Water Air Soil Pollut (2021) 232: 196
https://doi.org/10.1007/s11270-021-05148-6

Developmental Effects of Amoxicillin at Environmentally Relevant Concentration Using Zebrafish Embryotoxicity Test (ZET)

Edgar David González-González · Leobardo Manuel Gómez-Oliván · Hariz Islas-Flores · Marcela Galar-Martínez

Received: 25 February 2021 / Accepted: 14 April 2021 / Published online: 1 May 2021

Abstract Amoxicillin (AMX) is an antibiotic that has been added to the watch list of substances by the European Union, through the Water Framework Directive (WFD; 2000/60/EC) and its daughter regulation (Decision 2018/840) for which monitoring data have to be gathered with a possibility for future regulation. Previous studies have demonstrated that this antibiotic generates toxic effects, among which oxidative stress in aquatic organisms is noteworthy. The aim of this study was to evaluate the effect on embryonic development and the teratogenic effects induced by AMX at environmentally relevant concentrations in oocytes and embryos of *Danio rerio*. Furthermore, oxidative stress biomarkers were evaluated at 72 and 96 hpf. The LC_{50} was 14.192 μgL^{-1}, EC_{50} was 7.083 μgL^{-1}, and TI was 2.003. Biomarkers of cellular oxidation and antioxidant enzymes were modified in a concentration-dependent way with respect to the control group ($p<0.05$). The main malformations identified were tail malformation, pericardial edema, yolk sac malformation, scoliosis, pectoral fin absence, and no hatching. The results allow us to conclude that AMX at environmentally relevant concentrations is capable of inducing embryotoxic and teratogenic effects and oxidative damage. This compound represents a risk to aquatic organisms such as *Danio rerio*.
Clinical Trials Registration Not applicable.

Keywords Amoxicillin · Zebrafish · Embryotoxicity · Teratogenicity

E. D. González-González · L. M. Gómez-Oliván (✉) · H. Islas-Flores
Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan, Colonia Residencial Colón, CP, 50120 Toluca, State of Mexico, Mexico
e-mail: lmgomezo@uaemex.mx
e-mail: lgolivan74@gmail.com

M. Galar-Martínez
Laboratorio de Toxicología Acuática, Departamento de Farmacia, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Unidad Profesional Adolfo López Mateos, Av. Wilfrido Massieu s/n y cerrada Manuel Stampa, Col. Industrial Vallejo, CP 07700 Mexico City, Mexico

1 Introduction

In the last few years, there has been growing concern about the release of organic compounds of anthropogenic origin, known as emerging organic contaminants (EOCs), to the environment. These EOCs include a diverse group of thousands of chemical compounds, such as pharmaceuticals and personal care products (PPCPs), pesticides, hormones, surfactants, flame retardants, plasticizers, and industrial additives. Metabolites and intermediate degradation products of parent compounds are also included (Farré et al., 2008).

The major concern about these compounds is their impact on the water sources. In the case of pharmaceuticals, even very low amounts can exhibit undesirable effects in nontarget species. This is especially true for pharmacological classes such as antibiotics and hormones (Naidu et al., 2016).

Antibiotics are widely used for human and veterinary therapies, but in recent decades they have been used in ever-increasing quantities (Danner et al., 2019; Wang et al., 2020). According to a recent World Health Organization (WHO) study, the overall consumption of antibiotics ranged from 4.4 to 64.4 defined daily doses (DDD) per 1000 inhabitants per day. In most countries amoxicillin (AMX) and amoxicillin/clavulanic acid were the most frequently consumed antibiotics (WHO, 2018). Despite their benefits, antibiotics have been recently recognized as environmental pollutants in aquatic environments, due to their potential adverse effects on ecosystem and human health (Manaia, 2017; Wang et al., 2020).

Among the antibiotics, AMX is widely consumed worldwide, approximately 29.4% of total DDD in the region of the Americas (WHO, 2018). It is a semisynthetic penicillin class (β-lactam antibiotics) drug used in the treatment and prevention of bacterial respiratory, gastrointestinal, urinary, and skin infections in humans and animals (Bush, 2003; Rao et al., 2011).

During its metabolization, it is excreted 80–90% unchanged from the human body (Hirsch et al., 1998). With its high recommended daily dose (750–2250 mg day^{-1}) and its resistance to changes in forms during excretion, amoxicillin is expected to be in greater proportion than other PPCPs in the municipal wastewater, in terms of both levels and frequency (Mutiyar & Mittal, 2013).

The presence of AMX in the environment, especially in surface waters, rivers, hospital wastewater, effluents from waste stabilization ponds, freshwaters, urban wastewater, and drinking water, has been identified in many countries around the world in continents such as Europe, Africa, and Oceania. The identified concentrations of this antibiotic range from 0.000001 to 11.99 (μgL^{-1}). These findings confirm the ubiquitous presence of this antibiotic in water bodies around the world (Table 1).

It is well known that the metabolism of AMX has two major products: amoxicilloic acid and amoxicillin piperazine-2,5-dione (DIKETO). These compounds do not display antibiotic activity; however, the amoxicilloic acid could have potential allergic properties (Reyns et al., 2008).

AMX has only recently been included in the European Union watch list (through the Water Framework Directive (WFD; 2000/60/EC) and its daughter regulation (Decision 2018/840)) as a target compound for monitoring its risk to the aquatic environment (Loos et al., 2018). The importance of their inclusion lies in the fact that it has been identified as a substance potentially harmful for the aquatic environment but for which monitoring data has not been sufficient to establish their environmental risk.

Growth inhibition measurements on algae have shown the high sensitivity of these organisms to antibiotics with low NOEC, measured at 3.1 μgL^{-1} for clarithromycin (Yamashita et al., 2006) and at 0.78 μgL^{-1} for amoxicillin (Andreozzi et al., 2004).

Detrimental effects on nontarget organisms, including humans and plants, have also been observed (Gomes et al., 2019; Lawrence et al., 1996; Lowes et al., 2009), and aquatic ecosystems are particularly prone to the toxic effects of antibiotics. AMX was toxic (through mortality) to *Tilapia nilotica* fish and to *Culex pipiens* mosquito larvae at concentrations up to 10 μgL^{-1} (Yasser & Nabila, 2015).

Previous studies in our research group demonstrate that the AMX generate oxidative stress, genotoxicity, and cytotoxicity in *Cyprinus carpio* (Elizalde-Velázquez et al., 2017; Orozco-Hernández et al., 2019). Our hypothesis that the AMX is capable to induce alterations to embryonic development and teratogenicity in *D. rerio* is supported by oxidative stress involved in the etiology of defective embryo development (Guérin et al., 2001).

Zebrafish embryos have been used for the ecotoxicological assessment of metals, agrochemicals, and pharmaceuticals, among other chemicals. Several studies suggested that fish embryo toxicity (FET) tests with zebrafish are as sensitive as a toxicity test with adult fish (Lammer et al., 2009). Many advantages of FET can be listed: usage of a low volume of test solutions and, consequently, low volume of wastes; test organisms are easily obtained; and in a short period of time, mortality and developmental responses are assessed (de Oliveira et al., 2020).

The objective of this study was to evaluate the effect on embryonic development, teratogenic effects, and oxidative damage induced by AMX at environmentally relevant concentrations in oocytes and embryos of *Danio rerio*.

Table 1 Occurrence of AMX in different types of effluents and substrates in water bodies

Country	Water bodies	Concentrations (μgL^{-1})	References
Ghana	Hospital wastewater and effluents from waste stabilization ponds	0.003	Azanu et al. (2018)
France	River	0.068	Dinh et al. (2011)
UK	Surface waters	0.039–0.245	Kasprzyk-Hordern et al. (2008)
Australia		0.2	Watkinson et al. (2009); Fatta-Kassinos et al. (2011)
Brazil	River	0.127	Kim et al. (2018)
	Surface waters	0.017	Locatelli et al. (2011)
Tanzania	Hospital wastewater, urban wastewater, freshwaters	11.99	Kaseva et al. (2008); Ebele et al. (2017)
Italy	Wastewater treatment plants	0.0018–0.12	Andreozzi et al. (2004); Castiglioni et al. (2005)
		0.622	Zuccato et al. (2010)
Australia		0.03 and 0.05	Watkinson et al. (2007); Watkinson et al. (2009)
Malaysia	Drinking water	0.000001	Praveena et al. (2019)
Australia	Hospital effluents and urban wastewater	0.9 and 1.67	Kasprzyk-Hordern et al. (2008)

2 Material and Methods

2.1 Test Substance

Amoxicillin trihydrate (6-[2-amino-2-(4-hydroxyphenyl)-acetyl]amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid trihydrate) utilized in the study was purchased from Tokyo Chemical Industry Co., Ltd. The purity of the standard was 98%, $C_{16}H_{19}N_3O_5S{\cdot}3H_2O$, with a molecular weight of 365.40 (CAS number 61336-70-7).

