.3%. The treatment with the mixture haloperidol LC_{50} and PS-50 nm 10 µg/L induced the highest mortality rate, 53.3%, being the only treatment eliciting statistically differences from the control. In the assay with *P. perezi* tadpoles, no mortality was observed in tadpoles exposed to the control and to the PS-50 nm treatments. The mixture of haloperidol with 10 mg/L of PS-50 nm resulted in a much lower mortality (20%), that was not significantly different from the control but was significantly different from the single exposure to haloperidol. The mortality of single mixture of haloperidol and combined exposure with 10 µg/L was the same.

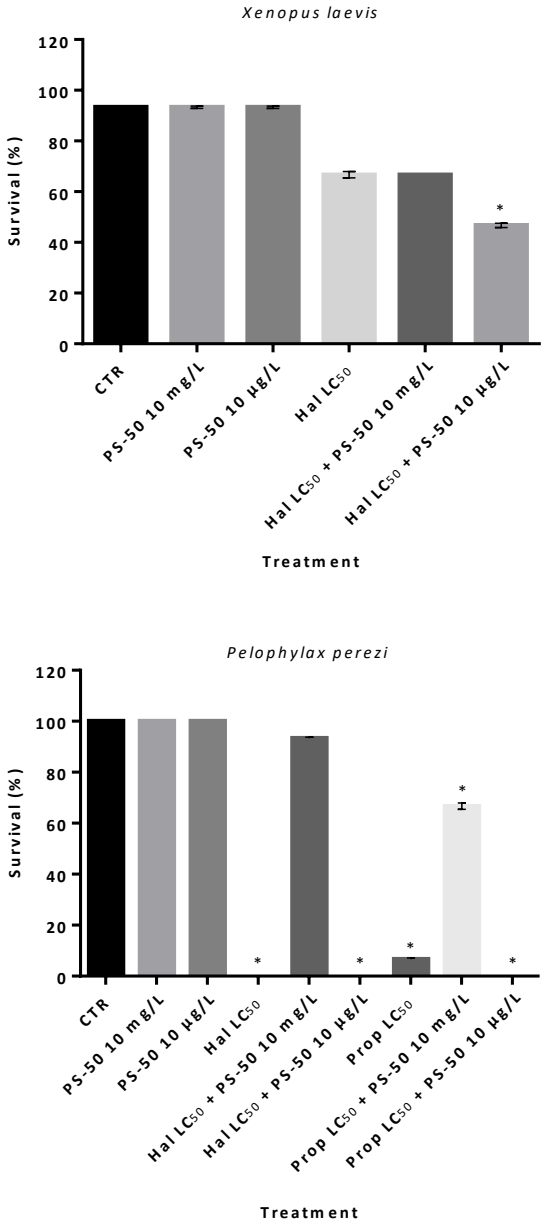


Figure 22. Average survival of *Xenopus laevis* (up) and *Pelophylax perezi* (bottom) tadpoles after being exposed for 96h to single and combined effects of PS-50 nm nanoplastics (10 mg/L and 10 µg/L) and the pharmaceuticals (propranolol and haloperidol). Error bars represent standard deviation. * indicates significant differences relatively to the control ($p<0.05$).

The information regarding malformations observed in both assays is presented in Figures 23, 24 and 25. For *X. laevis*, the number of malformations was low, with the highest percentage being in the individual exposure of haloperidol (40%), the most common being hyperpigmentation (Figure 24d). For *P. perezi*, the percentage of malformations was low with

the only exception being in the single exposure to propranolol, where the only survivor showed a malformation (specifically hyperpigmentation; Figure 25c) thus 100%.

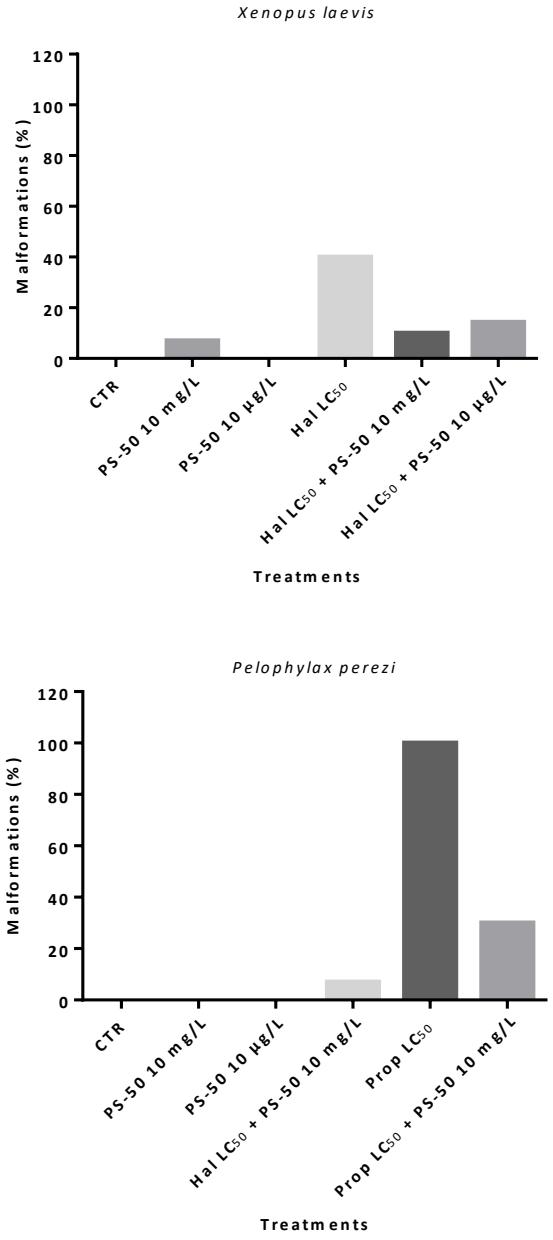


Figure 23. Proportion of tadpoles of *Xenopus laevis* (up) and *Pelophylax perezi* (bottom) with malformations after being exposed for 96 h to single and combined effects of PS-50 nm nanoplastics (10 mg/L and 10 µg/L) and the pharmaceutical (haloperidol and propranolol).

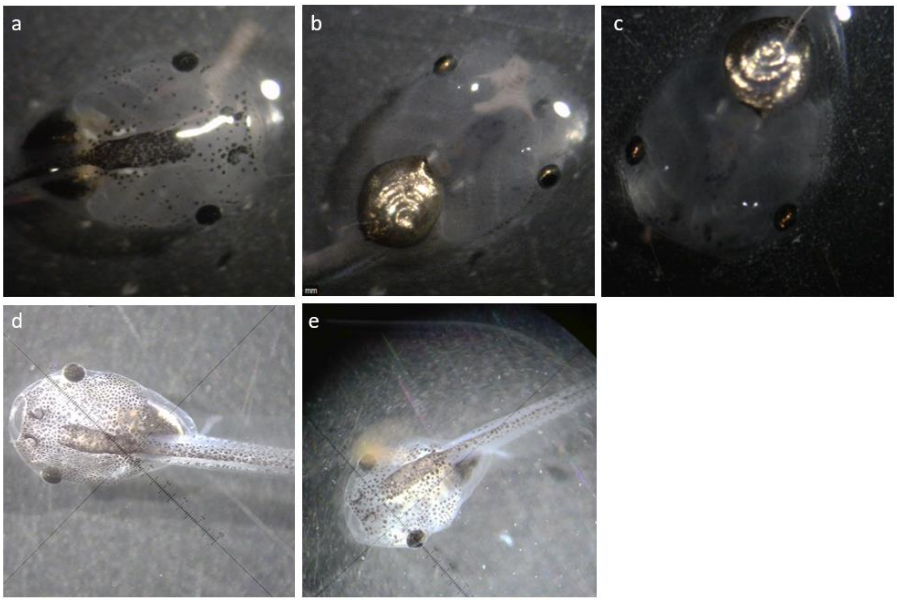


Figure 24. Photographs illustrating tadpoles of *Xenopus laevis* at the end of the tadpole toxicity assay. (a) and (b) control tadpole without malformations; (c) tadpole exposed 10 mg/L PS-50 nm without malformations; (d) tadpole exposed to LC_{50} haloperidol showing hyperpigmentation; (e) tadpole exposed to combined exposure to LC_{50} haloperidol and 10 mg/L of PS-50 nm also showing hyperpigmentation.

