Colegio de Postgraduados

Acute toxicity induced by treated sewage to zebrafish (*Danio rerio*)

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ABSTRACT

Objective: Acute toxicity markers in zebrafish caused by exposure to treated sewage from two sewage treatment plants were evaluated in order to obtain accurate and relevant information on the potential environmental health risks associated with these waters.

Design/methodology/approach: The acute toxicity assessment was performed according to the guidelines proposed by the Organization for Economic Cooperation and Development (OECD). Five proportions of each type of water treated with dechlorinated tap water (20, 40, 60, 60, 80 and 100%) were tested to determine the Median Lethal Dilution ($LDil_{50}$). The dechlorinated tap water was the negative control and as a positive control potassium dichromate at Mean Lethal Concentration (0.065 mg/L). The description of the teratogenic effects was carried out using the spine biomarker, which consists of dividing the fish into three sections to identify the damaged area.

Results: The results revealed that the mixture of treated sewage (mCl) and treated sewage disinfected with sodium hypochlorite (NaClO) caused toxic effects in zebrafish embryos. On the other hand, treated sewage from the Macroplanta de Bahía de Banderas (BBdClO) was not toxic at 96 hours of exposure.

Limitations on study/implications: It would be advisable to characterize the treated sewage from these two sewage treatment plants.

Findings/conclusions: These findings underscore the importance of assessing the toxicity of treated sewage and its short- and long-term effects on aquatic organisms, providing valuable information for water resource management and environmental protection.

Keywords: *Danio rerio*, toxicity, treated sewage.

Citation: Ponce-Palomera, K. O., Guerrero-Galván, S. R., Martínez-Cárdenas, L., Vega-Villasante, F., Rojas-García, A. E., & Badillo-Zapata, D. (2024). Acute toxicity induced by tread ed sewage to zebrafish (*Danio rerio* Hamilton-Buchanan). *Agro Productividad*. https://doi.org/10.32854/agrop.v17i3.2836

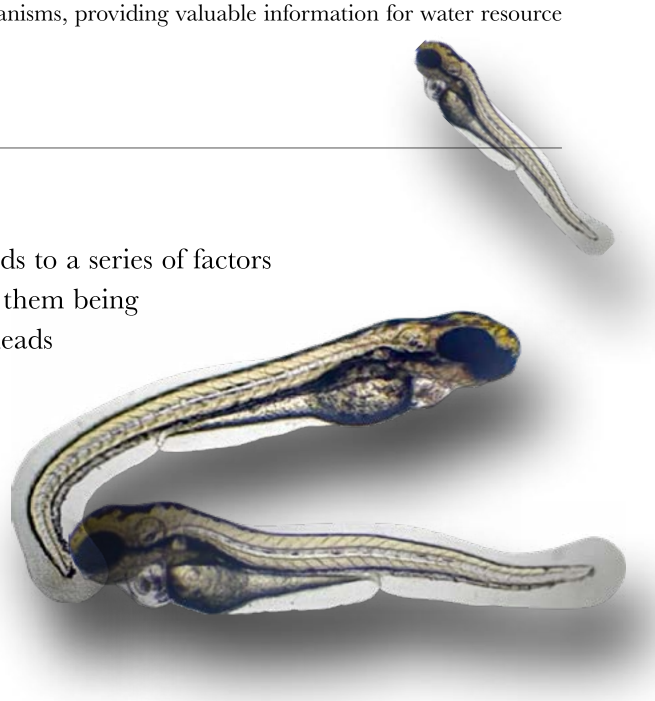
Academic Editors: Jorge Cadena Iñiguez and Lucero del Mar Ruiz Posadas
Guest Editor: Daniel Alejandro Cadena Zamudio

Received: December 16, 2023.
Accepted: February 22, 2024.
Published on-line: April 11, 2024.

Agro Productividad, 17(3). March. 2024. pp: 133-142.

INTRODUCTION

The increase in human population leads to a series of factors that contribute to water pollution, one of them being the excessive use of drinking water that leads to the generation of sewage that ends up in treatment plants and is subsequently discharged into the environment (Sayo *et al.*, 2020). Sewage treatment plants (WWTPs) were designed to treat sewage from urbanization;



however, they do not completely remove all pollutants (Papa *et al.*, 2016). The removal efficiency of these pollutants is going to depend on each type of treatment, even so, the removal efficiency of the treatments is minimal, due to the fact that the WWTPs are not well equipped (Deblonde *et al.*, 2011; Zhou *et al.*, 2019).

