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Comparative study of diclofenac-induced embryotoxicity and teratogenesis in *Xenopus laevis* and *Lithobates catesbeianus*, using the frog embryo teratogenesis assay: *Xenopus* (FETAX)

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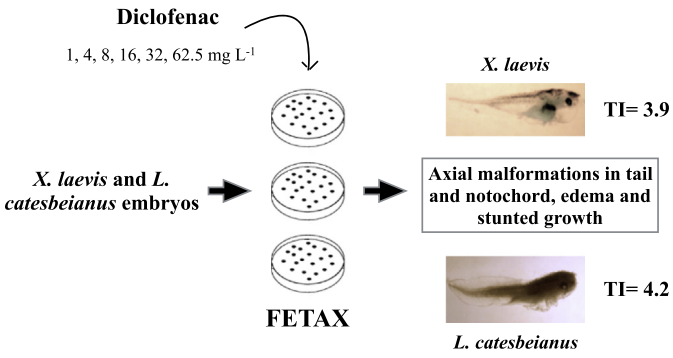
HIGHLIGHTS

- *L. catesbeianus* is marketed as a nutritional meat source in Mexico
- Sensitivity to diclofenac exposure was compared in *X. laevis* and L. *catesbeianus*
- Diclofenac induced embryotoxicity and teratogenesis on L. *catesbeianus* and *X. laevis*
- Axial malformations, edema, and growth inhibition were induced in both species
- *L. catesbeianus* embryos are more sensitive to diclofenac exposure than *X. laevis*

GRAPHICAL ABSTRACT



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ABSTRACT

Water is an increasingly deteriorated, limited natural resource due to population increase and industrialization. Also, the widespread use of pharmaceuticals in modern society leads to their presence in domestic, hospital and industrial effluents. Due to their analgesic properties, some of the most commonly used pharmaceuticals are non-steroidal anti-inflammatory drugs (NSAIDs). High concentrations of one these products, diclofenac (DCF), have been detected in effluents and water bodies of different countries, including Mexico. Diverse studies show that trace amounts (ng L^{-1} to μg L^{-1}) of this compound induce toxicity on aquatic organisms such as algae, microcrustaceans and fish. However, studies on its potential toxicity during development in species of commercial interest such as the American bullfrog *Lithobates catesbeianus* are scarce. The present study aimed to evaluate DCF-induced teratogenesis and embryotoxicity in *Xenopus laevis* and L. *catesbeianus*, a species marketed as a nutritional meat source in Mexico, using the frog embryo teratogenesis assay: *Xenopus* (FETAX). Oocytes in mid-blastula transition were exposed for 96 h to 1, 4, 8, 16, 32 and 62.5 mg DCF L^{-1}. The criteria evaluated were mortality, malformation and growth inhibition. The teratogenic index was 4.2 in L. *catesbeianus*, three-

Abbreviations: DCF, diclofenac; $EC_{50\ (malformation)}$, effective concentration inducing 50% malformation; FETAX, frog embryo teratogenesis assay: *Xenopus*; LC_{50}, median lethal concentration; MBT, mid-blastula transition; NSAID, nonsteroidal anti-inflammatory drugs; ROS, reactive oxygen species; TI, teratogenic index.

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fold higher than the reference limit (1.5), and 3.9 in *X. laevis*. Diclofenac induced diverse malformations in both species, the most frequent of these being axial malformations in the tail and notochord, edema and stunted growth. Results indicate that DCF is a potentially teratogenic compound and is toxic during development in *X. laevis* and L. *catesbeianus*, a species which, due to its sensitivity, can be used to evaluate the toxicity of pharmaceutical products, using FETAX.

1. Introduction

Water is a limited natural resource essential for life and environmental sustainment, which, as a result of rapid social and economic development, has undergone inadequate use and an alarming deterioration (Barceló and De Alda, 2008). In recent years, emerging contaminants (or microcontaminants) have aroused notable interest. These compounds are of diverse origin and chemical nature, and their presence and consequences have until recently gone unnoticed. They are present in water at low concentrations (ng L^{-1} to μg L^{-1}) and are considered harmful to human health and the environment since they can elicit diverse effects in living organisms, such as chronic toxicity, endocrine disruption, and bioaccumulation (VIrkutyte et al., 2010). These compounds have been termed "emerging" because they are still not environmentally regulated or are only now being subjected to regulation (Barceló and De Alda, 2008). A further particularity with regard to this type of contaminants is that, since they are continuously released into the environment due to high production and consumption levels, they need not be persistent to occur environmentally and induce deleterious effects on organisms (Petrović et al., 2003). Among emerging contaminants, those arousing the most concern and study in recent years are pharmaceutical products, nonsteroidal anti-inflammatory drugs (NSAIDs) being the most frequently detected ones in surface water (Buser et al., 1998; Corcoran et al., 2010). Diverse studies state that NSAIDs are a heterogeneous group of medications that are among the most commonly prescribed analgesics, anti-inflammatory agents and antipyretics, and include acetylsalicylic acid, acetaminophen, ibuprofen, diclofenac (DCF) and naproxen. DCF is consumed in the hundreds of tons annually (Buser et al., 1998) and, while non-persistent by reason of its physicochemical properties, due to its continuous release in wastewater discharges it is frequently detected in the environment, inducing diverse potentially negative effects on exposed organisms (Islas-Flores et al., 2013; Oviedo-Gómez et al., 2010; Saucedo-Vence et al., 2015).

NSAIDs are found at higher concentrations in the environment (ng L^{-1} to μg L^{-1}) than other pharmaceuticals (Sim et al., 2010) and environmental analyses of their presence have been carried out in diverse countries and are summarized in various reports (e.g. Richardson, 2007; Palmer et al., 2008; Wang et al., 2011). Their environmental presence is due not only to excreta, through which a significant part of the medication is eliminated from the body without being metabolized, it is also the result of manufacturing processes and the inadequate disposal of residues of these products (Boxall, 2004), not omitting their veterinary, agricultural, livestock and poultry industry use which has continued to grow in recent years (Patiño Menéndez et al., 2014).