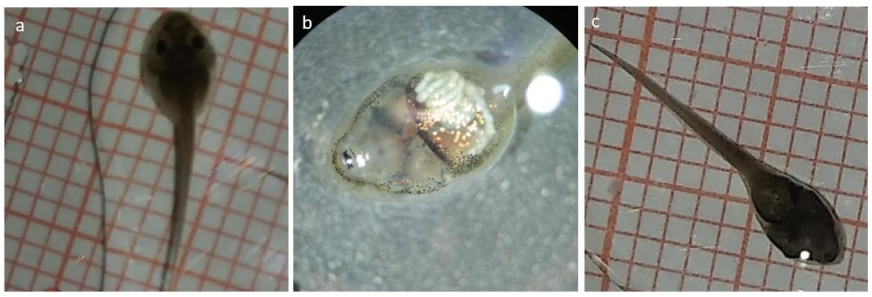


Figure 25. Photographs illustrating tadpoles of *Pelophylax perezi* at the end of the tadpole toxicity assay. (a) control tadpole without malformations; (b) tadpole exposed 10 mg/L PS-50 nm showing the presence of nanoplastics in the gut; (c) tadpole exposed to single exposure to LC_{50} propranolol showing hyperpigmentation.

The developmental stages of the tadpoles were not influenced by the performed treatments (Figure 26). More than 80% of *X. laevis* tadpoles in the control and both individual treatments of nanoplastics were in the development stage NF 48, whereas all tadpoles from the

single and mixture exposures with haloperidol were in a lower development stage, NF 47. In *P. perezi*, 20.0% of the tadpoles in the control were at the G 26 stage while in the PS-50 10 µg/L 13.3% of the tadpoles were at this stage. The remaining treatments with alive organisms had all tadpoles in the G 25.

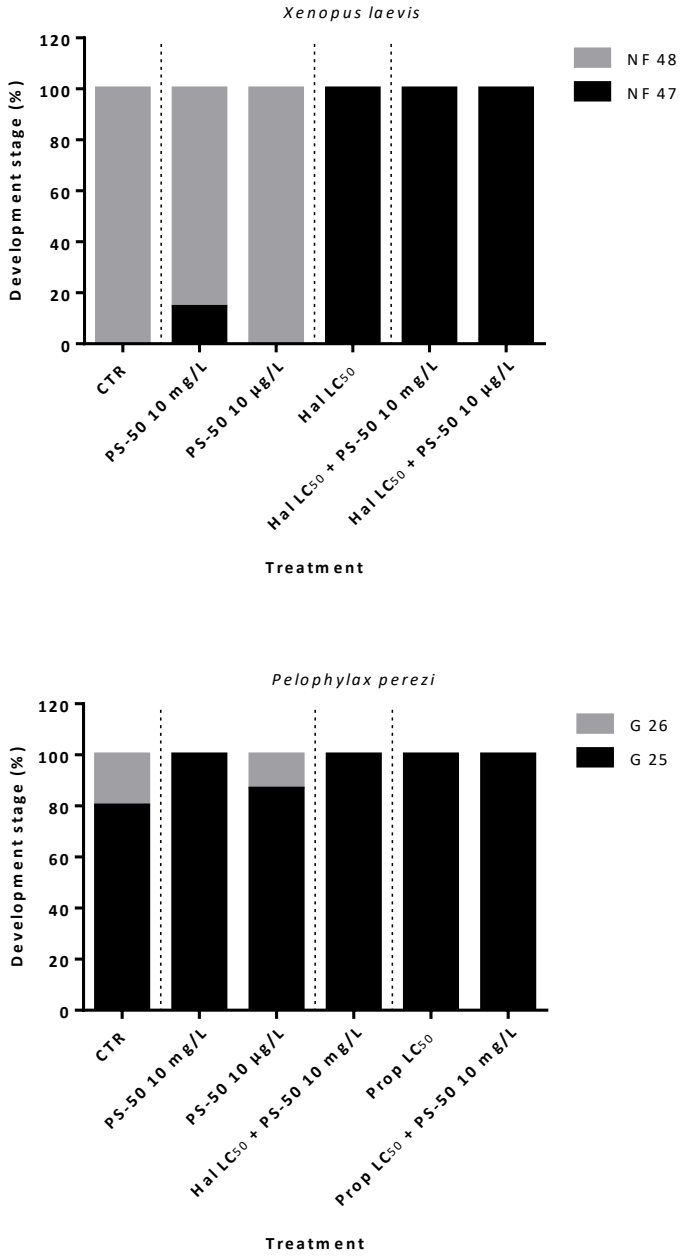
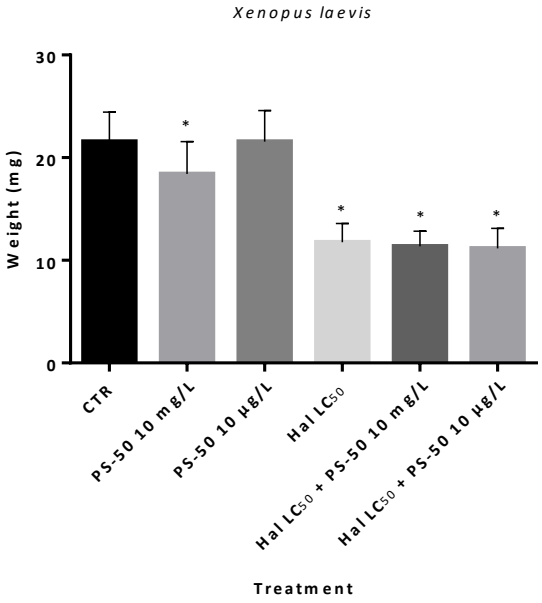


Figure 26. Percentage of *Xenopus laevis* (up) and *Pelophylax perezi* (down) tadpoles at the NF 47 or NF 48 and G 25 or G26 after being exposed for 96 h to single and combined treatments of nanoplastics of polysterene (PS-50 nm; 10 mg/L and 10 µg/L) and the pharmaceuticals (Hal - haloperidol and Prop - propranolol).

The weight of the *X. laevis* and *P. perezi* tadpoles in each treatment is represented in the Figure 27 ($F=42.57$, $p<0.0001$; $F=10.80$, $p<0.0001$, respectively). For *X. laevis*, the exposure of PS-50 10 µg/L (average weight of 21.6), was the only treatment that did not show significant reduction when compared to the control while all other treatments showed significant differences. For the exposure of PS-50 10 mg/L, the average weight was 18.4 mg, a higher average when compared with the other treatments with significant differences. The average weight of haloperidol single exposure, the mixture with 10 mg/L and 10 µg/L was 11.8, 11.4 and 11.2 respectively. For *P perezi*, the exposure to PS-50 10 mg/L resulted in significant increase of weight of the tadpoles (average weight of 18.01 mg) whilst the combined exposure to propranolol and the nanoplastic at 10 mg/L resulted in significant decrease of the average weight (average of 12.12 mg).