2.2 Zebrafish Maintenance and Breeding

Wild-type 4-month-old zebrafish specimens (0.95 ± 0.2 g in weight and 3.95 ± 0.5 cm in length) free of infection and disease symptoms were acquired from a local fish farmer (Aquanimals, MX) following the requirements cited in the Test Guideline No. 203 Fish, Acute Toxicity Testing (OECD 2013). Fish were kept in 120-L vessels (loading capacity of 1 L of water per fish) for 30 days prior the experiments with dechlorinated tap water at 26 ± 2 °C, pH ranging between 7.2 and 7.6, oxygen saturation above 80%, and natural photoperiods of 12-h dark and 12-h light. Specimens were fed with commercial flake food (Tetramin®) two times a day according to methodologies in Test Guideline No. 203 Fish, Acute Toxicity Testing (OECD 2013). After the acclimation period, the breeding fish were separated by sex and kept in separate tanks (that maintained the same characteristics as the acclimation tanks for 1 week prior to the mating of males and females, during which time the brood zebrafish were fed with Tetramin® and supplemented with *Artemia* sp. three times a day).

2.3 Collection of Eggs

For the production of eggs, three males and six females (with visible gravity) of size 4–4.5 cm were transferred from the stock population of breeding fish the evening before an experiment. The organisms were placed in breeding traps (4 × 4 mm mesh) in separate tanks. For this purpose, the tanks contained 12 L of water aerated, dechlorinated, and reconstituted with commercial salts Instant Ocean® at 9 mg L^{-1}. On the morning of the next day, the eggs were collected from the bottom of the tank using a 2-mm-diameter plastic Pasteur pipette. The eggs were washed with saline solution and observed using a stereomicroscope. The eggs selected for the study were in the middle blast stage and used immediately for embryolethality and teratogenesis tests (Beekhuijzen et al., 2015).

2.4 Embryo AMX Exposure

To evaluate the AMX toxicity to embryonic development alterations and teratogenic effects, the guidelines established by the OECD were followed in their test guideline Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013. Thirty embryos per triplicate were randomly placed in environmentally relevant concentrations (0.039, 1.746, 3.453, 5.160, 6.868, 8.575, 10.282, and 11.990 μgL^{-1}) and in a control system (drug-free). Exposures were performed in 24-well plates using 2 mL of drug solution, and medium was not exchanged during the experiment. Dead embryos, identified as coagulated eggs or heart beating absence, were removed every 24 h. The plates were maintained at a temperature of 27 ± 1 °C and during natural photoperiods of 12-h dark and 12-h light. At 96 hpf, surviving and dead embryos were counted, and percentage of mortality calculated. Percentages were used to establish a dose-mortality curve and to calculate median lethal concentration (LC_{50}). For the embryolethality and teratogenesis tests, the experiments were replicated in triplicate.

2.5 Embryo Survival Test

At 4-h post fertilization (hpf), embryos were randomly assigned and placed in 24-well plates for each concentration used. The concentrations of AMX used were environmentally relevant considering the reported in wastewater treatment plants, surface waters, rivers, and urban wastewater lakes. The concentrations used were 0.039, 1.746, 3.453, 5.160, 6.868, 8.575, 10.282, and 11.990 μgL^{-1}. For each concentration, 30 eggs were placed in each well, and the experiment was performed in triplicate (total=90 eggs). In addition, 30 embryos were used as a control for each replica of the experiment (total=90 eggs). After 96 hpf, the live, dead, and malformed embryos were counted. Lethal concentration 50 (LC_{50}) and effective concentration of malformation (EC_{50m}) were calculated. With the data obtained, a figure was made to determine the rate of live and malformed organisms.

2.6 Evaluation of Developmental Effects

Embryonic development was examined at selected time points, namely 12, 24, 48, 72, and 96 hpf, and morphological characteristics of the embryos were evaluated using a stereomicroscope. This evaluation was performed in each organism when compared with the reference embryo, according to Kimmel et al., (1995). Each embryo received a score for each of the factors to be evaluated, which are the following: tail development, eye development, somite formation, movement, heartbeat, blood circulation, head-body pigmentation, tail pigmentation, appearance of pectoral fin, buccal bump, and hatching. For each abnormality or delay presented in any of the characteristics to be evaluated, one unit was subtracted from the score (Hermsen et al., 2011). Six different categories of abnormalities were recorded including pericardial edema, yolk sac malformation, tail malformation, scoliosis, pectoral fin absence, and no hatching. Those most representative deformities were photographed for later description.

2.7 Calculating of the Teratogenicity Index (TI)

To characterize the teratogenic potential of the test substance (AMX), the teratogenicity index (TI) which was defined as the quotient of LC_{50} and EC_{50m} was calculated. If the TI was > 1, AMX was considered as teratogenic and if it was < 1 as embryolethal, according to criteria of Weigt et al. (2011).

2.8 Evaluation of Oxidative Stress

One gram of *Danio rerio* eggs were exposed to AMX environmentally relevant concentrations (0.039, 1.746, 3.453, 5.160, 6.868, 8.575, 10.282, and 11.990 μgL^{-1}) in systems prepared using six liters of water added with the antibiotic and a control. After 72 and 96 h, the embryos were homogenized in 1.5 mL of phosphate buffer (PBS) at pH 7.4. Samples were centrifuged at 15,000 g for 15 min at 4 °C. Cell oxidation biomarkers were determined: lipoperoxidation level (Buege & Aust, 1978), hydroperoxide content (Jiang et al., 1992), and activity of antioxidant enzymes superoxide dismutase (Misra & Fridovich, 1972) and catalase (Radi et al., 1991). The experiments were replicated fivefold.

2.9 Statistical Analyses

For the embryolethality test, embryos were quantified at each concentration. After 96 hpf, the live, dead, and malformed embryos were counted and a maximum likelihood linear regression analysis was performed to calculate lethal concentration 50 (LC_{50}) and effective concentration of malformation (EC_{50m}) with their 95%

confidence intervals (p<0.05). The Spearman-Karber method trimmed was used (US-EPA software ver 1.5). With the data obtained, a figure was made to determine the rate of live and malformed organisms (Fig. 2) (IBM SPSS Statics 26).

Embryotoxicity and teratogenicity were analyzed using one-way analysis of variance (ANOVA), followed by Fisher's LSD test. Significance was accepted when $p < 0.05$, using StatPlus 6.2.2.0. The validity criteria used in this study were principal two: the first was that the fertilization rate was ≥ 90%, and the second was that the test was considered valid if the control groups showed no > 10% of lethal teratogenic effects at 96 hpf.

Oxidative stress biomarkers were examined using one-way analysis of variance (ANOVA), followed by Fisher's LSD test with 95% confidence limit, to determine differences between means. StatPlus 6.2.2.0 was used.

3 Results

3.1 Embryolethality and Teratogenicity Data Induced by AMX

Table 1 shows the data on the percentages of mortality and malformations of the *D. rerio* embryos exposed to the different environmentally relevant concentrations of AMX. The number of dead and malformed embryos increases proportionally with the increase in concentration. The LC_{50} value was 14.192 μg L^{-1} with confidence intervals to 95% of [10.902–28.897] and the EC_{50m} was 7.083 μg L^{-1} with confidence intervals to 95% [5.051–8.658]. With these data the teratogenic index that was 2.003 was calculated. Weigt et al. (2011) established that if the TI of a given substance is greater than 1, the substance is teratogenic and if the TI is below 1, the substance produces mainly embryolethal effects. According to these criteria, AMX was classified as teratogenic.

Figure 1 shows mortality increase for concentrations from 0.039 to 11.990 μgL^{-1}, reaching 26.7% and 58.9%, respectively. Mortality was identified by embryonic coagulation and a missing heartbeat.

In addition, Fig. 2 indicates that as the concentration of AMX increases, the malformation rate also increases while the survival rate decreases. Malformations were observed in all the treatment groups during the exposure

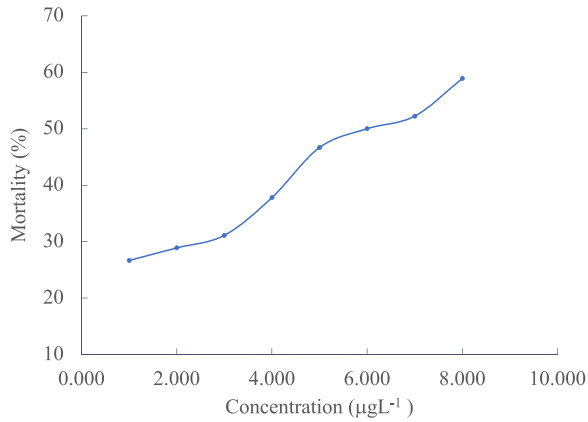


Fig. 1 Mortality of zebrafish embryos exposed to AMX

period in concentration-dependent relation. Some embryos survived even with malformations.