Diclofenac is a widely used pharmaceutical in various countries (Petrovic et al., 2008). It is the first-choice anti-inflammatory agent in 74 of 100 countries evaluated by McGettigan and Henry (2013). In Mexico, it is listed in the basic schedule of medications of the public health sector for the treatment of ophthalmological, rheumatological and trauma-related disorders, and has been ranked fourth in total consumption in a family medicine center in the State of Mexico (Gómez-Oliván et al., 2009). This pharmaceutical acts by reversible or irreversible inhibition of one or both isoforms of the enzyme cyclooxygenase (COX-1 and COX-2) which catalyzes the synthesis of diverse prostaglandins (Morrow and Roberts, 2001). These substances are involved in processes such as neurotransmission, ion transport across cell membranes, pain, inflammation, fever, sleep regulation, allergic reactions, muscle contraction, bronchoconstriction, circulatory system regulation and platelet aggregation (Arkhipova et al., 2005; Cha et al., 2006).

Concern about the potential environmental toxicity of DCF emerged some ten years ago with reports linking declining vulture populations in India with veterinary use of this medication (Oaks et al., 2004). Since then, it has been detected around the world at concentrations in the ng L^{-1} to μg L^{-1} range (Lonappan et al., 2016; Nebot et al., 2015; Pereira et al., 2015; Samaras et al., 2013; Tran et al., 2014; Yu et al., 2013). In Mexico only a small number of studies have been carried out in water bodies; those bodies in which DCF has been detected include: the Mezquital Valley irrigation system and the Tula Valley in the state of Hidalgo, with concentrations of 0.25–0.50 μg L^{-1} and 2.052–4.824 μg L^{-1} respectively (Gibson et al., 2010; Siemens et al., 2008); as well as the Lerma-Cutzamala water supply system, with 0.001 μg L^{-1} and 0.028–0.032 μg L^{-1} in ground and surface water respectively (Félix–Cañedo et al., 2013), and Madín Dam with 0.20–031 μg L^{-1} (González-González et al., 2014), both in the State of Mexico. In the latter state, DCF has also been found in industrial effluent from an NSAID manufacturing plant (104.63 μg L^{-1}) and in hospital wastewater (0.0065 μg L^{-1}) (Neri-Cruz et al., 2015; SanJuan-Reyes et al., 2015). As regards DCF-induced toxicity in aquatic organisms, acute toxicity assays show that phytoplankton, with a 96-h median lethal concentration (LC_{50}) of 14.5 mg L^{-1}, is more sensitive to DCF than zooplankton (96-h LC_{50} = 22.43 mg L^{-1}) (Ferrari et al., 2003). In *Oncorhynchus mykiss*, 28 days of exposure to 5 μg DCF L^{-1} induced chronic histopathologic effects such as renal lesions (degeneration of tubular epithelium, interstitial nephritis), while exposure to 1 μg DCF L^{-1} induced gill and subtle subcellular changes such as severe protein accumulation in tubular cells, macrophage infiltration, and structural alterations (dilation, vesiculation) of the endoplasmic reticulum in proximal and distal renal tubules (Schwaiger et al., 2004; Triebskorn et al., 2004). This pharmaceutical has also been shown to induce oxidative stress, and cyto- and genotoxicity in diverse aquatic organisms such as *Hyalella azteca, Daphnia magna* and *Cyprinus carpio* (Gómez-Oliván et al., 2014; Islas-Flores et al., 2013; Oviedo-Gómez et al., 2010). Furthermore, a log K_{ow} of 4.02 to 4.51 has been reported for DCF (Syracuse Science Center, 2002), and its bioaccumulation has been demonstrated in different compartments such as blood plasma, bile, liver, kidney, gills and muscle (Lahti et al., 2011; Mehinto et al., 2010; Kallio et al., 2010; Schwaiger et al., 2004; Saucedo-Vence et al., 2015) and may contribute to the risk of toxicity from exposure to this pharmaceutical in diverse aquatic organisms as it travels through the food chain.

In the past 25 years a dramatic decline in amphibian populations has occurred in many parts of the world, so much so that amphibians are now considered more threatened than mammals or birds (Beebee and Griffiths, 2005). This may be due to their exposure to hazardous levels of contaminants, which are usually present at higher concentrations in irrigation channels, ponds and swamps than in larger water bodies (Tejedo, 2003). As a result, frogs are considered valuable bioindicators, capable of integrating changes in aquatic and terrestrial habitats (Beiswenger, 1988). Ecotoxicological studies reveal that, unlike fish or macroinvertebrates, many amphibian species are particularly sensitive to chemical stress (Birge et al., 2000). Additionally, in the adult stage they feed on invertebrates and are themselves preyed upon by higher

vertebrates, constituting an important link in the aquatic and terrestrial food chains (Sparling et al., 2000). Furthermore, from an evolutionary viewpoint, amphibians are a "connecting" class between mammals and vertebrates of more ancient origin (cartilaginous and bony fish), and certain amphibians have been widely used as experimental models in the study of developmental alterations, genetics, molecular biology and so on, providing large amounts of data for understanding the action and biological mechanisms induced by chemical attack (Ankley et al., 2004; Robert and Ohta, 2009), and are consequently considered bioindicator species in toxicity studies.