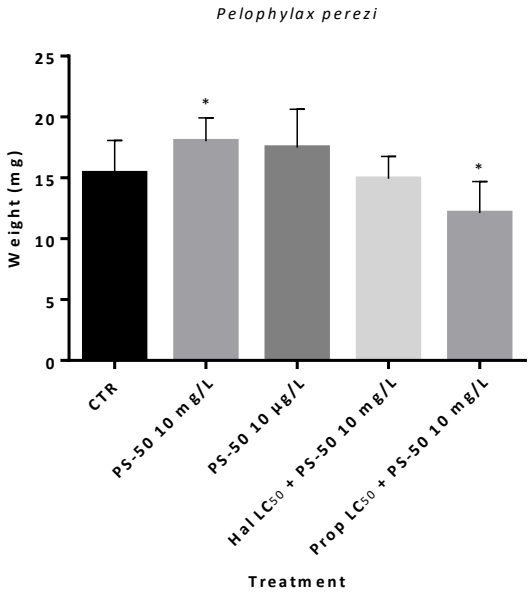


Figure 27. Average weight of tadpoles of *Xenopus laevis* (up) and *Pelophylax perezi* (bottom), after being exposed for 96h to single and combined treatments of nanoplastics of polysterene (PS-50 nm; 10 mg/L and 10 µg/L) and the pharmaceuticals (Hal - haloperidol and Prop - propranolol). Error bars represent standard deviation. * indicates significant differences relatively to the control ($p<0.0001$).

The body lengths of tadpoles of the two species at the end of the assay are represented in Figure 28. Total body length ($F=15.47$; $p<0.0001$) and snout-to-vent length ($F=46.38$; $p<0.0001$) for *X. laevis* tadpoles were significantly reduced in all treatments with the exception for the single exposure of nanoplastics at 10 µg/L. In the case of tail length ($F=4.383$; $p<0.0001$), tadpoles in haloperidol exposure both single and with mixtures of nanoplastics were significantly reduced when compared to the control. For the *P. perezi* tadpoles, the total body length ($F=11.36$, $p<0.0001$) for the treatments of single exposure of nanoplastics 10 mg/L and for the combined exposure of propranolol and PS-50 nm 10 mg/L was significantly different. Significant differences were also observed in snout-to-vent ($F=12.58$, $p<0.0001$) for both concentrations of single exposure to PS-50 nm. Finally, tail length ($F=8.514$; $p<0.0001$) was only significantly different to the control in the mixture of propranolol and PS-50 nm 10 mg/L.

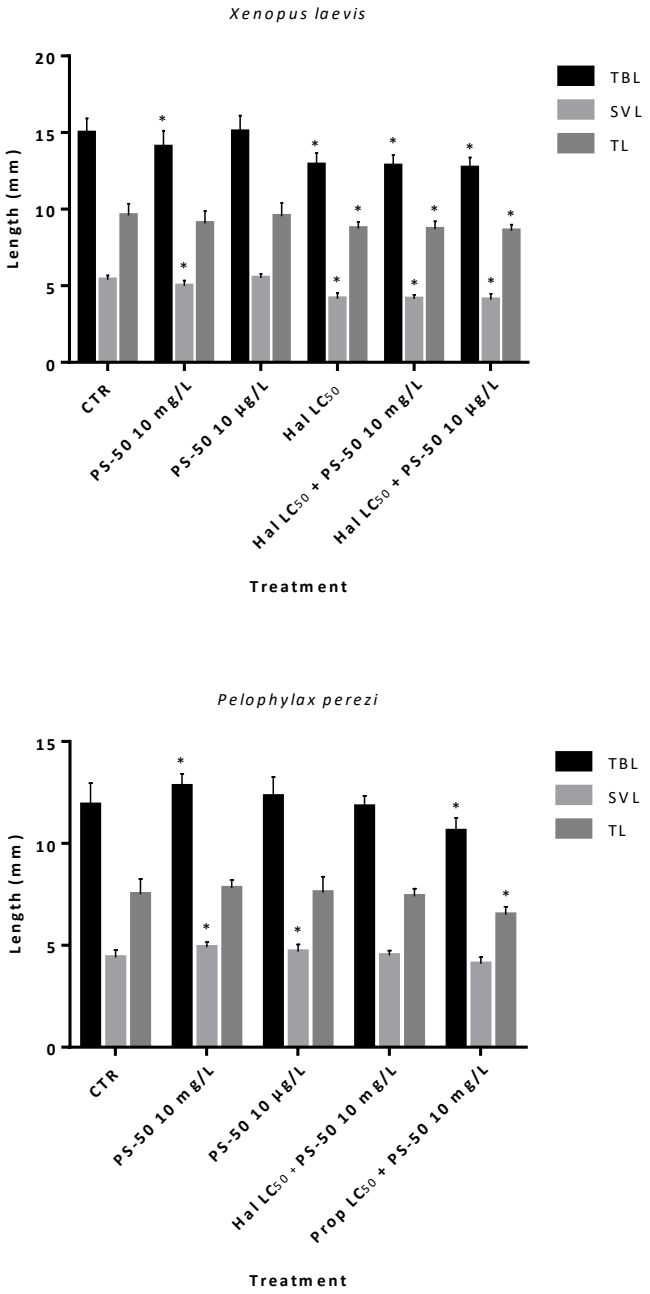
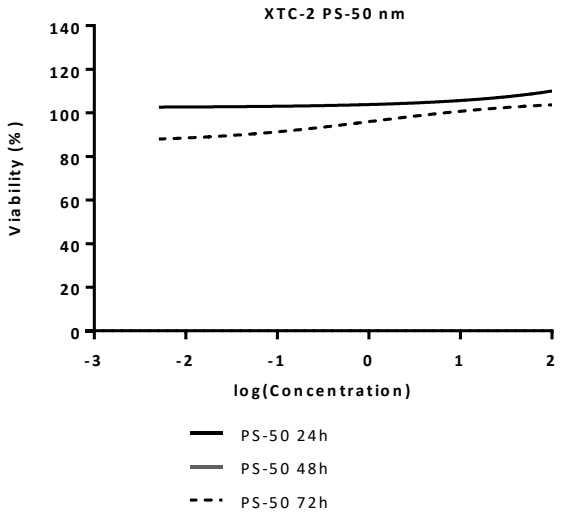


Figure 28. Average length of total body (TBL), snout-to-vent (SVL) and tail (TL) of tadpoles of *Xenopus laevis* (up) and of *Pelophylax perezi* (bottom), after being exposed for 96h to single and combined treatments of nanoplastics of polysterene (PS-50 nm; 10 mg/L and 10 µg/L) and the pharmaceuticals (Hal - haloperidol and Prop - propranolol). Error bars represent standard deviation. * indicates significant differences relatively to the control ($p<0.0001$).

3.7 *In vitro* cytotoxicity assays with nanoplastics

Lethal concentrations for the exposure of nanoplastics PS-50 nm in A6 cell line were not able to be interpolated from the data with the exception for 24h LC_{10} which resulted in the value of 0.036 mg/L. No significant differences were observed between the cytotoxicity curves at 24h and 48h (p=0.0997) and 24h and 72h (p=0.7902). However, significant differences were found between 48h with the 72h (p=0.0055). When producing a nonlinear regression for PS-50 nm effects in the XTC-2 line, the program was not able to show the regression for 48h time point. As a result, it's not represented in Figure 29.. Lethal concentration was only found for 72h LC_{10}: 0.042 mg/L. Significant differences in the curves were found between 24h and 48h (p=0.0001), 24h and 72h (p=0.0018), and 48h and 72h as well (p=0.0163).