Treated sewage contains two major groups of pollutants, called traditional pollutants and emerging pollutants (Feng *et al.*, 2022). Traditional pollutants are those whose toxic effects are known and studied; within this group we find heavy metals with toxicity potential. Emerging pollutants are those whose presence is recent in the environment and their toxic effects are not fully known; for example, pharmaceuticals and personal hygiene products, surfactants, pesticides, among others (Ahmed *et al.*, 2017; Von Sperling, 2007; Zhou *et al.*, 2019). Most of these xenobiotics and their active metabolites can cause adverse effects on aquatic organisms, a part of these compounds accumulates in aquatic tissues and pose a threat to both the ecosystem and humans (Babić *et al.*, 2017; Brar *et al.*, 2010). The persistence of these compounds in the environment depends largely on their chemical properties such as liposolubility, volatility, adsorption and low biodegradation, these characteristics also prevent sewage treatments from being more efficient for their removal (Robledo Zacarías *et al.*, 2017).

Moreover, treated sewage contains high concentrations of ammonium from human urine and fecal matter, and WWTPs use chlorine in the form of sodium hypochlorite and/or chlorine gas for water disinfection (Albolafio *et al.*, 2022). By-products are also generated from the reaction of chlorine with compounds found in water and are sometimes more toxic than the original compounds (Huang *et al.*, 2021).

Treated sewage has been shown to induce various toxic effects in zebrafish embryos (Garcia-Cambero *et al.*, 2019). Zebrafish is a very reliable model for toxicity assessment of treated sewage due to characteristics such as sensitivity, transparent embryonic development, well-characterized genetics, and its ability to represent the effects of toxicants found in aquatic systems (Nagel, 2002). The use of this model can provide information on potential risks to human health and the environment associated with treated sewage (Porretti *et al.*, 2022).

In Mexico, the composition of sewage discharges is regulated by the official Mexican standard NOM-001-SEMARNAT-2021, which establishes the permissible limits of pollutants in these discharges into receiving bodies owned by the nation. The treated sewage used in this study meets the requirements of the aforementioned standard. It is important to point out that the main economic activity in Puerto Vallarta is tourism, while manufacturing and transformation industries are absent. Therefore, there are no heavy metals, cyanide, arsenic and hexavalent chromium in the sewage (Ponce-Palomera *et al.*, 2022).

This work represents one of the first efforts in evaluating the toxicity of treated sewage in the region, markers of acute toxicity in zebrafish caused by exposure to treated sewage from two WWTPs were evaluated in order to obtain accurate and relevant information on the potential environmental health risks associated with treated sewage, which in turn guides decision-making in water resource management and environmental protection.

MATERIALS AND METHODS

Source of secondary treated sewage

The treated sewage was obtained from the Norte-II sewage treatment plant in Puerto Vallarta, Jalisco, and from the Macroplanta in Bahía de Banderas, Nayarit. The Norte-II has two operating units and one disinfects sewage with sodium hypochlorite (NaClO) and the other with gaseous chlorine (dCl_2), and a mixture (mCl) of both with a ratio of 30% and 70%, respectively, is discharged into the Ameca River. Macroplanta, it only disinfects the water with sodium hypochlorite (BBdClO). Spot sampling was conducted in August 2022 for mCl, April 2023 for NaClO and May 2023 for BBdClO. For toxicity tests, free chlorine was measured with a specific multiparameter ion meter (Hanna HI 83200®) and removed with a 4% sterile sodium thiosulfate solution to avoid chlorine toxicity.

Cultivation and collection of embryos

To obtain embryos, nine females and six males (3:2 ratio) were separated for one week. One night before spawning, they were placed in an oviposition tank to avoid cannibalism towards the embryos. Spawning occurred at dawn and embryos were collected at one hour post fertilization (hpf) with a glass siphon and rinsed with Hank's solution (Westerfield, 2007). Using a stereo transmission microscope, embryos were selected from one hpf of development and unfertilized eggs were separated.

Toxicity test

The evaluation of acute toxicity was performed according to the guidelines proposed by the OECD (Organization for Economic Cooperation and Development) in section #236 entitled: Fish Embryo Acute Toxicity (FET) Test. Plastic well plates were replaced by glass Petri dishes to avoid adsorption to polystyrene (OECD, 2013). Three types of treated water were tested: NaClO, mCl and BBdClO. To determine the Median Lethal Dilution ($LDil_{50}$), five proportions of each type of treated water were analyzed with dechlorinated tap water (20, 40, 60, 60, 80 and 100%). The dechlorinated tap water was the negative control and as a positive control 3,4-dichloroaniline was substituted with potassium dichromate at the Median Lethal Concentration (0.065 mg/L). Ten milliliters of each proportion of treated sewage and each control were placed in triplicate in Petri dishes containing twenty embryos each. Mortality was recorded every twelve hours for 96 hours and was defined as embryo coagulation or loss of heartbeat. The test was carried out in an incubator at 28 °C.