The frog embryo teratogenesis assay: *Xenopus* (FETAX) is a 96-h acute toxicity assay determining lethality, morphologic alterations and minimum growth-inhibitory concentration. It has been used in over 100 studies focused on assessment of physical substances, chemicals, mixtures and environmental water and soil samples (Boga et al., 2008; Brausch et al., 2010; Bruner et al., 2002; Hoke and Ankley, 2005; Prati et al., 2000). This assay uses the African clawed frog *X. laevis* due to its suitable biological characteristics for captive breeding (short reproductive cycle and fully aquatic habitat) and the fact that it is the most extensively studied and best known research amphibian (Robert and Ohta, 2009). Also, the characterization of its embryogenesis has been documented at the molecular and cellular levels, providing a reliable description of the different stages in its embryonic development (Nieuwkoop and Faber, 1956), plus there is a growing number of studies as a result of "modification" of the FETAX protocol in terms of the species used, most of them focusing on comparing *X. laevis* with endemic species (Fort et al., 2006; Mann and Bidwell, 2000).

The American bullfrog *Lithobates catesbeianus* (Ranidae) (Lutz and Avery, 1999), a species native to the northeastern US, has been introduced in Central and South America as a promissory species for production of animal protein. In 1993, it was introduced by the Agriculture and Livestock Department of the State of Mexico in the La Paz aquaculture center (Villa Guerrero, State of Mexico) for its production and commercialization. This center supplies animals to frog farms in the states of Mexico, Yucatán, Michoacán, Tamaulipas, San Luis Potosí and Jalisco (Andreu et al., 2001) where this species is marketed as a nutritional meat source, making it vulnerable to contaminants within its environment. While the species has been tested previously in a few studies using FETAX, toxicological studies are needed to characterize the environmental impact of DCF and to this end the use of bioindicator species that are consumed as meat sources, such as L. *catesbeianus*, is essential. Therefore, the aim of the present study was to evaluate DCF-induced teratogenesis and embryotoxicity during development in L. *catesbeianus*, a species of commercial interest in Mexico, using FETAX to compare it with *X. laevis*, as well as to obtain reliable data to evaluate the potential toxicity of this pharmaceutical product and determine the environmental impact of its presence in Mexican water bodies.

2. Materials and methods

2.1. Test specimens

Male and female adult L. *catesbeianus* frogs (7–15 cm in length from mouth to tail, with a mean weight of 250 and 320 ± 30 g respectively, and aged 2–3 years) were obtained from the La Paz aquaculture center (Villa Guerrero, State of Mexico). Males and females were maintained separately in 120-L aquaria containing 22 °C water, aquatic vegetation and access to a dry area, under simulated natural conditions of humidity (70–90%), a 12/12 h light/dark photoperiod and 23 ± 2 °C ambient temperature, and were fed Purina Nutripec® 4210 daily.

X. laevis frogs (males 7.5–10, females 10–12.5 cm long, weighing 50–60 and 150–200 ± 10 g respectively and aged 2–3 years) were obtained from the Aquanimals center in the state of Querétaro. Males and females were maintained separately in 120-L aquaria at 21 ± 3 °C temperature with a natural 12/12 h dark/light photopheriod, and were fed Purina CamaronEX® daily.

2.2. Test substance

Diclofenac (CAS # 1307-86-5, >99% purity) $C_{14}H_{11}Cl_2NO_2$, 296 Da, was obtained from Sigma-Aldrich (St Louis). The stock solution was prepared daily by dissolving 1 g DCF in FETAX solution.

2.3. Fertilization

Specimens of L. *catesbeianus* were made to fast for 24 h prior to fertilization. Such fasting is recommended to avoid contamination of the sperm sample with urine and/or feces, and favors adequate oviposition in artificial fertilization (Dreanno et al., 1998; Catcoparco et al., 2012; Poole and Grow, 2008). The fertilization method described by Afonso (2004) was used. Females were injected 1 mL buserelin acetate (Conceptal®, Intervet-Mexico) intraperitoneally and were subsequently placed together with the males in polyurethane boxes (0.60 × 0.40 × 0.12 m) containing 1 L FETAX solution (neutral pH, 24 ± 1 °C). A second hormone administration was performed 10 h after the first, at which time the water was replaced. Oocyte collection was made 26–30 h after the first hormone administration, by lateral compression at oviduct height in the anteroposterior direction. A clean, dry 8-L polyurethane box was used to collect oocytes.

Semen was collected 24 h after the first hormone administration in females, by inducing spermatogenesis in males via intraperitoneal injection of 0.1 mL buserelin acetate. After 2 h, collection was made by inserting a 1-mL pipette in the male cloaca and storing the semen in a graduated test tube. Oocyte fertilization was done by diluting this semen with 50 mL water (pH 6.8–7.4) and pouring it over the oocyte mass, shaking manually for about 2 min. Oocytes were incubated at 24 ± 1 °C until mid-blastula transition (MBT) was attained.

To induce natural ovulation and oocyte fertilization in *X. laevis*, 100 IU human chorionic gonadotropin (HCG, CHORAGON®, Ferring) was injected into the dorsal lymph sac of the female, which was then placed alone in a 40-L fish tank and provided with food. Five days after this first administration and one night prior to the experiment, females were injected 600 IU and males 100 IU HCG (this being the only induction in the male). Males and females were now placed together in the fish tank, where oocyte fertilization took place naturally immediately after laying. Oocytes were examined in a stereoscopic microscope to select fertilized specimens for analysis.

2.4. Evaluation of teratogenesis

Evaluation was performed in accordance with the procedures in the standard guide of the American Society for Testing Materials (ASTM, 2004). The same experimental conditions were used for both species.

The FETAX solution was prepared with 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$ and 75 mg $MgSO_4$ per 1 L deionized water. All reagents were purchased from Sigma-Aldrich (St Louis). The final pH of the solution was 7.6–7.9.