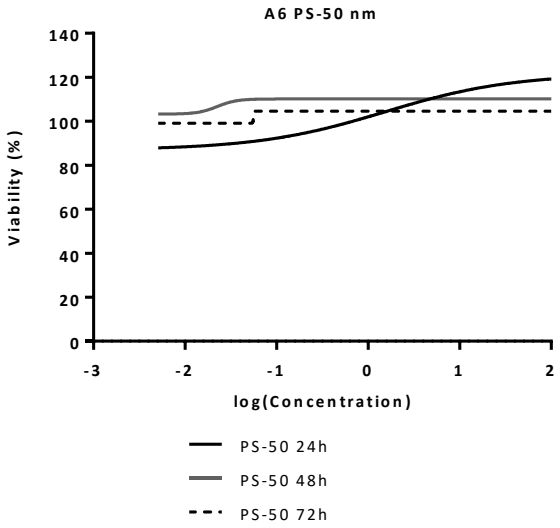


Figure 29. Cytotoxicity curves of XTC-2 (up) and A6 (down) cells when exposed to a range of concentrations of PS-50 nm nanoplastics at three time points (24, 48 and 72h).

3.8 *In vitro* cytotoxicity assays with combined exposures of propranolol and nanoplastics

Cytotoxicity curves of XTC-2 cells when exposed to a range of concentrations of propranolol alone or in combination with 10 mg/L and 10 µg/L of PS-50 nm at the three time points are represented in Figure 30. At the 24h time point, significant differences between propranolol individual exposure and propranolol + PS-50 10 mg/L mixture were observed, while no differences were found for the other combinations (propranolol and propranolol + PS-50 10 µg/L; propranolol + PS-50 10 mg/L and propranolol + PS-50 10 µg/L). At the 48h and 72h time points, all combinations showed significant differences. The lethal concentrations (LC_{10}, LC_{20} and LC_{50}) for each treatment and time point are presented in Table 5. For the combined exposure to propranolol and PS-50 nm 10 µg/L at 72h, the software was not able to calculate the confidence intervals for the lethal concentrations.

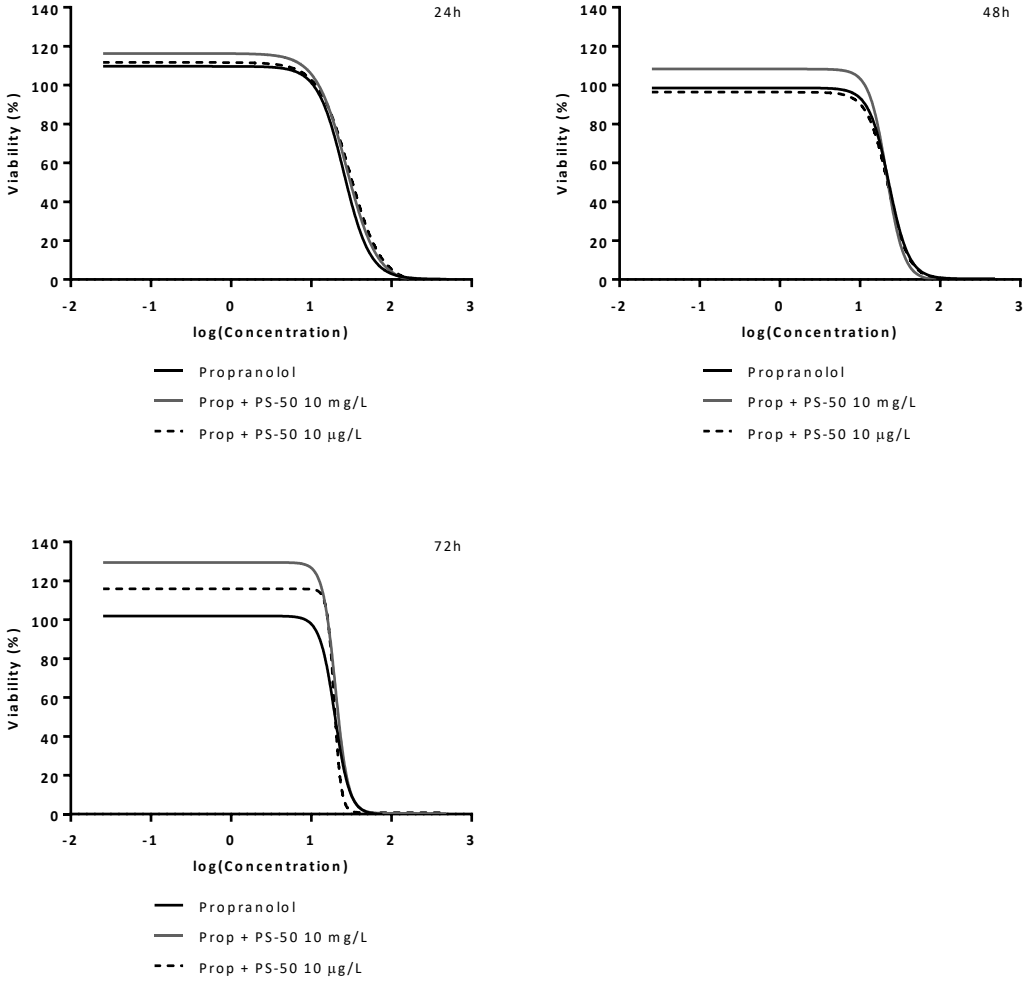
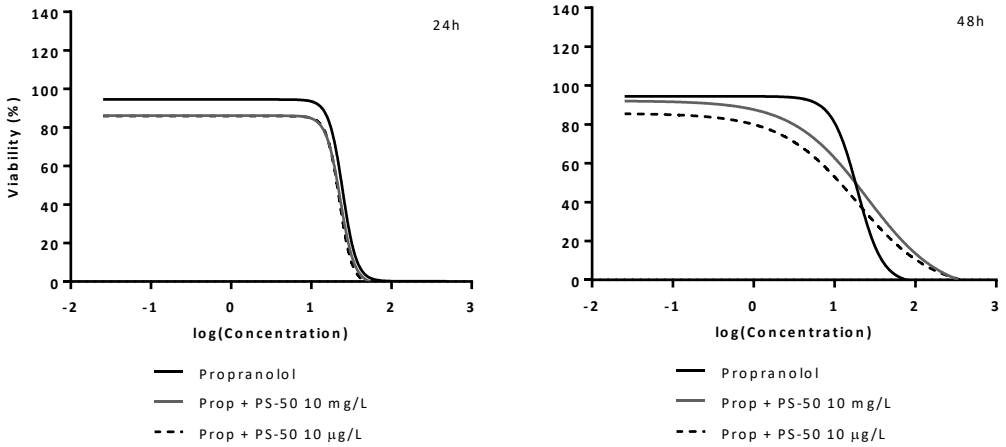


Figure 30. Cytotoxicity curves of XTC-2 cells when exposed to a range of concentrations of propranolol alone or in combination with 10 mg/L and 10 µg/L of PS-50 nm at the three time points (24, 48 and 72h).

Table 5. Estimated lethal concentrations to XTC-2 cell line after 24h, 48h and 72h exposure to propranolol - alone and combined with 10 mg/L and 10 µg/L of PS-50 nm. LCs were calculated through interpolation of a nonlinear regression with a four-parameter dose-response curve. Values missing indicate that LCs were out of the curve range.

