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Research Article

Toxicity of secondary treated sewage disinfected with chlorine gas and hypochlorite to zebrafish *Danio rerio*

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ABSTRACT. The sewage contains toxic chemical compounds that secondary treatment plants do not eliminate, and chlorine is usually added for disinfection before discharge. Chlorine reacts with sewage compounds forming other toxic compounds. The objective of this study was to evaluate the toxicity of treated sewage from a secondary treatment plant using *Danio rerio* embryos. Three types of treated sewage were tested, one disinfected with chlorine gas (dCl_2), another with sodium hypochlorite (dClO), and the plant discharge, which is a mixture of the two sewage disinfection methods (mCl) with a proportion of 70% of dCl_2 and 30% of dClO. To estimate the median lethal dilution treated sewage was diluted with dechlorinated tap water at 20, 40, 60, and 80%. Two additional points were made with pure treated sewage and pure dechlorinated tap water. Embryos were exposed 1 h after fertilization (hpf) to 144 hpf. The dCl_2 and the mCl were lethal at 60 and 72 hpf, respectively, while the dClO did not show lethality. The embryos exposed to the mCl showed heart failure and slower blood circulation. Those exposed to dCl_2 showed teratogenic effects such as pericardial edema and spinal curvature, while those exposed to dClO presented malformations such as incomplete eye development and otolith formation absence.

Keywords: *Danio rerio*; chlorine; disinfection; secondary treated sewage; teratogenesis

INTRODUCTION

In Mexico, there are around 2526 municipal sewage treatment plants, and 52.9% use secondary activated sludge treatment (CONAGUA 2018), consisting of the removal of solid waste, biological oxidation, sedimentation, and disinfection with chlorine (SEAPAL 2018). Disinfection is done with chlorine gas (dCl2), sodium hypochlorite (dClO), or calcium hypochlorite. Chlorine reacts with water forming hydrochloric and hypochlorous acid; both are corrosive substances that damage the integrity of cells, enzymes, and nucleic acids (Kozari et al. 2020).

Sewage treatment plants are built to protect the environment from various pollutants. However, they stay one of the principal sources of contamination of water bodies (Papa et al. 2016). The treated sewage contains cosmetics, pharmaceuticals, pesticides, and other substances. It is a complex mixture of several compounds (Sperling 2007) that disrupt the physiology of aquatic organisms (Brar et al. 2010). It can accumulate in the food chain, threatening higher trophic levels downstream from treatment plants, including humans, through the consumption of crustaceans and fish (Babić et al. 2016). Additionally, these compounds react with the hydrochloric and hypochlorous acid used in disinfection forming new substances that could be more toxic (Li-Sha et al. 2007).

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In Mexico, the composition of the sewage discharges is mandatory by the Mexican official standard NOM-001-SEMARNAT-2021, which establishes the permissible limits of pollutants in wastewater discharges in receiving bodies owned by the nation. The treated sewage used in the present study meets the requirements of the standard mentioned above. Furthermore, the main economic activity in Puerto Vallarta is tourism, and manufacturing and transformation industries are absent. No heavy metals, cyanide, arsenic, and hexavalent chromium are present in sewage. The main load in raw sewage is organic matter.

The fish embryos are sensitive to toxic substances and are used as a test tool for harmful evaluation (McIntyre et al. 2014). The use of zebrafish (*Danio rerio*) embryos is beneficial due to their small size, transparency, and rapid development (Lele & Krone 1996). Their ontogeny and the molecular biology tools developed for their study allow a glimpse of the effects of toxins found in the environment (Nagel 2002).

In the present study, zebrafish embryos were exposed to treated sewage to assess its lethal toxicity and predict the potential damages in the bodies of water that receive the treated sewage.