Under a microbiology laminar flow hood, 20 embryos in MBT were placed in 60-mm Petri dishes with 10 mL of FETAX solution and nominal concentrations of 1, 4, 8, 16, 32 or 62.5 mg DCF L^{-1} made by dilution of the stock solution (1000 mg L^{-1}). A DCF-free control group was also set up; the assay was performed in triplicate ($N = 60$ embryos per test concentration). Embryos were incubated at 22 ± 2 °C until the end of the assay. The medium was replaced daily and dead embryos were removed. The FETAX exposure time was 96 h. If 90% of control specimens had not reached stage 46 by 96 h, the assay was extended an additional 3 h to allow them to reach this stage.

At the end of the experiment, the LC_{50} was estimated. To obtain the 96-h LC_{50} and $EC_{50\ (malformation)}$ and their respective 95% confidence limits, the Probit method in the EPA v1.5 program (USEPA, 1985) was used to construct dose-response curves. Embryos were euthanized by placing them in a Petri dish with 0.06% MS-222 solution (lethal dose), and were then fixed in 3% formaldehyde for subsequent observation

of malformations, which were identified using the *Atlas of Abnormalities* (Bantle et al., 1991). Head-to-tail measurements were taken to estimate growth inhibition, and malformation was evaluated with the help of a stereoscope. Results were expressed as teratogenic index (TI) values [TI = $LC_{50}/EC_{50\ (malformation)}$], where $EC_{50\ (malformation)}$ is the effective concentration inducing 50% malformation, to determine whether the compound was toxic (TI ≥ 1.5).

2.5. Ecological risk assessment of diclofenac

An ecological risk assessment of DCF was made in both species using the Barnthouse et al. (1982) method, considering the following formula: RQ (risk quotient) = Environmental concentration/LC_{50}. To this end, the highest environmental concentration reported in studies carried out in Mexico (cited in the Introduction) and the LC_{50} values for both frog species obtained in the present study were used. The RQ values obtained were categorized as follows: RQ < 0.1 no adverse effects, 0.1 < RQ < 10 possible adverse effects, and RQ > 10 probable adverse effects.

All procedures were performed in accordance with ethical protocols for the maintenance, use and handling of test animals approved for use in the Universidad Autónoma del Estado de México (México). Provisions in the pertinent official Mexican norm were also taken into account (NOM-062-ZOO-1999, 1999, Technical specifications for the production, care and use of laboratory animals).

2.6. Statistical analysis

To obtain the 96-h LC_{50} and $EC_{50\ (malformation)}$ of the three replicates and their respective 95% confidence limits, the Probit method in the EPA v1.5 program (USEPA, 1985) was used to construct dose-response curves. The X^2 linear adjustment test was not significant at $P < 0.05$. Growth inhibition results were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's test, with P set at <0.05. The SPSS Statistics v22.0 program was used.

3. Results

Table 1 shows the results of FETAX in *X. laevis* and L. *catesbeianus* embryos exposed to DCF, listing the number and percentage of dead and malformed specimens found with each test concentration. The 96-h LC_{50} was 12.11 mg L^{-1} (CI, 3.61–46.06) in L. *catesbeianus* and 9.56 mg L^{-1} (CI, 1.70–38.78) in *X. laevis*. The highest concentration (62.5 mg L^{-1}) induced lethality in most embryos, showing a 95–100% mortality rate in both species.

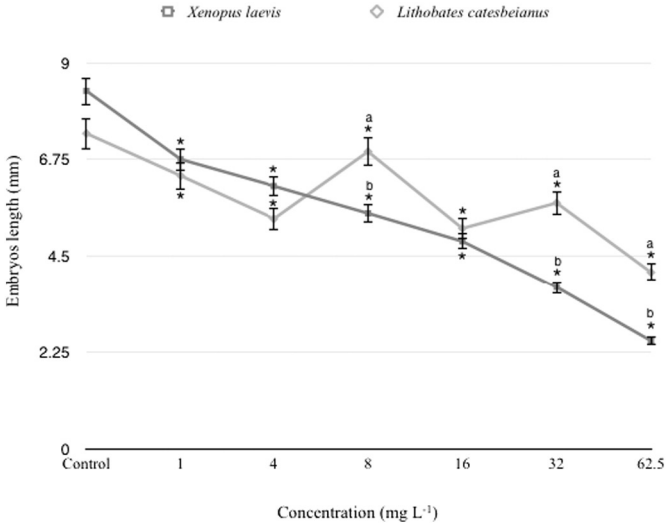

Fig. 1. Relationship between test concentration and mean embryo length as an indicator of growth, obtained in three replicates of *X. laevis* and L. *catesbeianus* exposed to diclofenac for 96 h. *Significantly different from the control group. Lowercase letters indicate a significant difference between [a]*X. laevis* and [b]*L. catesbeianus;* ANOVA and Dunnett's test ($P < 0.05$).

All concentrations induced a statistically significant reduction in embryo size. Fig. 1 shows the relationship between the test concentrations used and mean embryo length as an indicator of growth in *X. laevis* and L. *catesbeianus*. A significant difference relative to the control group was found with all concentrations in both species, with *X. laevis* showing greater size reduction.

3.1. Ecological risk assessment of DCF

As regards the risk quotient of DCF, the RQ values estimated for *X. laevis* and L. *catesbeianus* are shown in Table 2.

Figs. 2 and 3 show representative examples of malformed embryos of *X. laevis* and L. *catesbeianus* after exposure to the various concentrations. The developmental toxicity endpoints assessed include malformations (e.g. external, visceral, skeletal), variations (e.g. hypopigmentation) and growth (e.g. stunted body) which are listed in Fig. 4. Diverse types of malformations were found and increased in severity as the concentration increased. The most common ones were axial malformations in the tail and notochord, abdominal and facial edema, and hypopigmentation. The $EC_{50\ (malformation)}$ was 2.88 mg L^{-1} (CI, 0.927–5.343) in L. *catesbeianus* and 2.74 mg L^{-1} (CI, 0.003–8.405) in *X. laevis*.