XTC-2 MTT		Propranolol	Propranolol + PS-50 10 mg/L	Propranolol + PS-50 10 µg/L
24h	LC_{10}	14.4 (10.7 – 18.5)	16.0 (11.4 – 21.3)	15.8 (12.5 – 19.5)
	LC_{20}	17.6 (13.7 – 21.9)	19.3 (14.4 – 25.1)	19.7 (16.2 – 23.5)
	LC_{50}	27.3 (22.4 – 35.8)	30.8 (24.2 – 41.1)	32.6 (27.9 – 38.6)
48h	LC_{10}	11.7 (6.85 – 16.4)	14.4 (9.79 – 17.2)	10.2 (7.44 – 13.3)
	LC_{20}	15.1 (10.4 – 18.8)	16.5 (11.9 – 18.6)	14.0 (11.4 – 16.1)
	LC_{50}	22.8 (19.1 – 35.2)	22.3 (19.7 – 34.2)	22.0 (20.0 – 25.9)
72h	LC_{10}	12.8 (6.69 – 17.5)	17.2 (7.55 – 44.6)	17.1
	LC_{20}	14.9 (8.02 – 18.0)	18.4 (7.98 – 45.8)	17.8
	LC_{50}	19.8 (10.6 – 38.6)	21.8 (8.96 – 50.6)	19.9

Cytotoxicity curves of A6 cells when exposed to a range of concentrations of propranolol alone or in combination with 10 mg/L and 10 µg/L of PS-50 nm, at the three time points, are represented in Figure 31. At the 24h time point, significant differences were found between individual exposure to propranolol and the combined mixtures with nanoplastics ($p \leq 0.0032$). No significant differences were found between the combined exposures, Prop + PS-50 10 mg/L and Prop + PS-50 10 µg/L ($p=0.8141$). At the 48h time point, no significant differences were found between individual exposure and the mixture exposure ($p \geq 0.1040$), but significant differences between combined exposures were observed ($p=0.0007$). At the 72h time point, all comparisons were significantly different ($p \leq 0.0366$). The lethal concentrations (LC_{10}, LC_{20} and LC_{50}) for each treatment and time point are presented in Table 6.



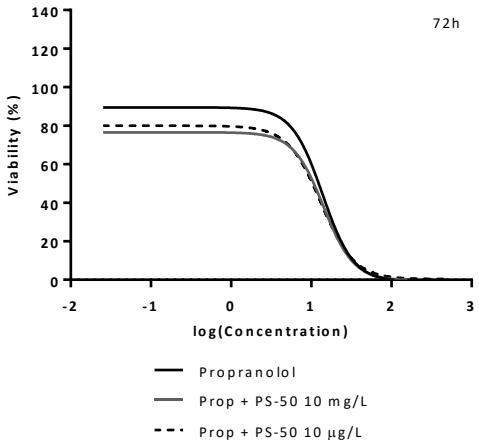


Figure 31. Cytotoxicity curves of A6 cells when exposed to a range of concentrations of propranolol alone or in combination with PS-50 nm nanoplastics (10 mg/L and 10 µg/L) at three time points (24, 48 and 72h).

Table 6. Estimated lethal concentrations to A6 cell line after 24h, 48h and 72h exposure to propranolol - alone and combined with 10 mg/L and 10 µg/L of PS-50 nm. LCs were calculated through interpolation of a nonlinear regression with a four-parameter dose-response curve. Values missing indicate that LCs were out of the curve range.

A6 MTT		Propranolol	Propranolol + PS-50 10 mg/L	Propranolol + PS-50 10 µg/L
24h	LC_{10}	13.6 (7.13 – 17.6)	-	-
	LC_{20}	17.6 (13.2 – 32.6)	13.6 (8.25 – 17.5)	13.9 (7.29 – 18.0)
	LC_{50}	24.1 (20.4 – 38.7)	21.5 (19.1 – 37.1)	21.0 (18.7 – 40.6)
48h	LC_{10}	6.46 (4.83 – 8.75)	0.444 (0.00 – 3.45)	-
	LC_{20}	10.3 (8.57 – 12.0)	3.12 (0.967 – 7.65)	0.997 (0.00 – 3.64)
	LC_{50}	18.2 (16.9 – 19.4)	18.1 (10.4 – 31.1)	11.7 (6.42 – 19.9)
72h	LC_{10}	-	-	-
	LC_{20}	5.37 (4.56 – 6.33)	-	-
	LC_{50}	12.4 (11.4 – 13.3)	10.7 (9.73 – 11.7)	10.3 (8.92 – 11.8)

3.9 *In vitro* cytotoxicity assays with combined exposures of haloperidol and nanoplastics

Cytotoxicity curves of XTC-2 cells when exposed to a range of concentrations of haloperidol alone or in combination with 10 mg/L and 10 µg/L of PS-50 nm, at the three time points, are represented in Figure 32. At the 24h time point, significant differences were found

between single exposure to haloperidol and the combined mixtures of haloperidol and nanoplastics (p≤0.0027), with no significant differences between combined exposures (p=0.3023). At 48h time, no significant differences were found between treatments (p≥0.7121). At the 72h time, significant differences between individual exposure to haloperidol and the combined mixtures of haloperidol and nanoplastics were found (p≤0.0083) with no significant differences between combined exposures (p=0.1128). The lethal concentrations (LC_{10}, LC_{20} and LC_{50}) for each treatment and time point are presented in Table 7.

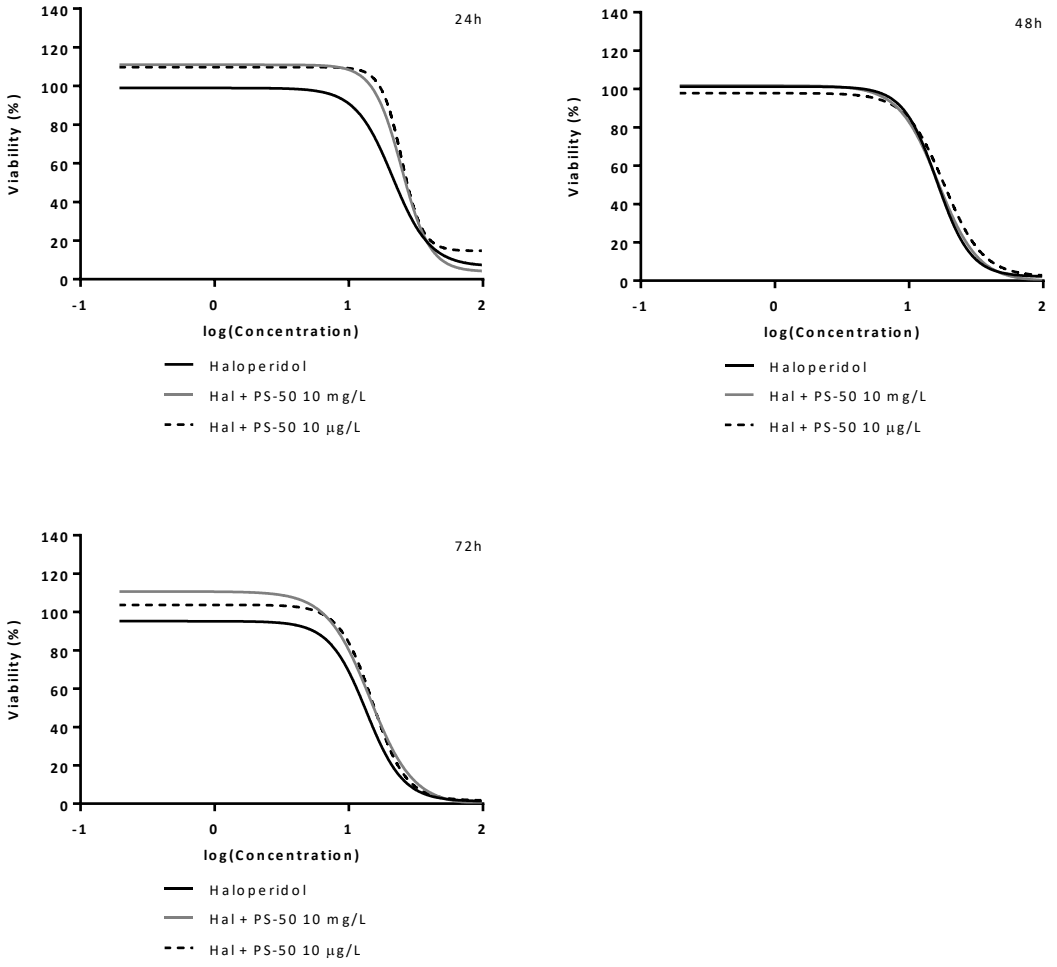


Figure 32. Cytotoxicity curves of XTC-2 cells when exposed to a range of concentrations of haloperidol alone or in combination with PS-50 nm nanoplastics (10 mg/L and 10 µg/L) at three time points (24, 48 and 72h).