The description of teratogenic effects was performed using the spine biomarker, which consists of dividing the fish into three sections to identify the damaged area (Castillo-Salas *et al.*, 2022; Gaytán-Oyarzún *et al.*, 2008; Olivares *et al.*, 2021). This description was only performed for mCl, 150 embryos were placed in Petri dishes in triplicate and the $LDil_{50}$ (88.13%) was added, observed at 72 hpf using a stereoscopic microscope and malformations were recorded.

Data analysis

The $LDil_{50}$ and its standard error were determined through the calculation of the Median Effective Dose (ED_{50}) described by Miller & Tainter, (1944). Pearson correlation analyses were performed to evaluate the relationship between $LDil_{50}$ and hours of exposure.

RESULTS

$K_2Cr_2O_7$ showed toxicity from 12 hours of exposure and the LC_{50} decreased over time. The LC_{50} of $K_2Cr_2O_7$ showed a negative logarithmic relationship with exposure time R^2=0.994, $p<0.05$ (Figure 1).

$LDil_{50}$ of mCl and NaClO were calculated, and $LDil_{50}$ at 96 hours were 88.13±9.55% and 36±3%, respectively. The $LDil_{50}$ data show an exponential relationship with exposure time R^2=0.8948, $p<0.05$ (Figure 2) and R^2=0.950, $p<0.05$ (Figure 3). On the other hand, BBdClO did not show toxicity at 96 h of exposure.

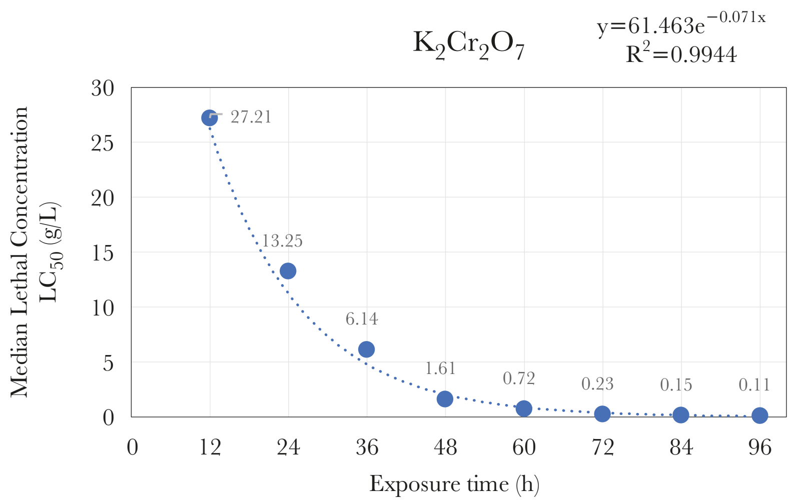


Figure 1. Median lethal concentration of potassium dichromate ($K_2Cr_2O_7$) for zebrafish embryos in relation to exposure time.

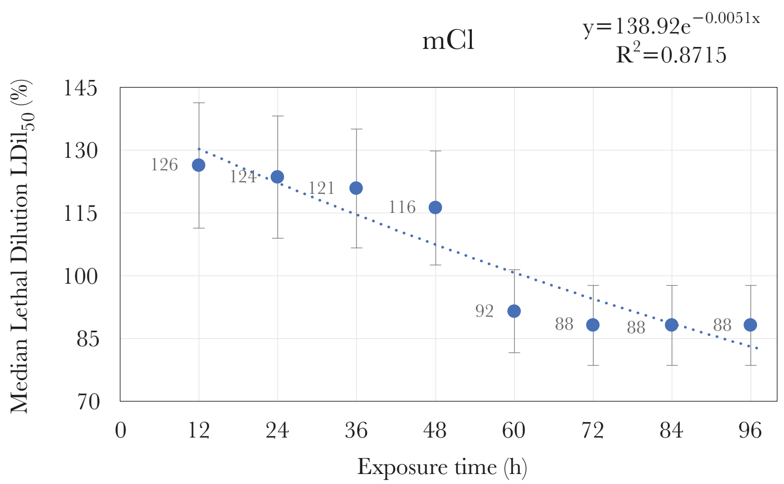


Figure 2. Median lethal dilution of mCl for zebrafish embryos in relation to exposure time. mCl: mixture of treated sewage disinfected with chlorine gas and sodium hypochlorite.