Table 1
Results of lethality and teratogenesis in *X. laevis* and L. *catesbeianus* embryos exposed for 96 h to diclofenac.

Concentration (mg L^{-1})	*Xenopus laevis*					*Lithobates catesbeianus*				
	Total number of embryos exposed	Total number of deaths	% mortality	Total number of malformed embryos	% malformation	Total number of embryos exposed	Total number of deaths	% mortality	Total number of malformed embryos	% malformation
0	60	0	0.0	0	0.0	60	0	0.0	0	0.0
1	60	13	21.6	19	31.6	60	11	18.3	16	26.6
4	60	18	30	21	35	60	16	26.6	18	30
8	60	24	40	25	41.6	60	20	33.3	28	46.6
16	60	27	45	30	50	60	24	40	34	56.6
32	60	41	68.3	41	68.3	60	41	68.3	45	75
62.5	60	60	100	60	100	60	57	95	60	100
LC_{50}	9.56 ± 0.8 (CI, 1.70–38.78)					12.10 ± 0.7 (CI, 3.61–46.06)				
$EC_{50\ (malformation)}$	2.74 ± 0.1 (CI, 0.01–8.41)					2.88 ± 0.1 (CI, 0.93–5.34)				
TI	3.5					4.2				

LC_{50}: median lethal concentration; $EC_{50\ (malformation)}$: effective concentration inducing 50% malformation; TI: teratogenic index.

Table 2
Risk quotient (RQ) values in *X. laevis* and *L. catesbeianus* embryos exposed to diclofenac for 96 h.

Mexican effluent type (reference)		Maximum concentration reported ($\mu g\ L^{-1}$)	RQ *X. laevis*	RQ *L. catesbeianus*
Municipal	Mezquital Valley (Siemens et al., 2008)	0.50	523×10^{-7}	413×10^{-7}
	Tula Valley (Gibson et al., 2010)	4.824	5046×10^{-7}	3987×10^{-7}
	Lerma-Cutzamala water supply system (Félix–Cañedo et al., 2013)	0.032	33×10^{-7}	26×10^{-7}
	Madín Dam (González-González et al., 2014)	0.31	324×10^{-7}	256×10^{-7}
Industrial (SanJuan-Reyes et al., 2015)		104.63	$109{,}446 \times 10^{-7}$	$86{,}471 \times 10^{-7}$
Hospital (Neri-Cruz et al., 2015)		0.0065	7×10^{-7}	5×10^{-7}

The TI values estimated after exposure to the different DCF concentrations were 3.5 in *X. laevis* and 4.2 in L. *catesbeianus*; values >1.5 indicate a higher potential of the toxicant to induce malformation (ASTM, 2004).

4. Discussion

The present study aimed to evaluate DCF-induced toxicity during embryonic development in *X. laevis* and L. *catesbeianus*. A study by Chae et al. (2015) reports LC_{50} values for *X. laevis* and *L. catesbeianus* embryos exposed to DCF that are approximately three-fold higher than the ones obtained in our study. This may be due to differences in the number of embryos exposed, since our study used twice as many specimens per test concentration and evaluated one more concentration ($8\ mg\ L^{-1}$). However, as regards other parameters such as malformation, similar results were obtained in both studies, as described below. On the other hand, few studies have evaluated the potential toxicity of other NSAIDs on amphibian development. Veldhoen et al. (2014) report an LC_{50} of $41.5\ mg\ L^{-1}$ in L. *catesbeianus* exposed to ibuprofen while Fort et al. (1992) recorded an LC_{50} of $191.1\ mg\ L^{-1}$ in *X laevis* exposed to acetaminophen. These results are consistent with previous findings in diverse aquatic species, in which DCF was found to have a lower LC_{50} than other NSAIDs and may therefore be considered more toxic (Gómez-Oliván et al., 2014; Islas-Flores et al., 2013). Embryo mortality in our study may be explained by the mechanism of action of DCF, which acts by blocking cyclooxygenase (Morrow and Roberts, 2001). This enzyme catalyzes arachidonic acid degradation in the production of prostaglandins. These eicosanoids act as autocrine and paracrine messengers and as important mediators during reproduction and in the immune system. Prostaglandins are also involved in inflammation, neurotransmission and transport of ions across cell membranes, as well as circulatory system regulation and vascular permeability (Arkhipova et al., 2005; Cha et al., 2006). Along with this, there is a reduction in the levels of leukotrienes, related to cell survival signaling (Öhd et al., 2000). Such DCF-induced biochemical changes may have contributed to embryo mortality in our study since, as Walker and McEldowney (2013) report, DCF in *X. tropicalis* binds to COX-2 via hydrogen bonds to the same amino acid residues as in the human form.

The RQ values estimated for *X. laevis* and L. *catesbeianus* using the equation proposed by Barnthouse et al. (1982) and the concentrations determined in municipal, industrial and hospital effluents in Mexico do not represent a risk for these species in the aforementioned environments. However, these concentrations have been shown to induce toxic responses such as oxidative stress and cyto- and genotoxicity in species such as *H. azteca*, *D. magna* and *C. carpio* (Gómez-Oliván et al., 2014, Islas-Flores et al., 2013, Oviedo-Gómez et al., 2010). Therefore,