Table 7. Estimated lethal concentrations to XTC-2 cell line after 24h, 48h and 72h exposure to haloperidol - alone and combined with 10 mg/L and 10 µg/L of PS-50 nm. LCs were calculated through interpolation of a nonlinear regression with a four-parameter dose-response curve. Values missing indicate that LCs were out of the curve range.

XTC-2 MTT		Haloperidol	Haloperidol + PS-50 10 mg/L	Haloperidol + PS-50 10 µg/L
24h	LC_{10}	10.4 (7.34 – 14.3)	17.6 (13.4 – 21.1)	19.6 (14.1 – 23.1)
	LC_{20}	13.8 (10.6 – 17.0)	19.9 (15.8 – 22.8)	21.6 (15.8 – 24.1)
	LC_{50}	22.1 (18.9 – 26.0)	26.6 (23.5 – 31.4)	27.2 (24.9 – 36.1)
48h	LC_{10}	8.90 (6.87 – 11.2)	8.24 (6.62 – 10.1)	8.19 (5.47 – 11.5)
	LC_{20}	11.1 (9.12 – 13.1)	10.6 (6.97 – 12.2)	11.2 (8.66 – 14.0)
	LC_{50}	16.5 (14.4 – 19.2)	16.7 (15.0 – 18.8)	18.3 (15.5 – 21.7)
72h	LC_{10}	5.49 (3.92 – 7.28)	8.30 (6.44 – 10.3)	8.85 (7.59 – 10.2)
	LC_{20}	8.02 (6.69 – 9.39)	10.0 (8.12 – 11.9)	10.7 (9.52 – 11.9)
	LC_{50}	13.2 (12.0 – 14.6)	15.4 (13.3 – 18.2)	15.5 (14.3 – 17.1)

Cytotoxicity curves of A6 cells when exposed to a range of concentrations of haloperidol alone or in combination with 10 mg/L and 10 µg/L of PS-50 nm, at the three time points, are represented in Figure 33. At the 24h time point, the single exposure of haloperidol was not significantly different than the combined exposure of haloperidol and PS-50 10 mg/L ($p=0.4781$) whereas significant differences were found between the other treatments ($p\leq0.0225$). A similar pattern was observed at 48h time point, with the single exposure and the combined exposure with PS-50 10 mg/L had a p-value of 0.9323, and the rest had a p-value lower than 0.0368. At the last time point, all comparisons were statistically different ($p\leq0.0297$). The lethal concentrations (LC_{10}, LC_{20} and LC_{50}) for each treatment and time point are presented in Table 8.

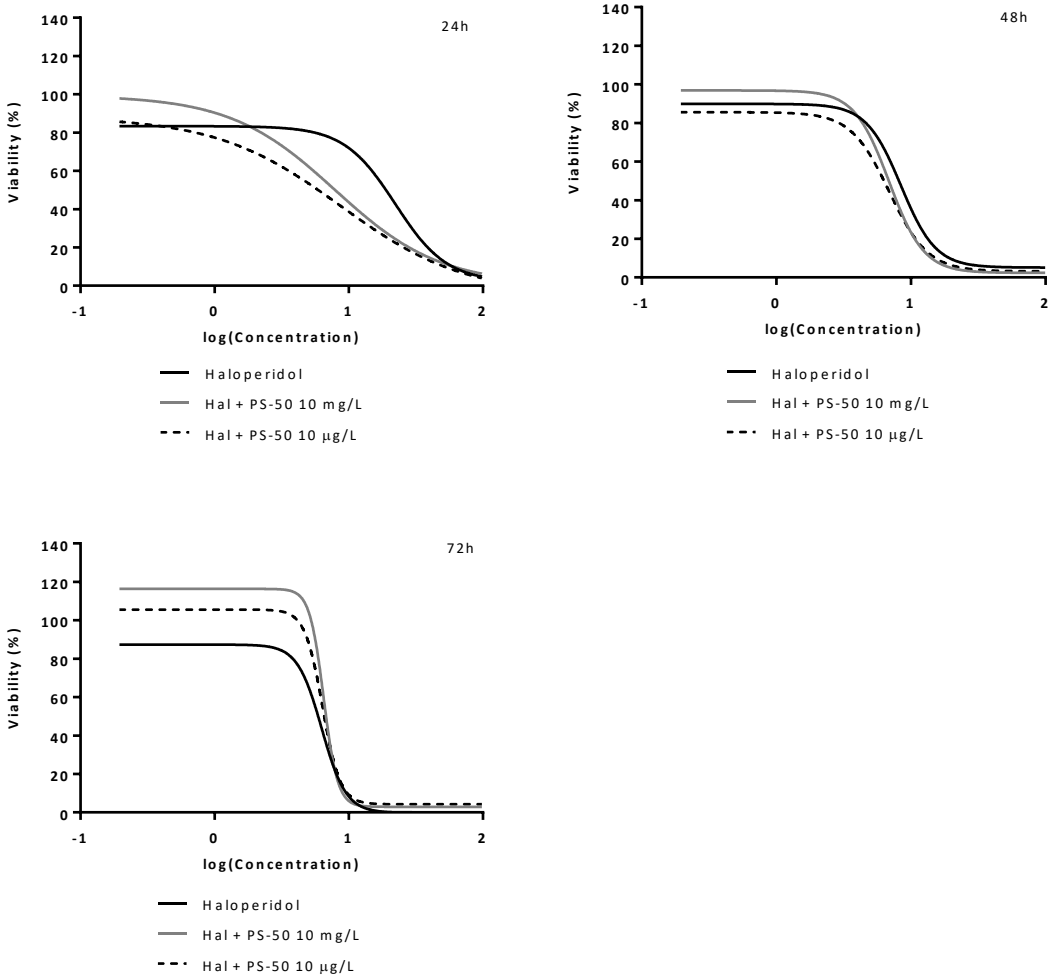


Figure 33. Cytotoxicity curves of A6 cells when exposed to a range of concentrations of haloperidol alone or in combination with PS-50 nm nanoplastics (10 mg/L and 10 µg/L) at three time points (24, 48 and 72h).

Table 8. Estimated lethal concentrations to A6 cell line after 24h, 48h and 72h exposure to haloperidol - alone and combined with 10 mg/L and 10 µg/L of PS-50 nm. LCs were calculated through interpolation of a nonlinear regression with a four-parameter dose-response curve. Values missing indicate that LCs were out of the curve range.