MATERIALS AND METHODS

Source of secondary treated sewage

The treated sewage was obtained from the Norte-II sewage treatment plant in Puerto Vallarta, Jalisco. The plant utilizes the activated sludge process and has two operating units. One plant unit disinfects the wastewater with sodium hypochlorite (dClO) and the other with chlorine gas (dCl_2); a mixture of both (mCl), with a proportion of 30 and 70%, is discharged into the Ameca River (SEAPAL 2018). Treated sewage of each operation unit and the outfall were sampled to perform the toxicity tests. For the toxicity test, the samples were previously filtered by nitrocellulose membranes of 0.45 µm pore (Millipore™) to prevent biological contamination and aerated overnight for oxygenation. The treated sewage and tap water were added with 0.5 mL sterile thiosulfate solution at 4% to remove free chlorine and verified with a Hanna HI 83200 Multiparameter Ion Specific Meter because free chlorine is lethal for the embryos and adults at the concentrations used in disinfection.

Nitrites, nitrates, and reactive soluble phosphorus were determined according to Strickland & Parsons (1972), ammoniacal nitrogen was determined according to Solórzano (1969), and pH was measured with an Orion Star™ A211 potentiometer.

Cultivation and collection of embryos

Adult zebrafish (*Danio rerio*) were bought in a local pet store and kept in an aquarium of 200 L, with aquarium filters, constant aeration, and a photoperiod of 12:12 h light:dark. They were fed twice daily with TetraColor™ commercial flakes. Average ± standard deviation along the experimental period was obtained for temperature 24 ± 1.7°C, pH of 7.1 ± 0.3, the conductance of 601 ± 48 µS, and dissolved oxygen of 80 to 90% of saturation.

For obtaining embryos, six females and eight males were set separately for a week. A night before spawning, males and females were placed in a fish tank with a horizontal mesh in the middle to prevent cannibalism toward embryos. The spawning occurred at dawn, and the embryos were collected at one hpf with a glass siphon and rinsed with Hank's solution (Westerfield 2007). A stereoscopic transmission microscope AmScope of 20 and 40x was used to select embryos of one hpf of development and separate the unfertilized eggs.

Toxicity test

Three kinds of treated sewage were tested, one disinfected with dCl_2, another with dClO, and a mCl. Five proportions of each were analyzed with dechlorinated tap water (20, 40, 60, 80, and 100%). Dechlorinated tap water was the negative control. Ten milliliters of each proportion of treated sewage and the negative control were triplicated in Petri dishes with 15 embryos each. Mortality, defined as the coagulation of the embryo or loss of heartbeat, was recorded every 12 h through six days. The heart rate was measured visually with a stopwatch at 48 hpf for the embryos exposed to mCl. The test was carried out at room temperature (28 ± 2°C) and natural photoperiod.

Data analysis

The median lethal dilution ($LDil_{50}$) and standard error were calculated according to Miller & Tainter (1944). Pearson correlation analyses were performed to evaluate the relationship between $LDil_{50}$ and hours of exposure, the Student's *t*-test to determine the statistical significance of the correlation, and the Tukey HSD multiple comparison tests. All tests were evaluated with a significance level of $P < 0.05$.

RESULTS

Lethality

The dCl_2 was the most toxic for zebrafish (*Danio rerio*) embryos; it was lethal after 60 h of exposure. For the embryos exposed to dClO, no lethality was observed during the 144 h of exposure. The mCl was lethal after 72 h (Table 1). The $LDil_{50}$ of sewage dCl_2 shows an inverse correlation with exposure time with a Pearson coefficient of $R^2 = 0.94$ with a $P < 0.05$ by Student test (Fig. 1). The mCl shows an inverse correlation with a Pearson coefficient of $R^2 = 0.93$ with a $P < 0.05$ by student test (Fig. 2).

Cardiotoxicity

The heart rate of embryos at 48 hpf was measured. Those exposed to 20 and 40% of mCl had a higher heart rate, and those exposed to dilutions of 60 and 80% showed a lower heart rate than the negative control (Fig. 3). A decreased blood rate was perceived in the embryos with lower heart rates.

Teratogenesis

Besides the lethal outcome, exposition to dCl_2 exhibited teratogenic effects on zebrafish larvae: spinal curvature, pericardial edema, and heart malformation showing a ventricle with slight contractions and an elongated cord-shaped atrium were observed (Fig. 4a). Those exposed to dClO showed a spinal curvature (Fig. 4b), otolith absence, and incomplete eye development without retinal formation (Fig. 4c). The negative control (Fig. 4d) had normal development, as described by Kimmel et al. (1995).