In Fig. 3, normal, dead, and malformed embryos are observed in each AMX concentration used in the experiment. From the concentration of 0.039 μgL^{-1} to 3.453 μgL^{-1} and from 5.160 to 8.575 μgL^{-1}, the embryos presented a similar number of malformations. The number of normal embryos decreased proportionally as the concentration of AMX. These data are consistent with Fig. 5, where we can observe that at the highest AMX concentration, a higher number of teratogenic alterations were observed.

3.2 Malformations of *D. rerio* Embryos Exposed to AMX

Figure 4 shows the averages of the scores obtained by the embryos of *D. rerio* in each of the proven concentrations of AMX. The highest score obtained was in the

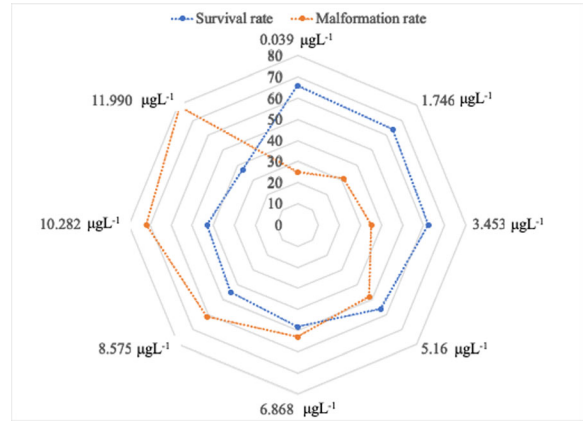


Fig. 2 Survival and malformation rate of zebrafish embryos exposed to AMX

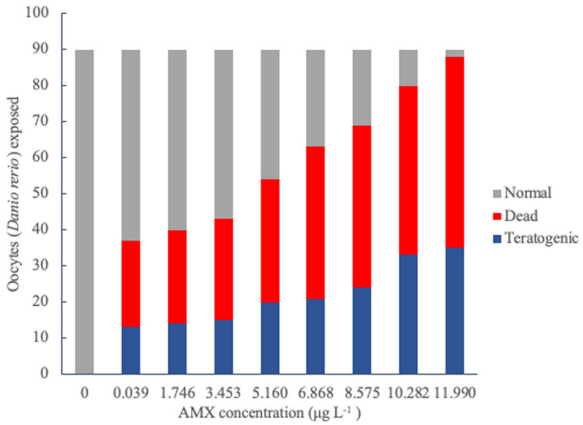


Fig. 3 Effects on zebrafish embryos exposed to different AMX concentrations

control since embryo development was normal. As the AMX concentrations increased, the score decreased due to all developmental alterations and teratogenic effects observed ($p<0.05$). All decreases were statistically significant with respect to the control score and at all exposure times.

Figure 5 illustrates the frequency of the teratogenic alterations observed in the embryos and larvae of *D. rerio* exposed to the different proportions of the industrial effluent. The most frequent were tail malformation, pericardial edema, yolk sac malformation, scoliosis, pectoral fin absence, and no hatching (Table 2). The highest concentrations of AMX 10.282 μgL^{-1} and 11.990 μgL^{-1} showed approximately 30–31% pectoral fin absence and 37–39% no hatching. In contrast, the lower concentrations of AMX 0.039 μgL^{-1} and 1.746 μgL^{-1} showed approximately 7–10% pectoral fin absence and 14–16% no hatching.

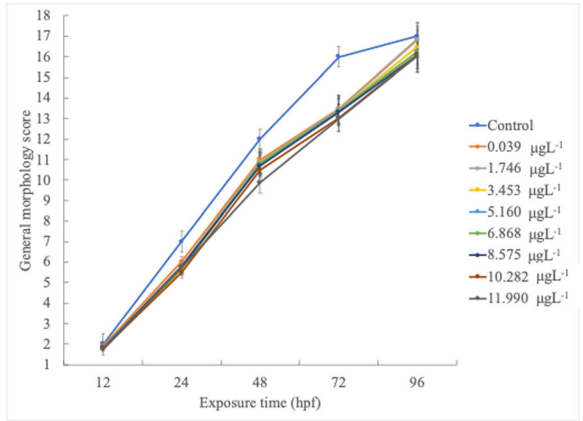


Fig. 4 Concentration-response curves of AMX in *Danio rerio* embryos

3.3 Oxidative Damage and Antioxidant Defenses Induced by AMX in *D. rerio* Embryos

Figures 6 and 7 show the biomarkers of cell oxidation and antioxidation at 72 and 96 hpf. As can be seen, the values of all biomarkers were increased with respect to the control group and the exposure time in a statistically significant manner and concentration dependent in *D. rerio* embryos. Increases of up to approximately 88% (at 72 hpf) and 58% (at 96 hpf) were observed for the hydroperoxides content (HPC) and 48% (at 72 hpf) and 49% (at 96 hpf) for the lipoperoxidation level (LPX) with respect to the control groups ($p<0.05$). In the case of antioxidant activity, increases of up to 105% (at 72 hpf) and 153% (at 96 hpf) and 541% (at 72 hpf) and 180% (at 96 hpf) were observed in the activities of the superoxide dismutase (SOD) and catalase (CAT) enzymes relative to the control group ($p<0.05$). These increases were time and concentration dependent in all cases.

4 Discussion

Antibiotics are biologically active and can inhibit the synthesis of proteins, nucleic acids, and cell walls (Faghih et al., 2017; Ma et al., 2016) and can also inhibit DNA replication and cell division (Sass & Brotz-Oesterhelt, 2013; van Eijk et al., 2017). Antibiotics are not completely removed after wastewater sewage treatment, and therefore aquatic environments are continuously exposed (Sinthuchai et al., 2016; Straub et al., 2012). Low concentrations of antibiotics cause nontarget toxicity to aquatic organisms, but the effects in fish are less often reported than in microorganisms (Fent et al., 2006; Santos et al., 2010).

In the aquatic environment, few studies have reported the effects of AMX in fish, probably because it is not described as highly toxic in scientific literature (AMX 96h-LC_{50} = 1000 mgL^{-1} for *Oryzias latipes* according to Park and Choi (2008)). In a study made by Oliveira et al. (2013), AMX caused premature hatching (48h-EC_{50} = 132.4 mgL^{-1}) and embryos of zebrafish presented abnormal development. The most frequent malformations were edemas and tail deformities. Although acute toxicity studies show that lethal concentrations of AMX in nontarget species are higher than 1000 mg L^{-1} such as *Daphnia magna*, *Moina macrocopa*, and *Oryzias latipes* (Kim et al., 2007;

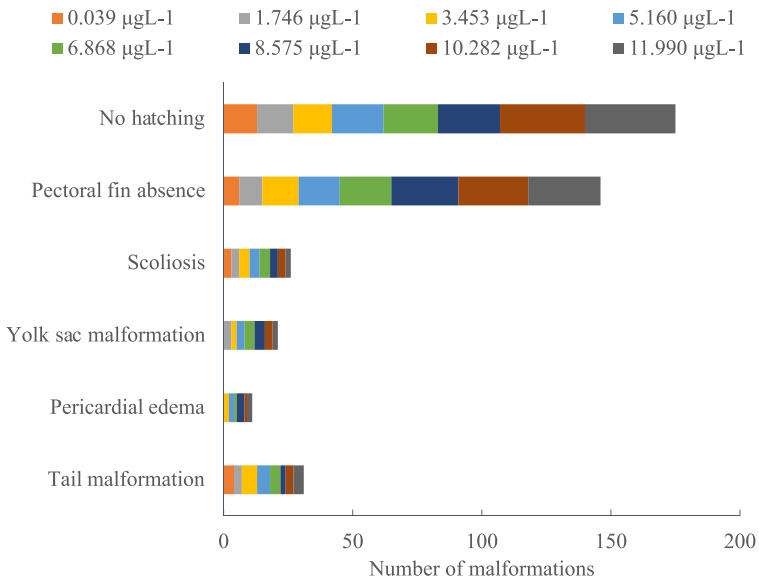


Fig. 5 Teratogenic malformations induced by exposure to AMX concentrations in *Danio rerio* embryos

Park & Choi, 2008). There are some studies that refer that this antibiotic can cause acute toxicity at lower concentrations such as *Synechococcus leopoliensis* (1.56 μgL^{-1}) (Busto et al., 2020) and *Spirodela polyrhiza* (0.089 μgL^{-1}) (Singh et al., 2018). This was one of the reasons why we decided to work with relevant amoxicillin concentrations (0.039 to 11.990 μgL^{-1}) since these are present in bodies of water and these are the indication of the presence of this antibiotic in the world. Our study shows that exposure to AMX for 96 h induces pectoral fin absence and no hatching (EC_{50m} = 7.083 μgL^{-1}), like most frequent malformations. One of the possible reasons for the disparity in EC50m found between Oliveira's study and our results is that AMX may have a hormesis effect. Some human and veterinary antibiotics are capable of inducing hormesis in plants (Agathokleous et al., 2018). Also the results obtained in our study could be related to the ability of AMX at concentrations of 0.039 and 1.67 μgL^{-1} to induce genotoxic and cytotoxic effects in species such as *Cyprinus carpio*, since these effects are often related to embryotoxic and teratogenic effects (Orozco-Hernández et al., 2019).