A6 MTT		Haloperidol	Haloperidol + PS-50 10 mg/L	Haloperidol + PS-50 10 µg/L
24h	LC_{10}	-	1.04 (1.00 – 1.91)	-
	LC_{20}	5.56 (0.00 - 13.6)	2.22 (1.40 – 3.34)	0.721 (0.216 – 1.15)
	LC_{50}	19.0 (12.3 – 27.9)	7.91 (5.99 – 10.7)	5.92 (4.79 – 7.24)
48h	LC_{10}	-	3.22 (2.40 – 4.16)	-
	LC_{20}	4.63 (3.62 – 5.81)	4.37 (3.64 – 5.10)	2.92 (2.11 – 3.82)
	LC_{50}	8.15 (7.12 – 9.35)	6.85 (6.19 – 7.64)	6.31 (5.64 – 7.01)
72h	LC_{10}	-	5.63 (3.92 – 6.15)	4.92 (3.56 – 5.85)
	LC_{20}	3.83 (2.49 – 5.27)	5.94 (4.14 – 6.25)	5.41 (3.97 – 6.01)
	LC_{50}	5.92 (4.63 – 6.89)	6.78 (6.33 – 10.0)	6.57 (5.08 – 8.98)

Discussion

This study aimed to increase the understanding of the potential effects of pharmaceuticals released into the aquatic environment on amphibians and to assess the sensitivity of different life stages of two species already used in ecotoxicity studies. Behaviour endpoints demonstrated a high sensitivity to the pharmaceuticals. Tadpoles and embryos from both species exposed to propranolol presented a reduction of spontaneous movements and eventually total lack of movement when a stimulus was applied. A reduction of movement has been previously reported in zebrafish embryos exposed to propranolol (at a range of concentrations between 5.07 and 40.6 mg/L) in a 4-day test (Fraysse et al., 2006). This is a significant effect, as under natural conditions, larvae and tadpoles that are not capable of response to external stimuli are more likely to be consumed by predators (Yu et al., 2013).

In this study, the ability of propranolol to induce edemas in embryos of *X. laevis*, mainly at the heart and proctodaeu areas was demonstrated. These findings are in line with other studies concerning effects of propranolol in the embryos of other species. Fraysse et al. (2006) reported that approximately 40% of zebrafish embryos exposed to 0.8 µM of propranolol developed a pericardial edema and presented a weak pigmentation. In *X. laevis* embryos (stage 10-11) exposed to 100 µM propranolol, Sullivan et al. (2016) reported that all organisms presented a miscoiled gut, a rectangular face and 90% of the embryos were hyperpigmented. As a result, it is possible to say that propranolol exposure of aquatic life stages can lead to severe effects in the development of embryos which may eventually impair their fitness, and furthermore, affect swimming ability, predator avoidance, feeding and food digestion (Yu et al., 2013). In the *X. laevis* tadpoles, the percentage of malformations present was very low (highest percentage was 13.3%) and all were haemorrhage. In the case of *P. perezi* tadpoles, although the percentage of malformations was higher, the type of malformation was the same. This seems to indicate that embryos were more prone to the development of malformations and higher variety of malformations than tadpoles when exposed to propranolol.

In the *in vitro* assays, the propranolol LC_{50} derived for A6 cell line, at the three time points were 24.1, 18.2 and 12.4 mg/L, for 24, 48 and 72h respectively, revealing an increase of toxicity with exposure duration. A similar pattern was observed for XTC-2 cell line, with propranolol LC_{50} values of 27.3, 22.8 and 19.8 for 24h, 48h and 72h respectively. The obtained data agrees with Zhou et al. (2016) study that reported decreased cell viability, in a concentration and time manner in A375 melanoma cell line. Overall, based on LC_{20} and LC_{50} values, data suggest a similar sensitivity of XTC-2 and A6 cell lines to propranolol. The data shows

that these cell lines are more sensitive than *Sparus aurata* fin cells lines (SAF-1) but less sensitive than *Dicentrarchus labrax* brain cell lines (DLB-1), that had 24h LC_{50} of 0.198 g/L and 0.002 g/L, respectively (Almeida et al., 2019). These values support the need to perform an adequate validation of the sensitivity of the tested models based on the type of xenobiotics tested.

Exposure of tadpoles to haloperidol revealed that this pharmaceutical can cause mortality at high concentrations and sub-lethal effects at lower concentrations. The haloperidol LC_{50} estimated for *X. laevis* and *P. perezi* tadpoles were in the same order of magnitude (1.45 mg/L for *X. laevis* and 2.20 mg/L for *P. perezi*), suggesting a similar sensitivity of the two species to this chemical. When comparing these results with those obtained for propranolol, where *X. laevis* revealed a higher sensitivity than *P. perezi*, it is perceivable that there is no clear pattern of sensitivity of *X. laevis* relatively to other amphibian species, as it varies with the tested chemical. This conclusion has already been reported in the scientific literature. For example, Yu et al. (2013), that compared the lethal and sublethal sensitivity of *X. laevis* to three types of pesticides with that of other seven anuran species found that *X. laevis* was more sensitive to pyrethroids and organophosphorus insecticides but were more tolerant to organochlorine insecticides.

In *X. laevis* tadpoles, the number of malformations observed after exposure to haloperidol was relatively low, with the highest percentage of malformations was found in organisms exposed to 0.606 and 0.910 mg/L, in around 40% of the tadpoles with most malformations being haemorrhages. The ability of this pharmaceutical to induce morphological malformations has already been reported for other species. Lin et al. (2019) reported that zebrafish embryos exposure to haloperidol (in a range between 10^{-7} to 10^{-3} µg/L) caused severe morphological malformations, with a common malformation being pericardium edema. Giacomini et al. (2006) determined that 9 µM haloperidol caused movement alterations (e.g., reduced swimming speed and increase of erratic movements). This last sub-lethal effect has also been reported as a side effect in humans taking this drug treatment. In the present study, a trend for decreased movement was present, as tadpoles were only able to move when a stimulus was applied at the higher concentrations.

A literature review did not yield any studies addressing the *in vitro* effects of haloperidol on aquatic species cell lines. Thus, this study provides relevant information for researchers addressing the ecotoxicity effects of haloperidol. In A6 cell line, increased toxicity was observed with exposure period increase with LC_{50} values of 19.0, 8.15 and 5.92 mg/L after 24, 48 and 72h, respectively. A similar pattern was observed in XTC-2 cell line, with LC_{50} values 22.1, 16.5 and

13.2 mg/L, from each time point. Although no considerable differences were found between LC_{50} values of the cell lines, XTC-2 appears to have a lower sensitivity to haloperidol. The difference in sensitivity can be associated with the type of cell and source tissue. In this sense, A6 cell line, being a kidney-derived epithelial cell line can present a higher sensitivity associated with a higher metabolic rate (Bhargava et al., 2017), that can help to metabolize xenobiotics more effectively and generate reactive metabolites.

Overall, the obtained data revealed that, for both pharmaceuticals, the lethal concentrations in the *in vivo* assays were in the milligrams per litre range. These concentrations are well above the concentrations reported in the aquatic environment, usually in the ng/L range. The data supports the idea that, under environmental conditions, the acute exposure impact is low. However, even in lower concentrations, effects on the development such as malformations, variations of length and weight could be observed, under combined exposures with other toxic contaminants and/or stressful situations associated with climate change and loss of habitat may impact amphibian population. In this sense, long-terms effects and combined exposure to other environmental contaminants should be addressed.