Pooled replicates of the controls show a hatching success >90% and a post-hatch survival at two weeks post-fertilization ≥80% are part of the test acceptability criteria proposed by the OECD (2013). Figure 4 shows the images of the teratogenic effects observed, the incomplete development of the eye and without otolith formation was only once, and pericardial edema with and without spinal curvature was observed more frequently. However, embryos with malformations in the negative control were always absent.

The major components of treated sewage used in the experiments were nitrite, nitrate, ammoniacal nitrogen, and soluble reactive phosphorus; in Table 2 are shown the geometric mean of them and mean pH.

In Mexico, the composition of the sewage discharges is mandatory by the Mexican official standard NOM-001-SEMARNAT-2021, which establishes the permissible limits of pollutants in wastewater discharges in receiving bodies owned by the nation. The treated sewage used in the present study meets the requirements of the standard mentioned above. The concentration of metals and arsenic was lower than the detection limit of the standard's required measurement technique (SEAPAL, 2022).

Table 1. Median lethal dilution ($LDil_{50}$) ± standard error of the treated sewage disinfected with chlorine gas (dCl_2) and a mixture of sewage disinfected with sodium hypochlorite and chlorine gas (mCl) related to exposure times. *No death observed, **No data.

Exposition time (h)	$LDil_{50}$ ± standard error (%)	
	dCl_2	mCl
12 to 60	*	*
72	31.68 ± 1.07	75.87 ± 0.13
84	30.52 ± 1.08	74.31 ± 0.12
96	30.52 ± 1.08	64.44 ± 0.12
108	30.13 ± 1.08	**
120	28.59 ± 1.08	**
132	27.12 ± 1.09	8.82 ± 0.20
144	26.61 ± 1.09	**

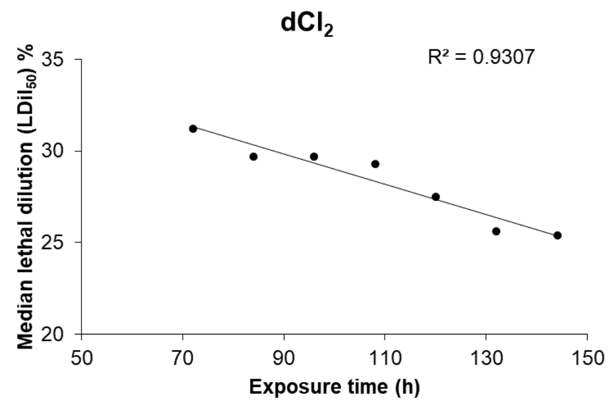

Figure 1. Median lethal dilution of treated sewage to zebrafish *Danio rerio* embryos related to the exposure time. dCl_2: disinfected sewage with chlorine gas. Pearson coefficient (R^2). Statistical significance calculated by t-Student test.

The composition of treated sewage for the NOM-001-SEMARNAT-2021 includes the concentration of fats and oils, total suspended solids, chemical, and biochemical oxygen demand, total nitrogen, arsenic, cadmium, cyanides, copper, chromium, mercury, nickel, lead, zinc, total and fecal coliforms, pH, floating matter, hexavalent chromium, barium, settleable solids, and methylene blue active substances.

DISCUSSION

Sánchez et al. (2021) describe teratogenic effects in *Danio rerio* by potassium dichromate at 12.5% lethal concentration (LC12.5) or less. The dCl_2 and mCl showed a lethal effect, and the dClO produced only teratogenesis. As described above, the plant has two operating units. The oldest one has the disinfection by Cl_2, the newest unit has the disinfection with NaClO,

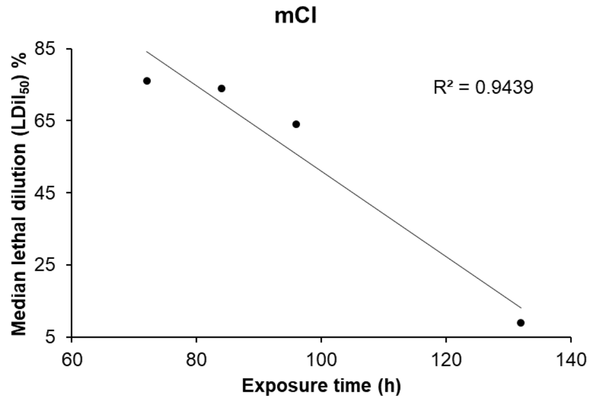

Figure 2. Median lethal dilution of treated sewage to zebrafish *Danio rerio* embryos related to the exposure time. mCl: mixture of disinfected sewage with chlorine gas and hypochlorite. Pearson coefficient (R^2). Statistical significance calculated by *t*-Student test.