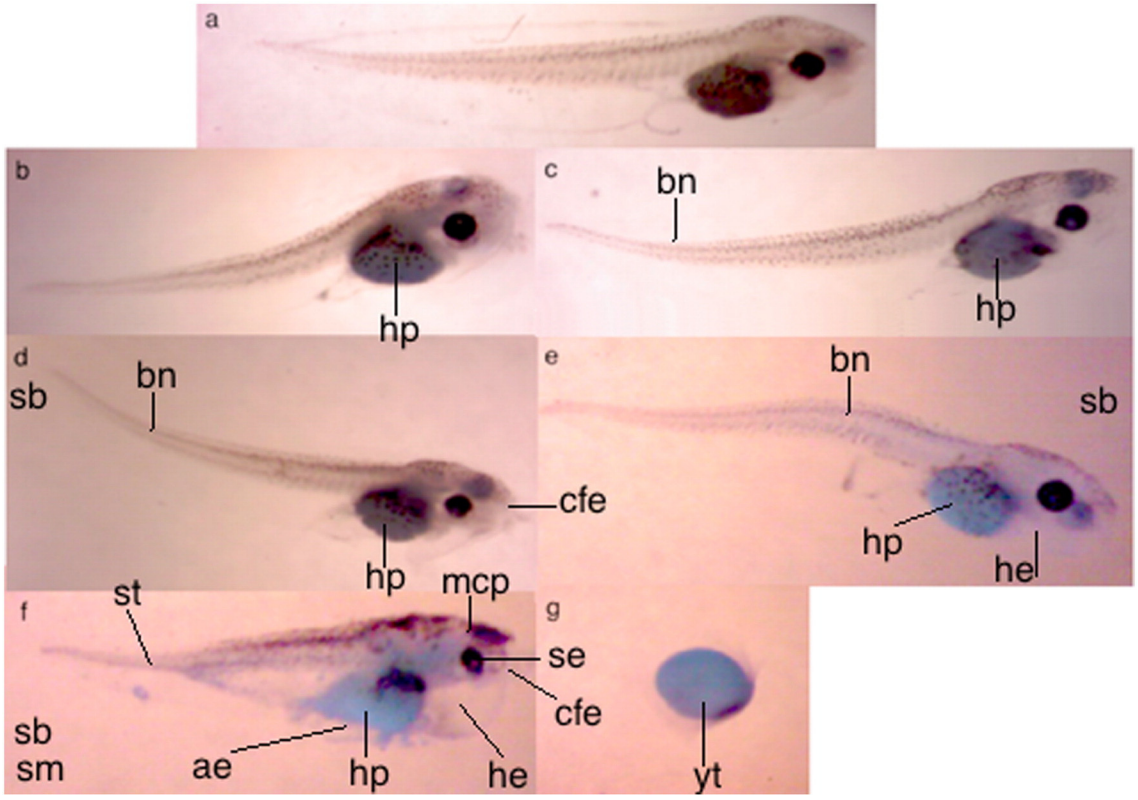

Fig. 2. Representative malformations in *X. laevis* embryos exposed for 96 h to diclofenac at the following concentrations ($mg\ L^{-1}$): (a) control, (b) 1, (c) 4, (d) 8, (e) 16, (f) 32, (g) 62.5. Abbreviations: ae, abdominal edema; bn, bent notochord; cfe, craniofacial edema; he, heart edema; hp., hypopigmentation; mcp, microcephaly; sb, stunted body; se, small eye; sm, severe malformations; st, short tail; yt, yolk tamponade.

concentrations of this magnitude should be tested to determine their potential effect on species evaluated under real conditions, which may represent the chemical, physical and biological processes that take place in the natural environment.

As regards malformation, $EC_{50\ (malformation)}$ values are congruent with the LC_{50} values and indicate that at lower concentrations, DCF induced slightly higher malformation in *X. laevis* embryos ($EC_{(malformation)} = 2.74$) than in L. *catesbeianus* (2.88), the main alterations being axial malformations in the tail and notochord, multiple edema, stunted growth and hypopigmentation (Figs. 2-4). The main DCF-induced malformations observed by Chae et al. (2015) were macrocephaly, microcardia, edema, gastrointestinal abnormalities and stunted growth, findings which are similar to ours (Fig. 4). The main metabolites of biotransformation of DCF are: 5,4-dihydroxydiclofenac, 3′-hydroxydiclofenac, 4′- and 3′-hydroxymethyl diclofenac, and 4′- and 5′-hydroxydiclofenac. The latter two are oxidized by benzoquinone imine intermediates, compounds that are highly toxic to aquatic organisms (Gómez-Oliván et al., 2009). In addition, diverse studies suggest that in the biotransformation of DCF, reactive oxygen species (ROS) and radical cations of DCF are formed (Doi et al., 2002). All these highly reactive species can induce teratogenesis, since they damage cell macromolecules such as DNA, lipids and proteins, also modifying multiple signaling pathways (Roede and Jones, 2010; Wells et al., 2009). Thus, diverse transcription factors are modified such as hypoxia inducible factor (HIF-1), activator protein 1 (AP-1), redox effector factor-1 (Ref-1) and nuclear factor (NF)-E2 related factor 1 (Nrf-1) that influence cell signaling pathways involved in proliferation, differentiation, and apoptosis are modified and, since gene expression during embryonic development is highly specific and conserved, any alteration induced by ROS or radical cations may lead to abnormal development, resulting in teratogenicity (Dennery, 2007).