The above findings make it evident that literature studies on the effect of AMX on aquatic flora and fauna are scarce, in addition to the fact that there are significant

Table 2 Mortality and malformation data in embryos of *Danio rerio* exposed to AMX

AMX concentration ($\mu g\ L^{-1}$)	Number of embryos exposed	Mortality (%)	Malformations (%)
0	90	0	0
0.039	90	26.7	28.9
1.746	90	28.9	35.6
3.453	90	31.1	47.8
5.160	90	37.8	55.6
6.868	90	46.7	60.0
8.575	90	50.0	68.9
10.282	90	52.2	77.8
11.990	90	58.9	81.1
		LC_{50} = 14.192	EC_{50} = 7.083
		CI = [10.902–28.897]	CI = [5.051–8.658]
		TI = 2.003	

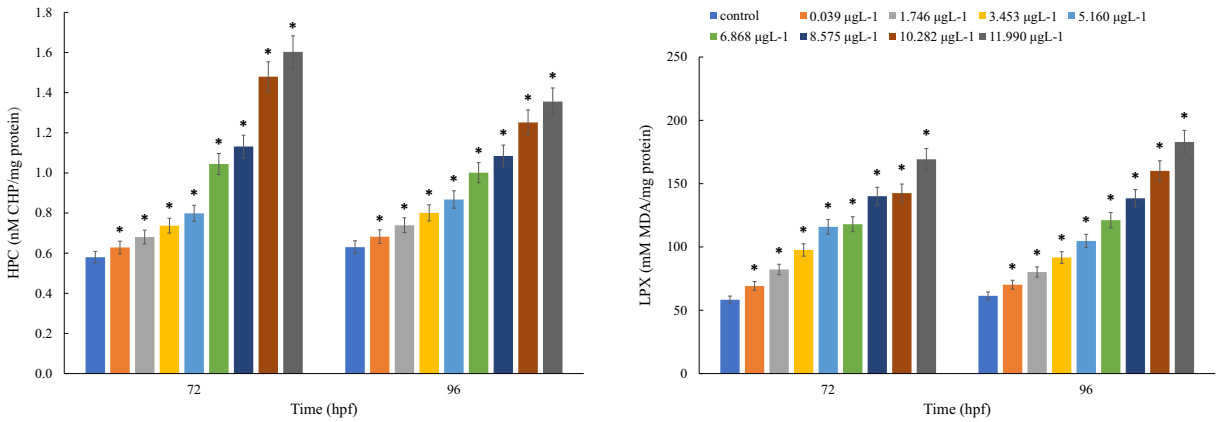


Fig. 6 Cell oxidative biomarkers, hydroperoxide content (HPC), and lipid peroxidation (LPX) in *Danio rerio* embryos exposed to AMX

differences using different bioindicators and endpoints of evaluation, so it is necessary to conduct further studies to clarify the toxic effects and mechanisms of action of this antibiotic.

Inhibition hatching was already described to kanamycin and a mixture of β-diketone antibiotics (Song et al., 2010; Wang et al., 2013). The hatching process is based on a combination of chemical, osmotic, and mechanical (muscular) mechanism (Denucé, 1985; Yamagami, 1981). Different toxic mechanisms could justify hatching delay in fish embryos, resulting from inhibition of the hatching enzyme chorionase, osmotic disturbances interfering with the activity of the hatching enzyme, and/or inability of the emerging larvae to break the egg shell (Dave & Xiu, 1991; Hallare et al., 2005).

Other malformations observed were tail malformation, pericardial edema, yolk sac malformation, and scoliosis.

In zebrafish, the heart is the first organ to develop and function; hence, it appears to be a primary target for developmental toxicity. The occurrence of pericardial edema is an indicator of abnormal cardiac development (Hill et al., 2005). Pericardial edema may also be an indicator of osmotic or metabolic dysfunction (Hill et al., 2004). Some results suggest that yolk sac edema can inhibit the supply of nutrients to the heart, resulting in pericardial edema (Mu et al., 2013). In a study conducted by Chowdhury et al. (2020), AMX was able to reduce zebrafish body length and alter the yolk sac by increasing its length and decreasing its height.

Spine deformities (such as scoliosis) might be the consequence of dysregulated Wnt signaling or decreased collagen in the spine column (Çelik et al., 2012). Spine deformities might also be linked to calcium and phosphorus ion depletion or deregulation, which are necessary for the normal development of zebrafish (Muramoto, 1981).

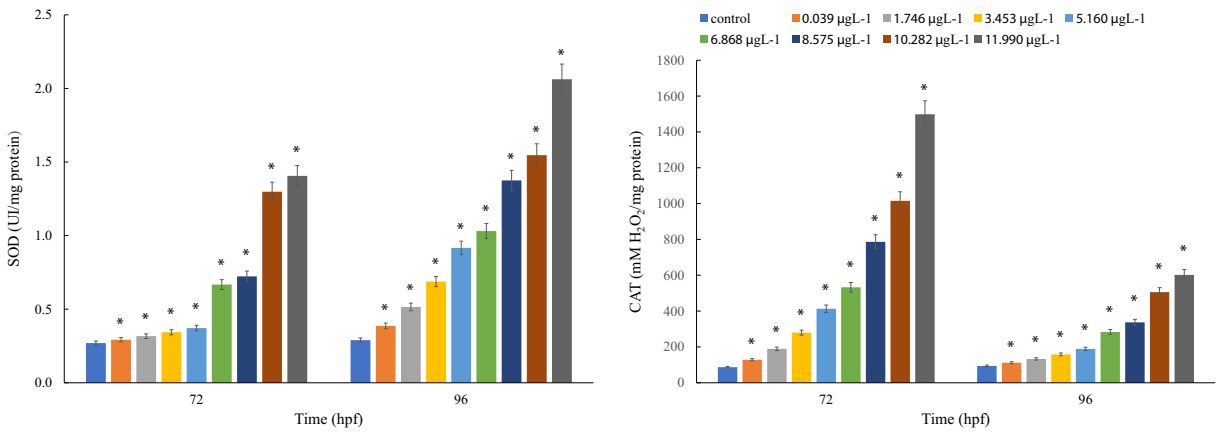


Fig. 7 Activity of antioxidant enzymes, superoxide dismutase (SOD), and catalase (CAT) in *Danio rerio* embryos exposed to AMX

In summary, these results demonstrate that AMX interferes with the normal development. It could be because AMX by acting as a hapten, leads to the activation of T-cell clones against penicillin-specific immunoglobulins, which bind to AMX antigens forming complexes that activate the immunoallergic response (Aldini et al., 2006, 2008; Kormoczi et al., 2001; Oettl & Stauber, 2007). Immune cell function is related to reactive oxygen species (ROS) production, but excessive amounts of ROS can become a source of tissue damage by attacking different cellular components and causing cell death by oxidative stress (Aderem & Underhill, 1999).

Teratogenic effects on the spine, tail, yolk sac, and heart were observed at concentrations lower than those causing lethality, indicating that AMX is a teratogen.

Several bactericidal agents, such as β-lactams, quinolones, and aminoglycosides, can induce ROS generation, which are highly damaging molecules that can inhibit most functions of oxygen-respiring cells (Kohanski et al., 2010).

The generation of oxidative stress induced by AMX, demonstrated by some authors, can alter the integrity of biomolecules such as lipids, proteins, and DNA, as well as being associated with disorders in organisms which could affect fertility, health, and the life cycle of fish, as has been demonstrated by Theodorakis et al. (2000), and provoking genotoxicity (Anlas & Ustuner, 2016) and cytotoxicity.

There is evidence that oxygen plays a key role in the metabolism of embryos and is critical in early embryonic development. Reactive oxygen species (ROS) have vital signaling functions and intervene in numerous physiological processes in the developing organism. During embryogenesis, ROS control cell division and the maturing of oocytes as well as the implantation and formation of blastocysts. When ROS are not duly regulated, they tend to generate oxidative stress and consequently embryotoxicity. Therefore, the antioxidant defense plays a vital role in the protection of organisms in formation, since pro-oxidant agents could elevate the concentration of ROS and induce oxidative damage in oocytes, mitochondrial alterations, depletion of the ATP content, DNA damage, lipoperoxidation, apoptosis, and/or a delay in the entire embryonic development process (Pašková et al., 2011).