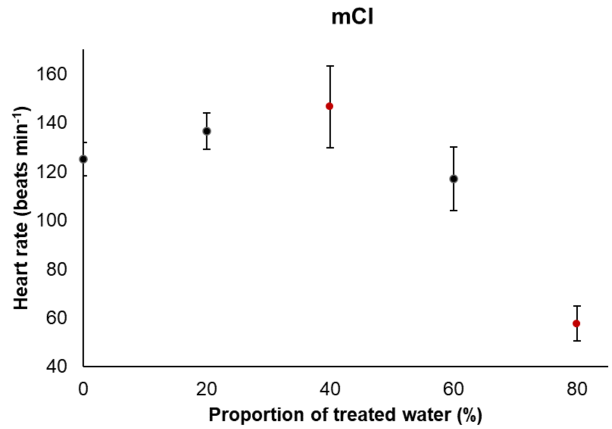

Figure 3. The heart rate of zebrafish *Danio rerio* embryos at 48 h of exposition, related to the dilution with dechlorinated tap water of the mCl (disinfected with chlorine gas 70% and sodium hypochlorite 30%). Red dots show significant differences with the negative control Tukey HSD test <0.05. n = 7×5.

and the plant effluent is mCl. Assuming that the composition of the treated effluents is almost the same in both operating units before disinfection, probably disinfection by-products by chlorine gas are more toxic than those produced with hypochlorite. Another possible explanation for the differences observed could be the efficiency of the treatment units; the oldest does not eliminate some substances responsible for the toxic effect, and the newest eliminates or decreases them, which is why no lethality was found.

Chlorine is used in large quantities for wastewater disinfection; however, few studies evaluate its toxicity in fish. Besides, it reacts with compounds found in wastewater, mainly ammonia formed from ammonium.

When chlorine gas reacts with water, it forms hydrochloric acid and hypochlorous acid, latter reacting with ammonia forms chloramines, which are more toxic than free chlorine, and 0.4 mg L^{-1} is sufficient to kill adult fish (Zillich 1972).

Various drugs and their metabolites have been found in municipal waters and sludge from treatment plants. Macrolide antibiotics have been shown to induce cardiotoxicity in zebrafish. At low concentrations, they cause tachycardia, and at high concentrations, they cause bradycardia (Yan et al. 2019). The cardiotoxicity found in mCl may be due to the presence of pharmaceutical products that alter the structure and function of the heart, resulting in pericardial edema and arrhythmias.

The heart rate of *D. rerio* is not fully defined. Kimmel et al. (1995) mention that the heart beats around 180 times per minute at 48 hpf. However, Barrionuevo & Burggren (1999) show that the heart rate depends on the temperature at which they develop and is constant trough embryonic development, obtaining that the heartbeat at 25, 28, and 31°C is 100, 125, and 150 beats min^{-1}, respectively and increases significantly at 10 days after fertilization. In this study, the control group had a heart rate of 125 ± 7 beats min^{-1}; instead, mCl caused an increase in heart rate at the lowest dilutions (20 and 40%) and a decrease in the highest dilutions (60 and 80%) in *D. rerio* embryos at 48 hpf.

Other drugs found in the aquatic environment, such as diclofenac, metoprolol, carbamazepine, and gemfibrozil, cause abnormalities in the embryonic development of zebrafish. Diclofenac causes deformation of the yolk sac and tail, metoprolol: causes scoliosis and developmental delay, and carbamazepine: causes pericardial edema and slow blood flow (Van den Brandhof & Montforts 2010). Gemfibrozil generates pericardial edema and delays hatching (Henriques et al. 2015). Most of these drugs are found in the basic table of public health in Mexico (Consejo de Salubridad General 2017) and reach the treatment plants through urine and fecal matter (Van den Brandhof & Montforts 2010). The drugs are probably not being eliminated properly, or chlorinated by-products are being generated in both units of the Norte II plant, which are not lethal but generate different teratogenic effects in zebrafish.