Among the emerging contaminants raising more concern in the aquatic environment are the micro(nano)plastics. In this study, the effects of polystyrene nanoplastics were tested as this polymer is among the most produced (PlasticsEurope, 2020) and detected in the environment (de Sá et al., 2018), with evidence of formation of nanoplastics as a result of plastic degradation (Wagner et al., 2014). In the *in vivo* assays*, P. perezi* tadpoles exposure to nanoplastics caused no mortality nor significant alterations in the sub-lethal parameters analysed, although its presence in the gut was clear (after visual inspection under a binocular microscope). In *X. laevis* tadpoles, the exposure to nanoplastics caused no significant mortality when compared to the control. While in the *P. perezi* the ingestion of the nanoparticles was clear, in *X. laevis* this presence was not obvious as in the end of the assay, most tadpoles were in the NF stage 48 and show a shining gold-coloured abdomen. This feature does not allow the definitive conclusion of the ingestion by the tadpoles without further tests, although a small difference in the colouring seemed to be evident.

This data supports the idea that polystyrene nanoplastics, without any additives or adsorbed contaminants present low danger in the development of amphibian early life stages. These finding are, however, not in agreement with the study of Tussellino et al. (2015). These authors reported that 50 nm polystyrene nanoplastics caused in *X. laevis* embryos mortality related to concentration exposed - 4.5, 9 and 18 mg/L, as well as malformations (e.g., alterations

of pigmentation distribution, edemas, malformations on the head, gut and tail). The same study reported that in embryos exposed by contact, mortality was inversely related to the concentration, with 4.5 mg/L having a higher mortality (52.8%) than 18 mg/L (18.2%). This decrease in concentration may be associated with the behaviour of nanoplastics that, at high concentrations may form aggregates and become less available for the organisms. In the same species, bigger plastic particles (3 µm) also did not elicit sub-lethal effects (e.g., on the body growth and swimming activity) in *X. laevis* tadpoles in concentrations up to 12.5 mg/L, despite the reported presence in the digestive tract of tadpoles at the highest (De Felice et al., 2018). Thus, characteristics of the tested particles (e.g., surface charge and/or presence of chemical residues of synthesis) may have been responsible for the differences between the present study and the study of Tussellino et al. (2015). When *X. laevis* embryos were exposed to three concentrations (ranging from 1 to 1000 µg/L) of PMMA nanoplastics, no effects were observed on mortality and malformations but at the highest concentration of the nanoplastics the body length was lower (Venâncio et al., 2022). In the same study, tadpoles exposed to the same concentrations of PMMA showed effects on body weight and body length were observed as well as malformation in the abdominal region, the externalization of the gut, at the concentration of 1000 µg/L of the nanoplastics. No effects on survival and feeding rate occurred in the tadpoles. In the *in vitro* assays, as observed *in vivo*, PS-50 nm nanoplastics presented limited toxicity, for A6 and XTC-2. For A6 cell line, only LC_{10} was interpolated for 24h - 0.036 mg/L whereas for XTC-2 only LC_{10} for 72h was calculated - 1.101 mg/L. The low toxicity of PS-50 nm nanoplastics *in vitro* was also reported by Almeida et al. (2019) that tested the effects of 100 nm particles in a concentration range from 0.001 to 10 mg/L in fish cell lines with no significant differences in terms of cell viability after 24h, despite the potential to alter antioxidant status. Future studies with amphibian cell lines should also consider the alteration of biochemical parameters as an endpoint to assess potential effects of xenobiotics.

Although the tested nanoplastics demonstrated a low risk to amphibians in individual exposures, in the combined exposure with propranolol, they caused significant effects on the survival of the *P. perezi* tadpoles. The LC_{50} obtained for propranolol in the individual exposure, when combined with 10 µg/L of PS nanoplastics caused 100% mortality, but when combined with a higher nanoplastic concentration, 10 mg/L, mortality rate was 67%. These results are in line with data reported by Tussellino et al., (2015), showing that in higher concentrations, aggregation of the nanoparticles may occur making it more difficult to enter the tadpole organism, whereas at lower concentrations nanoplastics are more dispersed in the medium allowing it to be more easily consumed. The observed effects may be related with a potential

"Trojan horse" effect of nanoplastics that may have increased the incorporation of the drug by the organisms. *In vitro*, in the A6 cell line, propranolol mixture with PS-50 nanoplastics had similar lethal concentrations for both plastic concentrations. The biggest difference was observed after 48h exposure, when the LC_{50} was 18.1 mg/L in the combination with 10 mg/L and 11.7 mg/L with 10 µg/L. *In vitro* data also support the idea of increased toxicity of propranolol in the presence of plastics.

The results from the combined *in vivo* exposures of haloperidol and nanoplastics is in agreement with the data for propranolol. When *X. laevis* was exposed to haloperidol combined with PS-50 10 mg/L, it caused the same mortality rate as the single exposure of the same pharmaceutical (33.3%). However, when haloperidol was exposed with a lower concentration of the nanoplastics the mortality rate increase to 53.3%. For *P. perezi,* the mixture of haloperidol with 10 µg/L PS nanoplastics a 100% of mortality was observed, whereas in the mixture with 10 mg/L lethality decreased considerably, with only 7% of mortality observed. The importance of plastic concentration was thus clearer in the exposure with haloperidol with highest concentration of plastics retaining the drug making it less available.

In vitro, in the A6 cell line, the combined exposures after 24h resulted in decreased LC_{50} for both plastic concentrations (haloperidol single exposure – 19.0; haloperidol + PS 10 mg/L - 7.91 and Hal + PS 10 µg/L - 5.92 mg/L) however after 72h a slight increase was found (haloperidol single exposure – 5.92; haloperidol and PS 10 mg/L – 6.78 and haloperidol and PS 10 µg/L – 6.57). In the XTC-2 cell lines, the combined exposures of haloperidol and the two different concentrations of nanoplastics resulted in an increase of LC_{50}, which does not support the finding for propranolol, suggesting drug specific interaction of the nanoplastics. Although the difference between the plastic concentrations tested is low, 10 µg/L elicited a higher LC_{50} than the 10 mg/L. Overall, A6 appears more sensitive to haloperidol than XTC-2.

Overall, the data from the present study demonstrate the difficulty in transposing data from *in vitro* studies to *in vivo*. Lethal concentrations calculated from the *in vivo* assays were in general lower than for those from *in vitro* assays, leading to conclude that cell lines tend to be more resistant than the tadpoles. Several factors may be responsible for these differences such as the ability to metabolize substances (that are cell specific) and potential protective role of serum against toxic effects, as reported for nanoparticles. In this sense, future studies could explore the role of serum in minimizing the effects of these substance and try to optimize the minimum amount of serum required to maintain cells viable.

Conclusion

The present work aimed to assess the effects of two pharmaceuticals and polystyrene nanoplastics, alone and in combined exposures, in early life stages of amphibian. Furthermore, the suitability of *in vitro* assays to act as a substitute for the *in vivo* assays was also evaluated.

Overall, the obtained *in vivo* data revealed that these pharmaceuticals alone may have little effect on amphibian population as the concentrations eliciting effects were considerably higher than those reported in the aquatic environment. The same conclusions apply for 50 nm PS nanoplastics that showed low toxicity in individual exposures. However, their presence can significantly alter the toxicity of other environmental contaminants like the pharmaceuticals tested in this study. Nanoplastics demonstrated a potential "Trojan horse" effect for these contaminants with a high potential to increase the toxicity at lower concentrations.

The *in vitro* studies confirmed the ability of nanoplastics to modulate the effects of the tested drugs. However, the LC values obtained *in vitro* are of a different order of magnitude than those obtained *in vivo*. Additional endpoints should be studied, such as oxidative stress and energetic metabolism to understand the mechanism associated with each contaminant. Furthermore, other chemicals should be tested *in vitro* and *in vivo* in order to establish assessment factors when extrapolating *in vitro* to *in vivo* data to determine environmentally safe concentrations for amphibians.

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