In this study, we corroborated that AMX can induce oxidative stress in zebrafish embryos in a concentration-dependent manner. Moreover, at 72 and 96 hpf, this antibiotic at all concentrations tested was capable to increase the level of cell oxidative biomarkers (LPX and HPC) with respect to the control group ($p<0.05$).

In 2007, a novel mechanism was proposed whereby bactericidal antibiotics (such as β-lactam) induce bacterial cell death. This mechanism involves the production of hydroxyl radicals and was reported to depend on metabolism-related NADH depletion, the tricarboxylic acid (TCA) cycle, the electron transport chain, damage of iron sulfur clusters in proteins, and stimulation of the Fenton reaction (Dwyer et al., 2007; Kohanski et al., 2007).

Reactive oxygen species (ROS) play a significant role in embryonic development. ROS modulate signaling pathways that promote differentiation, proliferation, and apoptosis (Dennery, 2007). Excess ROS lead to oxidative stress and the perturbation of redox-sensitive signaling pathways, some of which are associated with dysmorphogenesis (Ozolins & Hales, 1997). During mid-organogenesis, a period when the embryo is undergoing rapid cellular growth and differentiation leading to major structural changes, ROS may play an important role in mediating teratogenic insult (Schlisser et al., 2010).

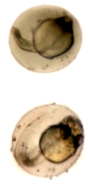
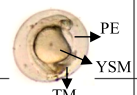
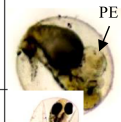
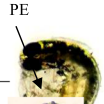
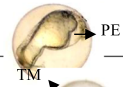
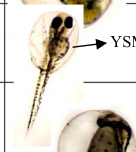
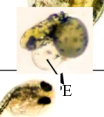
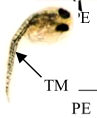
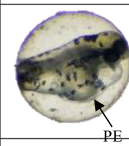
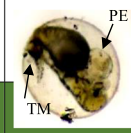
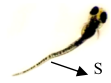
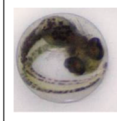
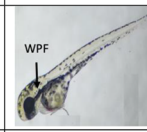
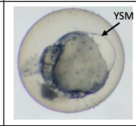
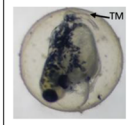
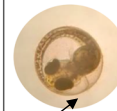
ROS contributes to embryonic maldevelopment by modifying redox-sensitive signaling pathways critical to development. During organogenesis, oxidative stress posttranslationally modified redox-regulated transcription factors with subsequent changes in gene expression in the embryo (Dennery, 2007).

Our results of embryolethality show that the malformations in the zebrafish embryos were more frequent at higher concentrations of AMX.

Two recent studies carried out by our group demonstrated that (1) AMX at concentrations of 10 ngL^{-1} and 10 μgL^{-1} generated an increase in the levels of lipoperoxidation, hydroperoxide content, and carbonylated protein content, as well as superoxide dismutase, catalase, and glutathione peroxidase in the brain, kidney, and gills of *Cyprinus carpio* (Elizalde-Velázquez et al., 2017) and (2) AMX in environmentally relevant concentrations, 0.039 μgL^{-1} and 1.67 μgL^{-1}, was capable of generating genotoxic alterations and cytotoxic effects in blood cells of the common carp (Orozco-Hernández et al., 2019).

The redox stress component of antibiotic lethality is hypothesized to derive from alterations to multiple core aspects of cellular physiology and stress response

Table 3 Main malformations in embryos of zebrafish identified by exposure to AMX at different concentrations

Concentration ng L^{-1}	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
Control					
0.039 µgL^{-1}					
1.746 µgL^{-1}					
3.453 µgL^{-1}					
5.160 µgL^{-1}					
6. 868 µgL^{-1}					
Concentration ng L^{-1}	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
8.575 µgL^{-1}					
10.282 µgL^{-1}					
11.990 µgL^{-1}				 	 

TM: tail malformation, **PE**: pericardial edema; **YSM**: yolk sac malformation; **S**: scoliosis; **WPF**: Whitouth pectoral fin ; **HR**: hatching retardation

activation. Specifically, this component includes alterations to central metabolism, cellular respiration, and iron metabolism initiated by drug-mediated disruptions of target-specific processes and resulting cellular damage (Dwyer et al., 2014).

Hatching is one of the important steps in embryogenesis (Yumnamcha et al., 2015). Embryo development rate is related to hatching (Dave & Xiu, 1991). These changes in hatching rate may be due to the toxic effect by ROS generate in the chorion. We think that this is related to the inhibition of important enzymes in the hatching process such as chorionase (Haendel et al., 2004).

ROS increases the oxidation of polyunsaturated fatty acids (Blahová et al., 2013). LPX has increased depending on AMX concentration. The results show that LPX correlates with changes in antioxidant levels (SOD and CAT).

Antioxidant enzymes such as SOD and CAT play a vital role in protecting the cells from oxidative stress. In the present study, SOD and CAT activity was found to be dramatically increased in AMX-treated embryos than that in the control.

AMX demonstrates toxicity in development. The development of many enzyme activities such as alkaline phosphatase (AKP), acid phosphatase (ACP), and antioxidant response are also affected by antibiotics (Zhou et al., 2018).

Increased amount of ROS can cause apoptosis during embryogenesis (Khazaei & Aghaz, 2017; Shi & Zhou, 2010). The apoptosis signal was detected as the result of the chemical toxic effect. Continuation of apoptosis signaling leads to lipid peroxidation and cellular death (Fulda et al., 2010; Weinberg et al., 2016).

Finally, a greater TI value is associated with a toxic agent that produces wide separations between the malformation and lethality dose-response curves. It is possible to have a toxic agent that causes severe malformations but not mortality; conversely, a potentially developmental toxic chemical can be so lethal that malformations are not observed (Reimers et al., 2004). AMX is a drug classified as pregnancy category B by the FDA, but our study showed little greater than 2 of TI value.

5 Conclusions

The data obtained in this study (LC_{50} = 14.192 μgL^{-1} and EC_{50} = 7.083 μgL^{-1}) showed a teratogenic index of 2.003, showing that AMX is an antibiotic that can generate embryotoxicity and teratogenicity. AMX at environmentally relevant concentrations 0.039–11.990 μgL^{-1} is capable of inducing alterations to embryonic development and teratogenic effects in oocytes and embryos of zebrafish (*Danio rerio*). The main malformations identified were tail malformation, pericardial edema, yolk sac malformation, scoliosis, pectoral fin absence, and no hatching (Table 3). Embryo malformations may be caused by reactive oxygen species–induced oxidative stress. The results allow us to conclude that AMX at environmentally relevant concentrations is capable of inducing embryotoxic and teratogenic effects and this compound represents a risk to aquatic organisms.

Author Contribution Edgar David González-González and Leobardo Manuel Gómez-Oliván performed all the exposure experiments. Leobardo Manuel Gómez-Oliván and Edgar David González-González were involved in the conception. Leobardo Manuel Gómez-Oliván, Edgar David González-González, and Hariz Islas-Flores were involved in the design and interpretation of the data, and the writing of the manuscript was with input from Marcela Galar-Martínez.

Funding This study was made possible by financial support from the Consejo Nacional de Ciencia y Tecnología (CONACyT, Project 300727).

Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics Approval The study was approved by the Ethics and Research Committee of the Universidad Autónoma del Estado de México, Toluca, Mexico (Approval ID: CEI.UAEMCQ.REC.132.2020).

Consent to Participate Not applicable.

Conflict of Interest The authors declare no competing interests.

Plant Reproducibility Not applicable.

References

Aderem, A., & Underhill, D. M. (1999). Mechanisms of phagocytosis in macrophages. *Annual Review of Immunology, 17*, 593–623. https://doi.org/10.1146/annurev.immunol.17.1.593.

Agathokleous, E., Kitao, M., & Calabrese, E. J. (2018). Human and veterinary antibiotics induce hormesis in plants: Scientific and regulatory issues and an environmental perspective. *Environment International, 120*, 489–495. https://doi.org/10.1016/j.envint.2018.08.035.

Aldini, G., Gamberoni, L., Orioli, M., Beretta, G., Regazzoni, L., Facino, R. M., & Carini, M. (2006). Mass spectrometric characterization of covalent modification of human serum albumin by 4-hydroxy-*trans*-2-nonenal. *Journal of Mass Spectrometry, 41*(9), 1149–1161. https://doi.org/10.1002/jms.1067.

Aldini, G., Vistoli, G., Regazzoni, L., Gamberoni, L., Facino, R. M., Yamaguchi, S., Uchida, K., & Carini, M. (2008). Albumin is the main nucleophilic target of human plasma: A protective role against pro-atherogenic electrophilic reactive carbonyl species? *Chemical Research in Toxicology, 21*(4), 824–835. https://doi.org/10.1021/tx700349r.