In treating activated sludge, the biological oxidation process is the essential step for drug removal. The elimination will depend on the sludge age and when residual water is retained (Fent et al. 2006). For example, naproxen is removed by more than 90% when

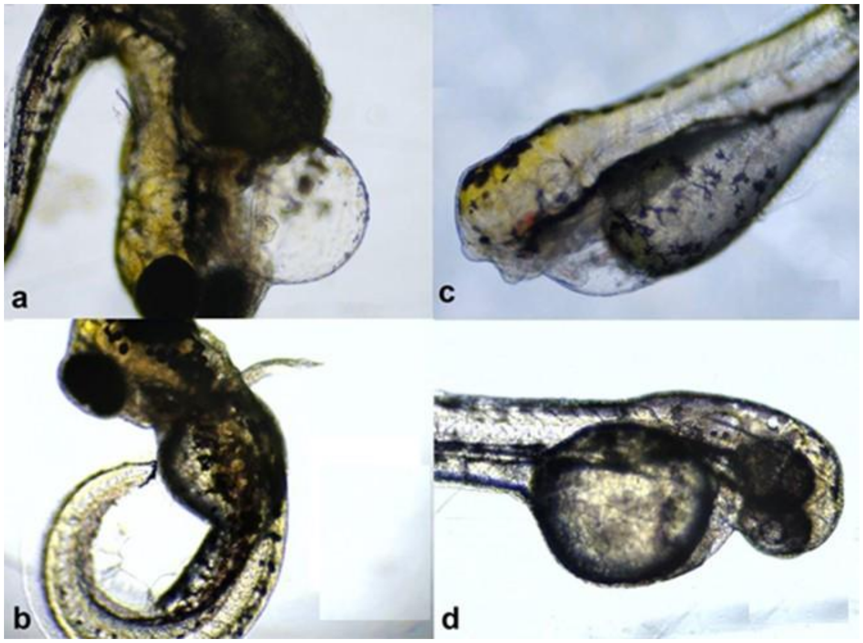

Figure 4. Teratogenic effects in zebrafish *Danio rerio* larvae exposed to treated sewage: a) pericardial edema, b) spinal curvature, c) incomplete eye development without otolith formation, d) control group.

water retention exceeds 12 h (Metcalfe et al. 2003); however, products such as carbamazepine are barely biodegradable, regardless of the water retention and the age of the sludge and go to bodies of water without change or as an active metabolite (Bessa et al. 2017). In the case of the North II Treatment Plant of SEAPAL Vallarta, the water retention time is around 5 h (SEAPAL 2022).

High concentrations of ammoniacal nitrogen in the treated sewage analyzed and its toxicity to the fishes (Randall & Tsui 2002) could explain the lethality and teratogenesis observed. The lethality occurred only after hatching. The chorion is permeable to this compound (Braun et al. 2009a); probably, the change of metabolic waste in embryos to larva is involved; because the embryos are ureotelic and larva ammoniotelic (Braun et al. 2009b). The protection of the chorion for its selective permeability probably protects the embryo from some of the toxic substances in the treated sewage (Pelka et al. 2017).

Ammoniacal nitrogen is present in two forms, non-toxic ammonium (ionized) and toxic ammonia (non-ionized). The proportion of ionized and non-ionized was calculated according to Emerson et al. (1975) using the geometric mean of ammoniacal nitrogen concentration and pH of Table 2. The pKa = 9.25, the estimated mean concentration of ammonia in treated sewage is 0.24 mg L^{-1}, and according to the $LDil_{50}$ at 72 h 31.7%, the concentration of ammonia and ammonium could be estimated as 0.076 and 2.21 mg L^{-1}, respectively. Toxic ammonia concentration for *D. rerio* is 0.532 mg L^{-1} at 72 h for adults (Ebrahimi et al. 2017), and the concentration of NH_4Cl of 7 mg L^{-1} reduces the ammonia excretion in embryos of 4 to 5 hpf (Braun et al. 2009a).