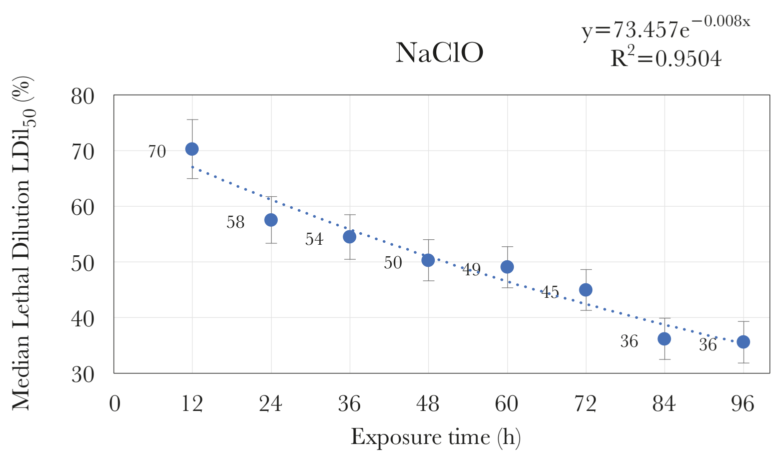


Figure 3. Median lethal dilution of NaClO for zebrafish embryos in relation to exposure time. NaClO: treated sewage disinfected with sodium hypochlorite.

The proportion of malformations was calculated (Figure 4) taking into account the spine biomarker. The results showed early and late malformations, as well as cardiac alterations at 72 hpf. For this, only $LDil_{50}$ 88.13% of mCl was taken and a very low proportion of these events was observed; total malformations represented 12 %. Only one type of early malformations was observed, which was the hook malformation and represented a proportion of less than 3%. As for late malformations, all those proposed by Sánchez-Olivares *et al.* (2021) were found, the most frequent being caudal fin malformation and double malformation, which represented 2 and 1.56%, respectively. Regarding cardiac alterations, a higher proportion of pericardial edema was found with 3.78%, which was the most prevalent effect and no yolk sac edema was observed. The control group had a normal development.

On the other hand, embryos exposed to half of the $LDil_{50}$ (22.5%) of NaClO showed low proportions of malformations. As for early malformations, only hook malformation was found with less than 1%. Late malformations were simple and curve malformations

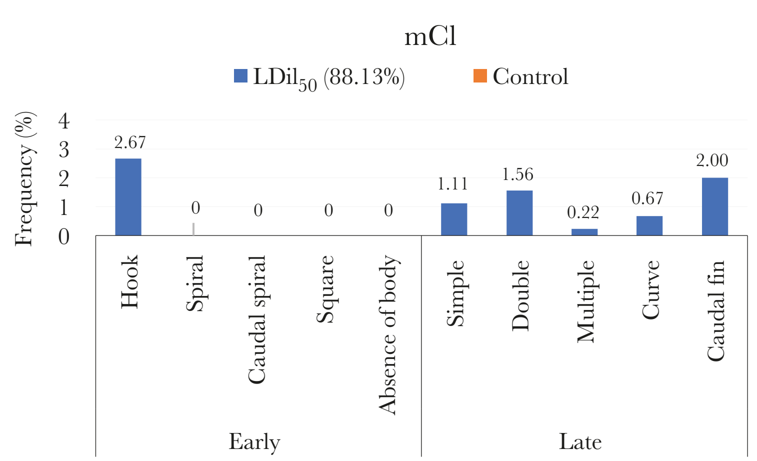


Figure 4. Frequency of malformations at 72 hours post-fertilization after exposure to the treated water mixture (mCl).

representing 3.33% and 2.2% respectively. As for cardiac alterations, they were found in a higher proportion compared to mCl. It is worth mentioning that, although it is not a malformation, almost half of the exposed embryos did not hatch at 72 hours. Total malformations represented 12.5%. The control group had normal development. Finally, 100% BBdClO did not present malformations in zebrafish development at 72 hours of exposure.

Photographs were taken of the malformations of zebrafish embryos exposed to mCl. Figure 5 shows the early malformations and cardiac alterations. Hook malformation (Figure 5A) which was the only early malformation observed, normal embryo development (Figure 5B) and pericardial edema (Figure 5C) consisting of water entering the pericardial zone.