Andreozzi, R., Caprio, V., Ciniglia, C., de Champdoré, M., Lo Giudice, R., Marotta, R., & Zuccato, E. (2004). Antibiotics in the environment: Occurrence in Italian STPs, fate, and preliminary assessment on algal toxicity of amoxicillin. *Environmental Science & Technology, 38*(24), 6832–6838. https://doi.org/10.1021/es049509a.

Anlas, C., & Ustuner, O. (2016). Genotoxic assessment of amoxicillin in rainbow trout (*Oncorhynchus mykiss*) by comet assay and micronucleus test. *Fresenius Environmental Bulletin, 25*(12), 5358–5364.

Azanu, D., Styrishave, B., Darko, G., Weisser, J. J., & Abaidoo, R. C. (2018). Occurrence and risk assessment of antibiotics in water and lettuce in Ghana. *Science of the Total Environment, 622-623*, 293–305. https://doi.org/10.1016/j.scitotenv.2017.11.287.

Beekhuijzen, M., de Koning, C., Flores-Guillén, M. E., de Vries-Buitenweg, S., Tobor-Kaplon, M., van de Waart, B., & Emmen, H. (2015). From cutting edge to guideline: A first step in harmonization of the zebrafish embryotoxicity test (ZET) by describing the most optimal test conditions and morphology scoring system. *Reproductive Toxicology, 56*, 64–76.

Blahová, J., Plhalová, L., Hostovský, M., Divišová, L., Dobšíková, R., Mikulíková, I., Štěpánová, S., & Svobodováa, Z. (2013). Oxidative stress responses in zebrafish (*Danio rerio*) after subchronic exposure to atrazine. *Food and Chemical Toxicology, 61*, 82–85. https://doi.org/10.1016/j.fct.2013.02.041.

Buege, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology, 52*, 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6.

Bush, K. (2003). *β-lactam antibiotics: Penicillin, and other β-lactam antibiotics. In: Finch RC, Greenwood D, Norrby SR, Whitley RJ. Antibiotic and chemotherapy: Anti-infective agents and their use in therapy* (8th ed.). Churchill Livingstone 1000 pp.

Busto, R. V., Roberts, J., Hunter, C., Escudero, A., Helwig, K., & Coelho, L. H. G. (2020). Mechanistic and ecotoxicological studies of amoxicillin removal through anaerobic degradation systems. *Ecotoxicology and Environmental Safety, 192*, 110207. https://doi.org/10.1016/j.ecoenv.2020.110207.

Castiglioni, S., Bagnati, R., Calamari, D., Fanelli, R., & Zuccato, E. (2005). A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. *Journal of Chromatography. A, 1092*(2), 206–215. https://doi.org/10.1016/j.chroma.2005.07.012.

Çelik, E. S., Kaya, H., & Yılmaz, S. (2012). Effects of phosalone on mineral contents and spinal deformities in common carp (*Cyprinus carpio*, L. 1758). *Turkish Journal of Fisheries and Aquatic Sciences, 12*, 259–264. https://doi.org/10.4194/1303-2712-v12_2_10.

Chowdhury, J., Mandal, T. K., & Mondal, S. (2020). Genotoxic impact of emerging contaminant amoxicillin residue on zebra fish (*Danio rerio*) embryos. *Heliyon, 6*, e05379. https://doi.org/10.1016/j.heliyon.2020.e05379.

Danner, M.-C., Robertson, A., Behrends, V., & Reiss, J. (2019). Antibiotic pollution in surface fresh waters: Occurrence and effects. *Science of the Total Environment, 664*(10), 793–804. https://doi.org/10.1016/j.scitotenv.2019.01.406.

Dave, G., & Xiu, R. Q. (1991). Toxicity of mercury, copper, nickel, lead, and cobalt to embryos and larvae of zebrafish *Brachydanio rerio*. *Archives of Environmental Contamination and Toxicology, 21*(1), 126–134. https://doi.org/10.1007/BF01055567.

de Oliveira, R. C. S., Oliveira, R., Rodrigues, M. A. C., de Farias, N. O., Sousa-Moura, D., Nunes, N. A., Andrade, T. S., & Grisolia, C. K. (2020). Lethal and sub-lethal effects of nitrofurantoin on zebrafish early-life stages. *Water, Air, and Soil Pollution, 231*(2), 54. https://doi.org/10.1007/s11270-020-4414-4.

Dennery, P. A. (2007). Effects of oxidative stress on embryonic development. *Birth Defects Research. Part C, Embryo Today, 81*, 155–162. https://doi.org/10.1002/bdrc.20098.

Denucé, J. M. (1985). How embryos escape from their envelopes: a new look at the (phylogenetically) old problem of hatching. *Meded Kon Acad Wet Belgie, 46*, 1–30.

Dinh, Q. T., Alliot, F., Moreau-Guigon, E., Eurin, J., Chevreuil, M., & Labadie, P. (2011). Measurement of trace levels of antibiotics in river water using on-line enrichment and triple-quadrupole LC-MS/MS. *Talanta., 85*(3), 1238–1245. https://doi.org/10.1016/j.talanta.2011.05.013.

Dwyer, D. J., Kohanski, M. A., Hayete, B., & Collins, J. J. (2007). Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. *Molecular Systems Biology, 3*(1), 3–91. https://doi.org/10.1038/msb4100135.

Dwyer, D. J., Belenky, P. A., Yang, J. H., MacDonald, I. C., Martell, J. D., Takahashi, N., Chan, C. T. Y., Lobritz, M. A., Braff, D., Schwarz, E. G., Ye, J. D., Pati, M., Vercruysse, M., Ralifo, P. S., Allison, K. R., Khalil, A. S., Ting, A. Y., Walker, G. C., & Collins, J. J. (2014). Antibiotics induce redox-related physiological alterations as part of their lethality. *Proceedings of the National Academy of Sciences of the United States of America, 111*(20), E100–E109. https://doi.org/10.1073/pnas.1401876111.

Ebele, A. J., Abdallah, M. A.-E., & Harrad, S. (2017). Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. *Emerging Contaminants, 3*(1), 1–16. https://doi.org/10.1016/j.emcon.2016.12.004.

Elizalde-Velázquez, A., Martínez-Rodríguez, H., Galar-Martínez, M., Dublán-García, O., Islas-Flores, H., Rodríguez-Flores, J., Castañeda-Peñalvo, G., Lizcano-Sanz, I., & Gómez-Oliván, L. M. (2017). Effect of amoxicillin exposure on brain, gill, liver, and kidney of common carp (*Cyprinus carpio*): The

role of amoxicilloic acid. *Environmental Toxicology, 32*(4), 1102–1120. https://doi.org/10.1002/tox.22307.

Faghih, O., Zhang, Z., Ranade, R. M., Gillespie, J. R., Creason, S. A., Huang, W., Shibata, S., Barros-Álvarez, X., Verlinde, C. L. M. J., Hol, W. G. J., Fan, E., & Buckner, F. S. (2017). Development of methionyl-tRNA synthetase inhibitors as antibiotics for Gram-positive bacterial infections. *Antimicrobial Agents and Chemotherapy, 61*(11). https://doi.org/10.1128/AAC.00999-17.

Farré, M., Pérez, S., Kantiani, L., & Barceló, D. (2008). Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. *TrAC, 27*(11), 991–1007. https://doi.org/10.1016/j.trac.2008.09.010.

Fatta-Kassinos, D., Meric, S., & Nikolaou, A. (2011). Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. *Analytical and Bioanalytical Chemistry, 399*(1), 251–275. https://doi.org/10.1007/s00216-010-4300-9.

Fent, K., Weston, A. A., & Caminada, D. (2006). Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology, 76*(2), 122–159. https://doi.org/10.1016/j.aquatox.2005.09.009.

Fulda, S., Gorman, A. M., Hori, O., & Samali, A. (2010). Cellular stress responses: cell survival and cell death. *International Journal of Cell Biology, 2010*, 214074. https://doi.org/10.1155/2010/214074.

Gomes, M. P., Richardi, V. S., Bicalho, E. M., da Rocha, D. C., Navarro-Silva, M. A., Soffiatti, P., Garcia, Q. S., & Sant'Anna-Santos, B. F. (2019). Effects of ciprofloxacin and roundup on seed germination and root development of maize. *Science of the Total Environment, 651*(Pt 2), 2671–2678. https://doi.org/10.1016/j.scitotenv.2018.09.365.

Guérin, P., El Mouatassim, S., & Ménézo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reproduction Update, 7*(2), 175–189. https://doi.org/10.1093/humupd/7.2.175.

Haendel, M. A., Tilton, F., Bailey, G. S., & Tanguay, R. L. (2004). Developmental toxicity of the dithiocarbamate pesticide sodium metam in zebrafish. *Toxicological Sciences, 81*(2), 390–400. https://doi.org/10.1093/toxsci/kfh202.