Crebelli et al. (2005) analyzed the formation of mutagenic by-products in wastewater disinfected with peracetic acid and sodium hypochlorite. They concluded that wastewater disinfection does not influence the production of genotoxic by-products. However, the results obtained in other studies indicate that disinfection with chlorine can be very toxic due to the generation of by-products that are lethal or teratogenic for *D. rerio*. This toxicity will depend on the region and the daily variation of the wastewater treated, so it would be advisable to eliminate chlorine as a wastewater disinfectant and replace it with other less harmful to reduce damage to ecosystems.

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REFERENCES

Babić, S., Barišić, J., Bielen, A., Bošnjak, I., Klobučar, R.S., Ujević, I., et al. 2016. Multilevel ecotoxicity assessment of environmentally relevant bisphenol a concentration using the soil invertebrate *Eisenia fetida*. Journal of Hazardous Materials, 318: 477-486.

Barrionuevo, W.R. & Burggren, W.W. 1999. O_2 consumption and heart rate in developing zebrafish (*Danio rerio*): influence of temperature and ambient O_2. American Journal of Physiology, 276: 505-513.

Bessa, V.S., Moreira, I.S., Tiritan, M.E. & Castro, P.M. 2017. Enrichment of bacterial strains for the biodegradation of diclofenac and carbamazepine from activated sludge. International Biodeterioration and Biodegradation, 120: 135-142.

Brar, S.K., Verma, M., Tyagi, R. & Surampalli, R. 2010. Engineered nanoparticles in wastewater and wastewater sludge e evidence and impacts. Waste Management, 30: 504-520.

Braun, M.H., Steele, S.L., Ekker, M. & Perry, S.F. 2009a. Nitrogen excretion in developing zebrafish (*Danio rerio*): a role for Rh proteins and urea transporters. American Journal of Physiology-Renal Physiology, 296: 994-1005.

Braun, M.H., Steele, S.L., Ekker, M. & Perry, S.F. 2009b. The responses of zebrafish (*Danio rerio*) to high external ammonia and urea transporter inhibition: nitrogen excretion and expression of rhesus glycoproteins and urea transporter proteins. Journal of Experimental Biology, 212: 3846-3856.

Comisión Nacional del Agua (CONAGUA). 2018. Infraestructura hidráulica. In: Comisión Nacional del Agua (Eds.). Estadísticas del agua en México. Secretaría de Medio Ambiente y Recursos Naturales, Ciudad de México, pp. 100-132.

Consejo de Salubridad General. 2017. Cuadro básico y catálogo de medicamentos. Comisión Interinstitucional del Cuadro Básico y Catálogo de Insumos del Sector Salud, Ciudad de México.

Crebelli, R., Conti, L., Monarca, S., Feretti, D., Zerbini, I., Zani, C., et al. 2005. Genotoxicity of the disinfection by-products resulting from peracetic acid- or hypochlorite-disinfected sewage wastewater. Water Research, 39: 1105-1113.

Ebrahimi, E., Alishiri, M. & Malekpouri, P. 2017. Lethal concentration of ammonia and tolerable range of pH in zebrafish (*Danio rerio*). Journal of Aquatic Ecology, 7: 145-152.

Emerson, K., Ruso, R.C., Lund, R.E. & Thurston, R.V. 1975. Aqueous ammonia equilibrium calculations: effect of pH and temperature. Journal of the Fisheries Research Board of Canada, 32: 2379-2383.

Fent, K., Weston, A.A. & Caminada, D. 2006. Ecotoxicology of human pharmaceuticals. Aquatic Toxicology, 7: 122-159.

Henriques, J., Almeida, A., Andrade, T., Koba, O., Golovko, O., Soares, A., et al. 2015. Effects of the lipid regulator drug gemfibrozil: a toxicological and behavioral perspective. Aquatic Toxicology, 170: 355-364.

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.