Figure 6 shows the late malformations, the single malformation (Figure 6A) consists of a single malformation of the spine in the cephalic area, double malformation (Figure 6B) with two malformations of the spine in the cephalic and central area, and multiple malformation (Figure 6C) consisting of several malformations of the spine in the three divisions of the fry's body. Curved malformation (Figure 6D), the fry's body turns to one side or the other preventing correct swimming, and finally caudal fin malformation (Figure 6E), in which the caudal fin is affected and bends upwards, downwards or to the sides.

DISCUSSION

For this study, the Median lethal concentration (LC_{50}) of potassium dichromate ($K_2Cr_2O_7$) at 72 hours was taken for zebrafish as a positive control; the LC_{50} was obtained from the study by Sánchez-Olivares *et al.* (2021), which shows that 0.065 mg/L of $K_2Cr_2O_7$ causes the death of half of the exposed organisms. However, when used in this study, this concentration had no effect on the organisms. For this reason, the LC_{50} of $K_2Cr_2O_7$ was calculated and an LC_{50} of 234.08±168.32 mg/L at 72 hours was obtained. The OECD establishes that acute toxicity tests must be carried out up to 96 hours; for this reason, the LC_{50} was changed to 96 hours as a positive control and was 110.25±68.03 mg/L. On the

Figure 5. Early malformations and cardiac alterations in zebrafish embryos exposed to mCl. A) hook, B) control, C) pericardial edema.

Figure 6. Late malformations in zebrafish embryos exposed to mCl. A) single, B) double, C) multiple, D) curve, E) caudal fin, F) control.

other hand, Menares Toro, (2017) obtains a LC_{50} of 651 mg/L of this same compound at 96 hours, which is almost six times higher than that obtained in this work. The author mentions that $K_2Cr_2O_7$ does not induce toxic effects on zebrafish at 48 hours of exposure, because the chorion, due to its selective permeability, protects the embryo from the entry of this compound and proposes to perform toxicity tests after hatching, however, in the present study there is mortality of organisms from 12 hours of exposure, suggesting the entry of $K_2Cr_2O_7$ into the embryo.

da Silva *et al.* (2021) obtains a LC_{50} of 32.5% in zebrafish embryos exposed to municipal treated sewage from Aguascalientes. Babić *et al.* (2017) demonstrates that mortality of embryos exposed to treated water starts after 24 hours and increases with exposure as occurs in this study. Guerrero-Jiménez *et al.* (2017) finds an annual LC_{50} of 60.71% in the rotifer *Lecane quadridentata* and does not find toxicity in the cladoceran *Dahpnia magna*, therefore, the toxicity of sewage is specific due to metabolic and physiological differences in each species. In this study BBdClO did not present acute toxicity to zebrafish embryos, so the toxicant removal process could be adequate for this WWTP.

Galus *et al.* (2013) found high percentages of deformity in embryos when exposed to treated urban sewage. These malformations are very common in zebrafish embryos when exposed to various pollutants separately, such as, for example, hexavalent chromium (Olivares *et al*., 2021), microplastics (De Marco *et al*., 2022), pharmaceuticals (Pohl *et al*., 2019), polycyclic aromatic hydrocarbons such as benzo(a)pyrene (Elfawy *et al*., 2021), organophosphate pesticides (Pamanji *et al*., 2016) and endocrine disrupting substances such as ethinylestradiol (Ramírez-Montero *et al*., 2022). All these substances are found in significant concentrations in treated effluents and perhaps are the ones that give rise to these malformations. It has been shown that some of these substances such as benzo(a) pyrene and profenes delay hatching in zebrafish, probably some of these compounds are involved in the delay in hatching of embryos exposed to NaClO in the present work, but further studies would be needed to know which pollutant is the cause of this effect.

CONCLUSIONS

This study highlights the importance of analyzing the toxicity of treated sewage and its impact on aquatic organisms, as well as its relevance to human and ecosystem health. The results indicate that treated sewage cause acute adverse effects in zebrafish. These findings support the need for improved sewage treatment processes. It is of concern that toxicity was observed in the embryonic development of zebrafish, the malformations and cardiac alterations suggest that contaminants present in the water may interfere with critical processes in the development of aquatic organisms. On the other hand, constant monitoring of water quality in receiving bodies in order to know the impact of these treated sewages on aquatic organisms is required.

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