Teratogenesis is a functional, biochemical or morphologic alteration induced during embryonic development and detected during gestation, at birth or subsequently (Roura and Rodriguez, 2007). Teratogenic compounds often but not always induce growth inhibition at much lower concentrations than those required to induce malformation (Bantle et al., 1989). In the present study, DCF induced stunted growth in embryos of *X. laevis* and L. *catesbeianus* beginning at the lowest concentration (19.07% and 13.37% respectively with 1 mg L^{-1}). Such size reduction was found to be concentration dependent in both species and was greater in *X. laevis* than L. *catesbeianus* (69.94% vs 43.93% respectively with 62.5 mg L^{-1}). Diverse studies using other NSAIDs also found significant growth inhibition (Chae et al., 2015; Fort et al., 1992; Veldhoen et al., 2014). While Singh et al. (2015) found that exposure to DCF in a rat embryo culture reduced overall growth and developmental parameters such as embryo weight, head-to-tail length and number of somites in vitro in a concentration-dependent pattern, and that the mechanism of teratogenicity of DCF may be oxidative stress induction leading to an abrupt increase in apoptosis, while the uncontrolled pattern of this cell death process results in diverse malformations whose severity depends on pharmaceutical concentration. Previous studies have reported DCF induction of oxidative stress and genotoxicity in aquatic organisms

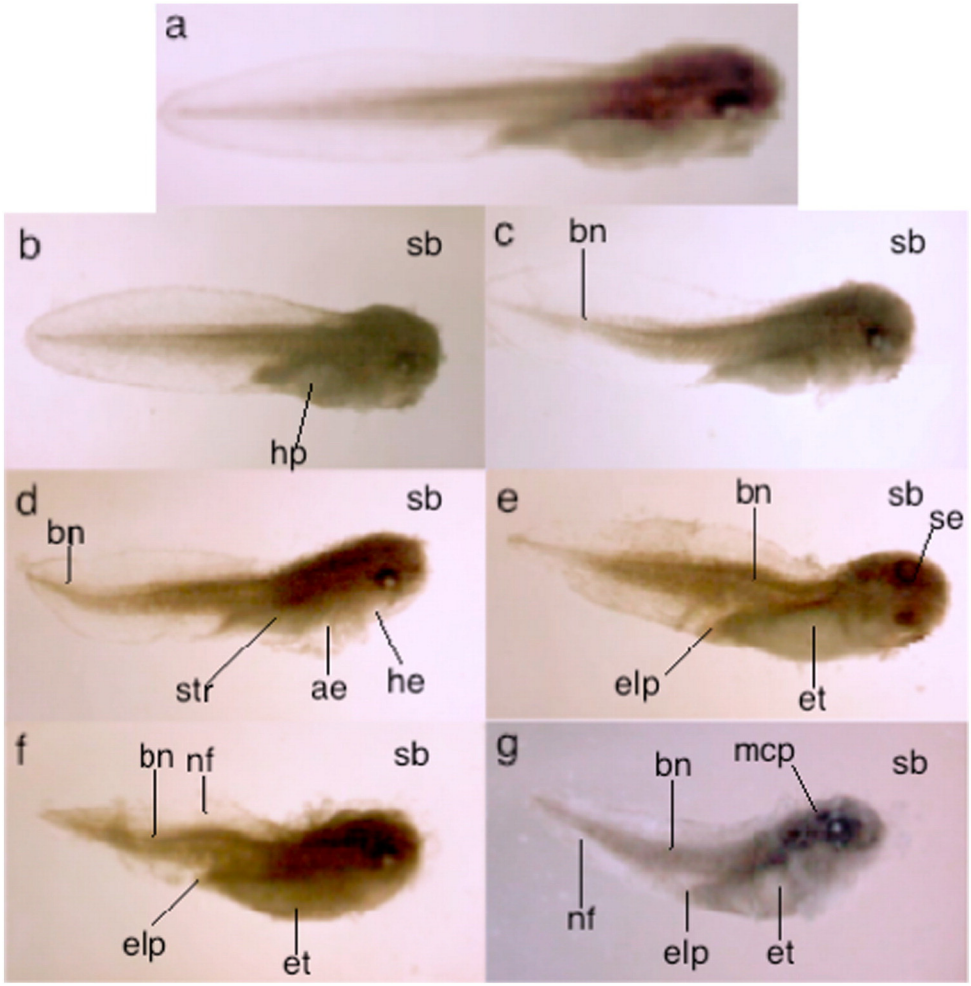

Fig. 3. Representative malformations in L. *catesbeianus* embryos exposed for 96 h to diclofenac at the following concentrations (mg L^{-1}): (a) control, (b) 1, (c) 4, (d) 8, (e) 16, (f) 32, (g) 62.5. Abbreviations: ae, abdominal edema; bn, bent notochord; elp, enlarged proctodeum; et, enlarged trunk; he, heart edema; hp., hypopigmentation; mcp, microcephaly; nf, narrow fin; sb, stunted body; str, stretched trunk.

such as *D. magna*, *H. azteca* and *C. carpio* (Gómez-Oliván et al., 2014; Islas-Flores et al., 2013; Oviedo-Gómez et al., 2010). Oxidative stress, an imbalance between ROS production and antioxidant defense mechanisms of the cell, induces irreversible oxidation of DNA, proteins and lipids, leading to inactivation of multiple enzymes and cell death (Islas-Flores et al., 2013). It can also affect gene expression by interfering with the activity of redox-sensitive transcription factors as well as signal transduction via thiol oxidation (Sahambi and Hales, 2006). During the prenatal period, this can give rise to birth defects and stunted growth and, in severe cases, mortality (Hansen, 2006; Trocino et al., 1995; Wells et al., 1997). Free radicals can also react with polyunsaturated fatty acids to yield electrophilic aldehydes (malondialdehyde) and 4-hydroxy-2-nonelal, which can form adducts with residues of cysteine, lysine and histidine, and affect protein function with possible induction of teratogenesis (Roede and Jones, 2010).