Hallare, A. V., Schirling, M., Luckenbach, T., Köhler, H.-R., & Triebskorn, R. (2005). Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. *Journal of Thermal Biology, 30*(1), 7–17. https://doi.org/10.1016/j.jtherbio.2004.06.002.

Hermsen, S. A. B., van den Brandhof, E.-J., van der Ven, L. T. M., & Piersma, A. H. (2011). Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their *in vivo* potencies. *Toxicology In Vitro, 25*(3), 745–753. https://doi.org/10.1016/j.tiv.2011.01.005.

Hill, A. J., Bello, S. M., Prasch, A. L., Peterson, R. E., & Heideman, W. (2004). Water permeability and TCDD-induced edema in zebrafish early-life stages. *Toxicological Sciences, 78*(1), 78–87. https://doi.org/10.1093/toxsci/kfh056.

Hill, A. J., Teraoka, H., Heideman, W., & Peterson, R. E. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicological Sciences, 86*(1), 6–19. https://doi.org/10.1093/toxsci/kfi110.

Hirsch, R., Ternes, T. A., Haberer, K., Mehlich, A., Ballwanz, F., & Kratz, K.-L. (1998). Determination of antibiotics in different water compartments via liquid chromatography-electrospray tandem mass spectrometry. *Journal of Chromatography. A, 815*(2), 213–223. https://doi.org/10.1016/S0021-9673(98)00335-5.

Jiang, Z. Y., Hunt, J. V., & Wolff, S. P. (1992). Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Analytical Biochemistry, 202*(2), 384–389. https://doi.org/10.1016/0003-2697(92)90122-N.

Kaseva, M. E., Mwegoha, W. J. S., Kihampa, C., & Matiko, S. (2008). Performance of a waste stabilization pond system treating domestic and hospital wastewater and its implications to the aquatic environment-a case study in Dar es Salaam, Tanzania. *Journal of Building and Land Development, 15*(1-2), 76–88.

Kasprzyk-Hordern, B., Dinsdale, R. M., & Guwy, A. J. (2008). Multi-residue method for the determination of basic/neutral pharmaceuticals and illicit drugs in surface water by solid-phase extraction and ultra-performance liquid chromatography-positive electrospray ionisation tandem mass spectrometry. *Journal of Chromatography. A, 1161*(1-2), 132–145. https://doi.org/10.1016/j.chroma.2007.05.074.

Khazaei, M., & Aghaz, F. (2017). Reactive oxygen species generation and use of antioxidants during *in vitro* maturation of oocytes. *International Journal of Fertility and Sterility, 11*(2), 63–70. https://doi.org/10.22074/ijfs.2017.4995.

Kim, Y., Choi, K., Jung, J., Park, S., & Kim, P. -., & Park, J. (2007). Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea. *Environment International, 33*(3), 370–375. https://doi.org/10.1016/j.envint.2006.11.017.

Kim, C., Ryu, H.-D., Chung, E. G., & Kim, Y. (2018). Determination of 18 veterinary antibiotics in environmental water using high-performance liquid chromatography-Q-Orbitrap combined with on-line solid-phase extraction. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences, 1084*, 158–165. https://doi.org/10.1016/j.jchromb.2018.03.038.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics 203*, 253–310. https://doi.org/10.1002/aja.1002030302.

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell, 130*(5), 797–810. https://doi.org/10.1016/j.cell.2007.06.049.

Kohanski, M. A., DePristo, M. A., & Collins, J. J. (2010). Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Molecular Cell, 37*(3), 311–320. https://doi.org/10.1016/j.molcel.2010.01.003.

Kormoczi, G. F., Wölfel, U. M., Rosenkranz, A. R., Hörl, W. H., Oberbauer, R., & Zlabinger, G. J. (2001). Serum proteins modified by neutrophil-derived oxidants as mediators of neutrophil stimulation. *Journal of Immunology, 167*(1), 451–460. https://doi.org/10.4049/jimmunol.167.1.451.

Lammer, E., Carr, G. J., Wendler, K., Rawlings, J. M., Belanger, S. E., & Braunbeck, T. (2009). Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology, 149*(2), 196–209. https://doi.org/10.1016/j.cbpc.2008.11.006.

Lawrence, J. W., Claire, D. C., Weissig, V., & Rowe, T. C. (1996). Delayed cytotoxicity and cleavage of mitochondrial DNA in ciprofloxacin-treated mammalian cells. *Molecular Pharmacology, 50*(5), 1178–1188.

Locatelli, M. A. F., Sodré, F. F., & Jardim, W. F. (2011). Determination of antibiotics in Brazilian surface waters using liquid chromatography-electrospray tandem mass spectrometry. *Archives of Environmental Contamination and Toxicology, 60*, 385–393. https://doi.org/10.1007/s00244-010-9550-1.

Loos, R., Marinov, D., Sanseverino, I., Napierska, D., & Lettieri, T. (2018). *Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List*. Publications Office of the European Union 268 pp.

Lowes, D. A., Wallace, C., Murphy, M. P., Webster, N. R., & Galley, H. F. (2009). The mitochondria targeted antioxidant MitoQ protects against fluoroquinolone-induced oxidative stress and mitochondrial membrane damage in human Achilles tendon cells. *Free Radical Research, 43*(3), 323–328. https://doi.org/10.1080/10715760902736275.

Ma, C., Yang, X., & Lewis, P. J. (2016). Bacterial transcription as a target for antibacterial drug development. *Microbiology and Molecular Biology Reviews, 80*(1), 139–160. https://doi.org/10.1128/MMBR.00055-15.

Manaia, C. M. (2017). Assessing the risk of antibiotic resistance transmission from the environment to humans: Non-direct proportionality between abundance and risk. *Trends in Microbiology, 25*(3), 173–181. https://doi.org/10.1016/j.tim.2016.11.014.

Misra, H. P., & Fridovich, I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *The Journal of Biological Chemistry, 247*(10), 3170–3175. https://doi.org/10.1016/S0021-9258(19)45228-9.

Mu, X., Pang, S., Sun, X., Gao, J., Chen, J., Chen, X., Li, X., & Wang, C. (2013). Evaluation of acute and developmental effects of difenoconazole via multiple stage zebrafish assays. *Environmental Pollution, 175*, 147–157. https://doi.org/10.1016/j.envpol.2012.12.029.

Muramoto, S. (1981). Vertebral column damage and decrease of calcium concentration in fish exposed experimentally to cadmium. *Environmental Pollution Series A, Ecological and Biological, 24*(2), 125–133. https://doi.org/10.1016/0143-1471(81)90074-X.

Mutiyar, P. K., & Mittal, A. K. (2013). Occurrences and fate of an antibiotic amoxicillin in extended aeration-based sewage treatment plant in Delhi, India: a case study of emerging pollutant. *Desalination and Water Treatment, 51*(31-33), 6158–6164. https://doi.org/10.1080/19443994.2013.770199.

Naidu, R., Arias, E. V. A., Liu, Y., & Jit, J. (2016). Emerging contaminants in the environment: Risk-based analysis for better management. *Chemosphere, 154*, 350–357. https://doi.org/10.1016/j.chemosphere.2016.03.068.

OECD (2013). Test No. 236: Fish Embryo Acute Toxicity (FET) Test. Guidelines for the testing of chemicals, section 2. https://doi.org/10.1787/9789264203709-en.

Oettl, K., & Stauber, R. E. (2007). Physiological and pathological changes in the redox state of human serum albumin critically influence its binding properties. *British Journal of Pharmacology, 151*(5), 580–590. https://doi.org/10.1038/sj.bjp.0707251.

Oliveira, R., McDonough, S., Ladewig, J. C. L., Soares, A. M. V. M., Nogueira, A. J. A., & Domingues, I. (2013). Effects of oxytetracycline and amoxicillin on development and biomarkers activities of zebrafish (*Danio rerio*). *Environmental Toxicology and Pharmacology, 36*(3), 903–912. https://doi.org/10.1016/j.etap.2013.07.019.

Orozco-Hernández, J. M., Gómez-Oliván, L. M., Heredia-García, G., Luja-Mondragón, M., Islas-Flores, H., SanJuan-Reyes, N., Galar-Martínez, M., García-Medina, S., & Dublán-García, O. (2019). Genotoxic and cytotoxic alterations induced by environmentally-relevant concentrations of amoxicillin in blood cells of *Cyprinus carpio*. *Chemosphere, 236*, 124323. https://doi.org/10.1016/j.chemosphere.2019.07.054.

Ozolins, T. R., & Hales, B. F. (1997). Oxidative stress regulates the expression and activity of transcription factor activator protein-1 in rat conceptus. *The Journal of Pharmacology and Experimental Therapeutics, 280*(2), 1085–1093.

Park, S., & Choi, K. (2008). Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology, 17*(6), 526–538. https://doi.org/10.1007/s10646-008-0209-x.

