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 (dCl₂), 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 dCl₂ 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 dCl₂ 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 dCl₂ 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).
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 (dCl₂); 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 dCl₂, 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 (LD₅₀) and standard error were calculated according to Miller & Tainter (1944). Pearson correlation analyses were performed to evaluate the relationship between LD₅₀ 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 dCl₂ 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 LD150 of sewage dCl2 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 dCl2 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 (LD150) ± standard error of the treated sewage disinfected with chlorine gas (dCl2) and a mixture of sewage disinfected with sodium hypochlorite and chlorine gas (mCl) related to exposure times.

<table>
<thead>
<tr>
<th>Exposition time (h)</th>
<th>LD150 ± standard error (%)</th>
<th>dCl2</th>
<th>mCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 to 60</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>72</td>
<td>31.68 ± 1.07</td>
<td>75.87 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>84</td>
<td>30.52 ± 1.08</td>
<td>74.31 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>30.52 ± 1.08</td>
<td>64.44 ± 0.12</td>
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</tr>
<tr>
<td>108</td>
<td>30.13 ± 1.08</td>
<td>**</td>
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</tr>
<tr>
<td>120</td>
<td>28.59 ± 1.08</td>
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<tr>
<td>132</td>
<td>27.12 ± 1.09</td>
<td>8.82 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>26.61 ± 1.09</td>
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**DISCUSSION**

Sánchez et al. (2021) describe teratogenic effects in Danio rerio by potassium dichromate at 12.5% lethal concentration (LC12.5) or less. The dCl2 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 Cl2, the newest unit has the disinfection with NaClO,
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 react 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 \textit{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 \textit{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 \( \text{pKa} = 9.25 \), the estimated mean concentration of ammonia in treated sewage is 0.24 mg L\(^{-1}\), and according to the \( \text{LDI}_{50} \) 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\(_4\)Cl 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


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