Kozari, A., Paloglou, A. & Voutsa, D. 2020. Formation potential of emerging by-products during ozonation and chlorination of sewage effluents. Science of the Total Environment, 700: 1-10.

Lele, Z. & Krone, P. 1996. The zebrafish as a model system in developmental, toxicological and transgenic research. Biotechnology Advances, 14: 57-72.

Li-Sha, W., Hong-Yi, H. & Chao, W. 2007. Effect of ammonia nitrogen and dissolved organic matter fractions on the genotoxicity of wastewater effluent during chlorine disinfection. Environmental Science and Technology, 41: 160-165.

Metcalfe, C.D., Miao, X.S., Koenig, B.G. & Struger, J. 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower great lakes, Canada. Environmental Toxicology and Chemistry, 22: 2881-2889.

McIntyre, J.K., Davis, J.W., Incardona, J.P., Stark, J.D., Anulacion, B.F. & Scholz, N.L. 2014. Zebrafish and clean water technology: assessing soil bioretention as a protective treatment for toxic urban runoff. Science of the Total Environment, 500-501: 173-180.

Miller, L.C. & Tainter, M.L. 1944. Estimation of LD_{50} and its error by means of log-probit graph paper. Experimental Biology and Medicine, 4: 261-264.

Nagel, R. 2002. DarT: the embryo test with the zebrafish *D. rerio* - a general model in ecotoxicology and toxicology. Alternatives to Animal Experimentation, 19: 38-48.

Organización para la Cooperación y el Desarrollo Económicos (OCDE). 2013. Test No. 236: fish embryo acute toxicity (FET) test. In: OECD Guidelines for the testing of chemicals. OECD Publishing, Paris, pp. 1-22.

Papa, M., Ceretti, E., Viola, G.C., Feretti, D., Zerbini, I., Mazzoleni, G., et al. 2016. The assessment of WWTP performance: towards a jigsaw puzzle evaluation? Chemosphere, 145: 291-300.

Pelka, K.E., Henn, K., Keck, A., Sapel, B. & Braunbeck, T. 2017. Size does matter - Determination of the critical molecular size for the uptake of chemicals across the chorion of zebrafish (*Danio rerio*) embryos. Aquatic Toxicology, 185: 1-10.

Randall, D.J. & Tsui, T.K. 2002. Ammonia toxicity in fish. Marine Pollution Bulletin, 45: 17-23.

Sánchez-Olivares, M.A., Gaytán-Oyarzun, J.C., Gordillo-Martínez, A.J., Prieto-Garcia, F. & Cabrera-Cruz, R.B. 2021. Toxicity and teratogenicity in zebrafish *Danio rerio* embryos exposed to chromium. Latin American Journal of Aquatic Research, 49: 289-298.

Sistema de Servicios de Agua Potable (SEAPAL). 2018. Cómo funciona. [https://www.seapal.gob.mx/como-funciona/]. Reviewed: December 18, 2021.

Sistema de Servicios de Agua Potable (SEAPAL). 2022. Operations management, personal communication. SEAPAL Vallarta, Puerto Vallarta.

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Solórzano, L. 1969. Determination of ammonia in natural waters by the phenol hypochlorite method. Limnology and Oceanography, 14: 799-801.

Sperling, M. 2007. Wastewater characteristics, treatment, and disposal. IWA Publishing, London.

Strickland, J.D. & Parsons, T.R. 1972. A practical handbook of seawater analysis. Ministry of Supply and Services, Ottawa.

Van den Brandhof, E. & Montforts, M. 2010. Fish embryo toxicity of carbamazepine, diclofenac, and metoprolol. Ecotoxicology and Environmental Safety, 73: 1862-1866.

Westerfield, M. 2007. The zebrafish book: a guide for the laboratory use of zebrafish (*Danio rerio*). University of Oregon, Oregon.

Yan, Z., Huang, X., Xie, Y., Song, M., Zhu, K. & Ding, S. 2019. Macrolides induce severe cardiotoxicity and developmental toxicity in zebrafish embryos. Science of the Total Environment, 649: 1414-1421.

Zillich, J.A. 1972. Toxicity of combined chlorine residuals to freshwater fish. Journal of Water Pollution and Control, 44: 212-220.