Pašková, V., Hilscherová, K., & Bláha, L. (2011). Teratogenicity and embryotoxicity in aquatic organisms after pesticide exposure and the role of oxidative stress. *Reviews of Environmental Contamination and Toxicology, 211*, 25–61. https://doi.org/10.1007/978-1-4419-8011-3_2.

Praveena, S. M., Mohd Rashid, M. Z., Mohd Nasir, F. A., Sze Yee, W., & Aris, A. Z. (2019). Occurrence and potential human health risk of pharmaceutical residues in drinking water from Putrajaya (Malaysia). *Ecotoxicology and Environmental Safety, 180*, 549–556. https://doi.org/10.1016/j.ecoenv.2019.05.051.

Radi, R., Turrens, J. F., Chang, L. Y., Bush, K. M., Crapo, J. D., & Freeman, B. A. (1991). Detection of catalase in rat heart mitochondria. *The Journal of Biological Chemistry, 266*(32), 22028–22034. https://doi.org/10.1016/S0021-9258(18)54740-2.

Rao, R., Kaur, S. P., & Nanda, S. (2011). Amoxicillin: A broad-spectrum antibiotic. *International Journal of Pharmacy and Pharmaceutical Sciences, 3*(3), 30–37.

Reimers, M. J., Flockton, A. R., & Tanguay, R. L. (2004). Ethanol- and acetaldehyde-mediated developmental toxicity in zebrafish. *Neurotoxicology and Teratology, 26*(6), 769–781. https://doi.org/10.1016/j.ntt.2004.06.012.

Reyns, T., Cherlet, M., De Baere, S., De Backer, P., & Croubels, S. (2008). Rapid method for the quantification of amoxicillin and its major metabolites in pig tissues by liquid chromatography-tandem mass spectrometry with emphasis on stability issues. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences, 861*(1), 108–116. https://doi.org/10.1016/j.jchromb.2007.11.007.

Santos, L. H. M. L. M., Araujo, A. N., Fachini, A., Pena, A., Delerue-Matos, C., & Montenegro, M. C. B. S. M. (2010).

Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. *Journal of Hazardous Materials, 175*(1-3), 45–95. https://doi.org/10.1016/j.jhazmat.2009.10.100.

Sass, P., & Brotz-Oesterhelt, H. (2013). Bacterial cell division as a target for new antibiotics. *Current Opinion in Microbiology, 16*(5), 522–530. https://doi.org/10.1016/j.mib.2013.07.006.

Schlisser, A. E., Yan, J., & Hales, B. F. (2010). Teratogen-induced oxidative stress targets glyceraldehyde-3-phosphate dehydrogenase in the organogenesis stage mouse embryo. *Toxicological Sciences, 118*(2), 686–695. https://doi.org/10.1093/toxsci/kfq287.

Shi, X. J., & Zhou, B. (2010). The role of Nrf2 and MAPK pathways in PFOS-induced oxidative stress in zebrafish embryos. *Toxicological Sciences, 115*(2), 391–400. https://doi.org/10.1093/toxsci/kfq066.

Singh, V., Pandey, B., & Suthar, S. (2018). Phytotoxicity of amoxicillin to the duckweed Spirodela polyrhiza: Growth, oxidative stress, biochemical traits and antibiotic degradation. *Chemosphere, 201*, 492–502. https://doi.org/10.1016/j.chemosphere.2018.03.010.

Sinthuchai, D., Boontanon, S. K., Boontanon, N., & Polprasert, C. (2016). Evaluation of removal efficiency of human antibiotics in wastewater treatment plants in Bangkok, Thailand. *Water Science and Technology, 73*(1), 182–191. https://doi.org/10.2166/wst.2015.484.

Song, C., Gao, N.-Y., & Gao, H.-W. (2010). Transmembrane distribution of kanamycin and chloramphenicol: Insights into the cytotoxicity of antibacterial drugs. *Molecular BioSystems, 6*(10), 1901–1910. https://doi.org/10.1039/B921810F.

Straub, J. O., Gysel, D., Kastl, U., Klemmer, J., Sonderegger, M., & Studer, M. (2012). Environmental risk assessment for ancillary substances in biotechnological production of pharmaceuticals. *Environmental Toxicology and Chemistry, 31*, 681–687. https://doi.org/10.1002/etc.1733.

Test No. 236: Fish Embryo Acute Toxicity (FET) Test. (2013). *OECD Guidelines for the Testing of Chemicals, Section 2*. OECD.

Theodorakis, C. W., Swartz, C. D., Rogers, W. J., Bickham, J. W., Donnelly, K. C., & Adams, S. M. (2000). Relationship between genotoxicity, mutagenicity, and fish community structure in a contaminated stream. *Journal of Aquatic Ecosystem Stress and Recovery, 7*, 131–143. https://doi.org/10.1023/A:1009971330138.

van Eijk, E., Wittekoek, B., Kuijper, E. J., & Smits, W. K. (2017). DNA replication proteins as potential targets for antimicrobials in drug-resistant bacterial pathogens. *The Journal of Antimicrobial Chemotherapy, 72*(5), 1275–1284. https://doi.org/10.1093/jac/dkw548.

Wang, H., Che, B., Duan, A., Mao, J., Dahlgren, R. A., Zhang, M., Zhang, H., Zeng, A., & Wang, X. (2013). Toxicity evaluation of β-diketone antibiotics on the development of embryo-larval zebrafish (*Danio rerio*). *Environmental Toxicology, 29*(10), 1134–1146. https://doi.org/10.1002/tox.21843.

Wang, G., Zhou, S., Han, X., Zhang, L., Ding, S., Li, Y., Zhang, D., & Zarin, K. (2020). Occurrence, distribution, and source track of antibiotics and antibiotic resistance genes in the main rivers of Chongqing City, southwest China. *Journal of Hazardous Materials, 389*, 122110. https://doi.org/10.1016/j.jhazmat.2020.122110.

Watkinson, A. J., Murby, E. J., & Costanzo, S. D. (2007). Removal of antibiotics in conventional and advanced wastewater treatment: Implications for environmental discharge and wastewater recycling. *Water Research, 41*(18), 4164–4176. https://doi.org/10.1016/j.watres.2007.04.005.

Watkinson, A. J., Murby, E. J., Kolpin, D. W., & Costanzo, S. D. (2009). The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. *Science of the Total Environment, 407*(8), 2711–2723. https://doi.org/10.1016/j.scitotenv.2008.11.059.

Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., & Broschard, T. H. (2011). Zebrafish (*Danio rerio*) embryos as a model for testing proteratogens. *Toxicology, 281*(1-3), 25–36. https://doi.org/10.1016/j.tox.2011.01.004.

Weinberg, J. M., Bienholz, A., & Venkatachalam, M. A. (2016). The role of glycine in regulated cell death. *Cellular and Molecular Life Sciences, 73*(11-12), 2285–2308. https://doi.org/10.1007/s00018-016-2201-6.

World Health Organization (WHO). (2018). *WHO report on surveillance of antibiotic consumption 2016-2018 early implementation*. World Health Organization 127 pp.

Yamagami, K. (1981). Mechanisms of hatching in fish: Secretion of hatching enzyme and enzymatic choriolysis. *American Zoologist, 21*(2), 459–471. https://doi.org/10.1093/icb/21.2.459.

Yamashita, N., Yasojima, M., Nakada, N., Miyajima, K., Komori, K., Suzuki, Y., & Tanaka, H. (2006). Effects of antibacterial agents, levofloxacin and clarithromycin, on aquatic organisms. *Water Science and Technology, 53*(11), 65–72. https://doi.org/10.2166/wst.2006.338.

Yasser, E.-N., & Nabila, E. D. (2015). Toxicity of amoxicillin and erythromycin to fish and mosquitoes. *Ecotoxicology and Environmental Contamination, 10*(1), 13–21. https://doi.org/10.5132/eec.2015.01.03.

Yumnamcha, T., Roy, D., Devi, M. D., & Nongthomba, U. (2015). Evaluation of developmental toxicity and apoptotic induction of the aqueous extract of *Millettia pachycarpa* using zebrafish as model organism. *Toxicological and Environmental Chemistry, 97*(10), 1363–1381. https://doi.org/10.1080/02772248.2015.1093750.

Zhou, L., Limbu, S. M., Shen, M., Zhai, W., Qiao, F., He, A., Du, Z.-Y., & Zhang, M. (2018). Environmental concentrations of antibiotics impair zebrafish gut health. *Environmental Pollution, 235*, 245–254. https://doi.org/10.1016/j.envpol.2017.12.073.

Zuccato, E., Castiglioni, S., Bagnati, R., Melis, M., & Fanelli, R. (2010). Source, occurrence and fate of antibiotics in the Italian aquatic environment. *Journal of Hazardous Materials, 179*(1-3), 1042–1048. https://doi.org/10.1016/j.jhazmat.2010.03.110.