In addition to growth inhibition, teratogenic compounds induce severe malformation, while non-teratogenic compounds usually cause death but not malformation (Bantle et al., 1989). Generally, TI values <1.5 indicate low teratogenic potential, i.e. little or no distance in dose-response curves between concentrations that induce malformation without embryo mortality and those with lethal effects (Fort et al., 1992). In the present study, TI values in *X. laevis* (3.5) and L. *catesbeianus* (4.2) exceeded this reference parameter, indicating a greater distance between malformation and the lethal response and therefore, a higher probability that embryos will be malformed in the absence of mortality. A similar TI value (2.64) was reported by Chae et al. (2015) for DCF in *X. laevis*, while the higher TI values in L. *catesbeianus* found in the latter study as well as ours may indicate a higher risk of malformation during exposure compared to *X. laevis* and that this pharmaceutical is teratogenic to both species. These results may be explained by the fact that *X. laevis* has a higher surface-to-volume ratio than L. *catesbeianus* and therefore relatively greater absorption of DCF, as can be seen in Fig. 1, leading possibly to increased mortality. Ortiz-Santaliestra et al. (2006) examined how developmental stage influences the effect of ammonium nitrate on embryonic and larval stages of anuran amphibians, finding that lethal effects increased with concentration and duration of exposure, with significant differences in sensitivity in relation to developmental stage. Furthermore, the present study found higher tolerance to DCF exposure in L. *catesbeianus* than in *X. laevis*, since embryos of the latter species showed lower mortality at the different concentrations but a larger number of malformations (Table 1). As reported by Herkovits and Fernandez (1978), tissue and organ development begin at fertilization and continue through metamorphosis. Incomplete tissue and organ differentiation may make individuals in earlier larval stages more sensitive to pollutants.

5. Conclusion

Diclofenac is teratogenic during embryonic development in *X. laevis* and L. *catesbeianus*, inducing growth inhibition and diverse malformations including mainly axial malformations in the tail and notochord, edema and hypopigmentation. The species L. *catesbeianus* is suitable for use as a bioindicator in FETAX due to its importance in the food chain, economic and culinary interest, and high sensitivity to pollutants. The estimated ecological risk assessment values suggest that DCF does not represent a risk for these amphibian species in Mexican ecosystems. However, further studies should be carried out using environmental concentrations, since different types of damage including oxidative stress and cyto- and genotoxicity have been reported in other aquatic organisms at these concentrations.

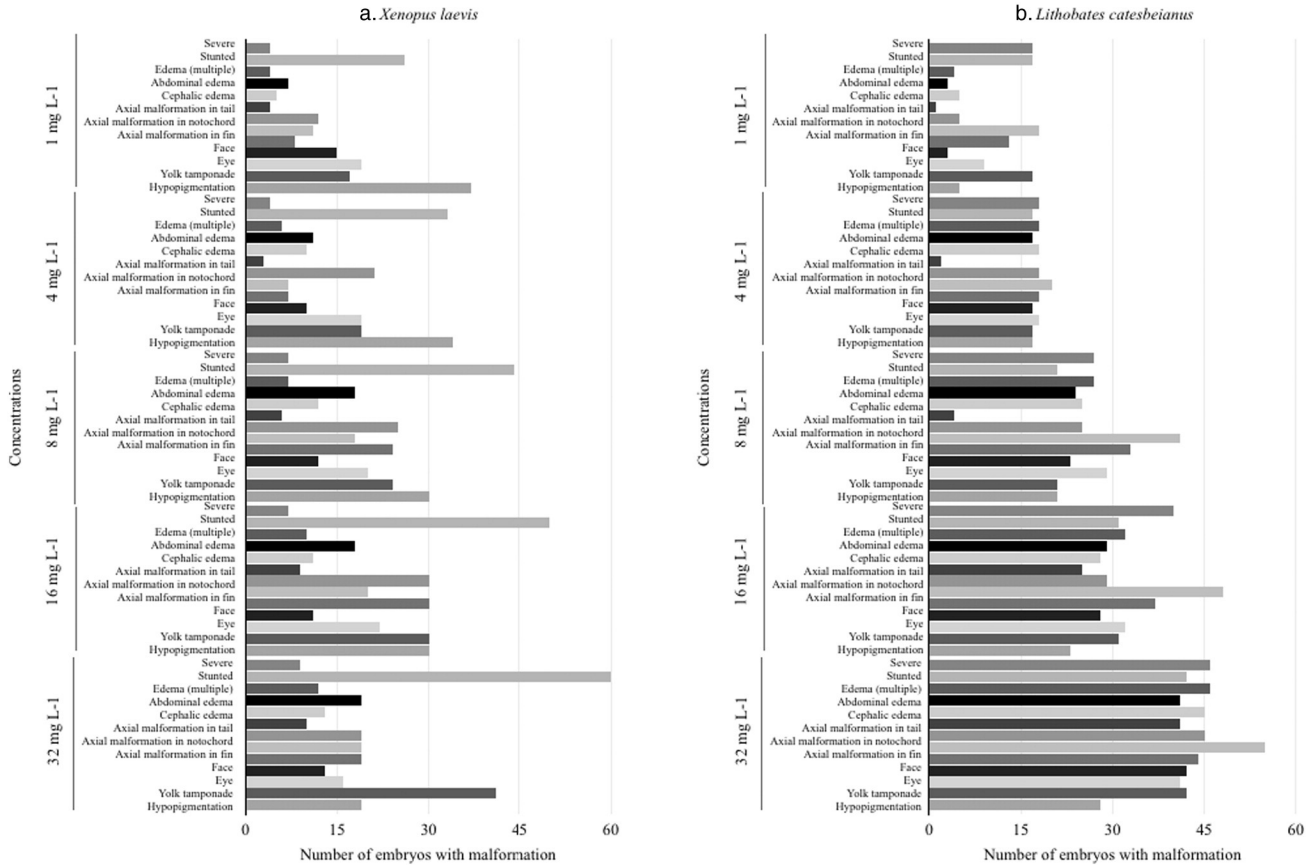

Fig. 4. Types and frequency of malformations in (a) *X. laevis* and (b) *L. catesbeianus* embryos after 96 h of exposure to different diclofenac concentrations.

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