UNIVERSIDAD REGIONAL AMAZÓNICA IKIAM

FACULTAD CIENCIAS DE LA TIERRA Y AGUA

CARRERA DE HIDROLOGÍA

DETERMINACIÓN DE LA CONCENTRACIÓN LETAL Y LA GENOTOXICIDAD PRODUCIDA POR EL SULFAMETOXAZOL, TRICLOSAN, NICOTINA Y EL ESTRADIOL EN ALEVINES DE *OREOCHROMIS NILOTICUS*

Proyecto de investigación previo a la obtención del título de:

INGENIERA EN CIENCIAS DEL AGUA

AUTORA

MARLENE JAKELINE CEVALLOS CAMPOZANO

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AUTORA: MARLENE JAKELINE CEVALLOS CAMPOZANO

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Napo-Ecuador

2022

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DEDICATORIA

A mi madre y a mi avi, Milys Campozano y Gumersindo Collado, por haberme forjado como la persona que soy en la actualidad; muchos de mis logros y sueños los inspiraron ustedes entre los que se incluye este.

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RESUMEN

La preocupación por la contaminación de los compuestos emergentes (CEs) se debe a que se sabe que estos compuestos tienen efectos biológicos, como la disrupción endocrina, en las especies acuáticas incluso a bajas concentraciones. Este estudio se centra en la exposición de alevines de *Oreochromis niloticus* a concentraciones elevadas de CEs, (sulfametoxazol, nicotina, estradiol y triclosán) para encontrar la CL50 (dosis letal/concentración letal para el 50% de los organismos), la CENO (concentración de efecto no observado) y el tiempo de supervivencia (TS) tras 10 días de exposición. Además, se evaluó la genotoxicidad inducida por los AE. Los AE más tóxicos fueron el triclosán (CL50 =0,03 µg L^{-1}; CENO= 0,003 µgL^{-1}) y el estradiol (CL50 =5,7 µg L^{-1}; CENO= 0,2 µg L^{-1}), en contraste con los menos tóxicos: sulfametoxazol (CL50 =103 µg L^{-1}; CENO= 1,3 µg L^{-1}) y nicotina (CL50 = 206 µg L^{-1}; CENO= 10 µg L^{-1}). El ST para el estradiol y el triclosán fue inferior a 10 días de exposición, mientras que el sulfametoxazol utilizó concentraciones más altas para causar mortalidad. En cuanto a los ensayos de genotoxicidad, no fue posible realizar ensayos con triclosán y estradiol debido a la elevada mortalidad. El sulfametoxazol y la nicotina mostraron un aumento de la frecuencia de anomalías nucleares en las concentraciones más altas. El triclosán y el estradiol fueron los compuestos más tóxicos para los alevines de *Oreochromis niloticus* y las dosis no letales causaron genotoxicidad. Esto hace que se alerte sobre el uso y la falta de regulación de estos compuestos.

Palabras clave: letal, ensayos, contaminación, especies acuáticas, emergentes.

ABSTRACT

The concern about the contamination of emerging compounds (ECs) stems from the fact that these compounds are known to have biological effects, as endocrine disruption, on aquatic species even at low concentrations. This study focuses on exposure *Oreochromis niloticus* fry to increased concentrations of ECs, (sulfamethoxazole, nicotine, estradiol, and triclosan) to finding the LC50 (lethal dosage/lethal concentration for 50% of organisms), CENO (concentration of unobserved effect), and survival time (ST) after 10 days of exposure. In addition, we assess the genotoxicity induced by the ECs. The most toxic ECs were triclosan (LC50 =0.03 µg L^{-1}; CENO= 0.003 µg L^{-1}) and estradiol (LC50 =5.7 µg L^{-1}; CENO= 0.2 µg L^{-1}), in contrast to the less toxic sulfamethoxazole (LC50 =103 µg L^{-1}; CENO= 1.3 µg L^{-1}) and nicotine (LC50 = 206 µg L^{-1}; CENO= 10 µg L^{-1}). The ST for estradiol and triclosan was less than 10 days of exposure, while sulfamethoxazole requires higher concentrations to cause mortality. Regarding the genotoxicity assays, it was not possible to perform assays with triclosan and estradiol due to the high mortality. Sulfamethoxazole and nicotine showed an increase in the frequency of nuclear anomalies at the highest concentrations. Triclosan and estradiol were the most toxic compounds to *Oreochromis niloticus* fry and non-lethal doses cause genotoxicity. This triggers an alert about the use and lack of regulation of these compounds.

Key words: lethal, assays, contamination, aquatic species, emerging.

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DETERMINATION OF LETHAL CONCENTRATION AND GENOTOXICITY PRODUCED OF SULFAMETHOXAZOLE, TRICLOSAN, NICOTINE AND ESTRADIOL IN OREOCHROMIS NILOTICUS FRY

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1. Introduction

Emerging contaminants (ECs) are chemical compounds that are commonly present in water, are not regulated and have different origin and chemical nature (Janet Gil et al., 2012). This group includes compounds such as pharmaceuticals and personal care products that have adverse effects on the endocrine systems of humans and wildlife, so they are included within the group of endocrine disrupting compounds (Rodriguez-Narvaez et al., 2017). They are generally found in low concentrations ranging from ng L^{-1} to µg L^{-1} (Pal et al., 2010). The main sources of ECs entering the aquatic ecosystem are point sources of pollution, such as: domestic and industrial wastewater discharges; as well as from diffuse sources (Janet Gil et al., 2012).

ECs enter the aquatic environment through wastewater discharges after being ingested by humans and/or animals and excreted unmetabolized compounds(Hilton & Thomas, 2003). These compounds and their metabolites are biologically active substances, which generate changes in the physiological state of a wide range of aquatic organisms (Wilkinson et al., 2022). Authors such as Brodin et al. (2013) and Arnold et al. (2014) demonstrate that environmental concentrations of these compounds affect the behavior, growth and reproduction of aquatic organisms, for example, (Brodin et al. (2013) reported and alteration in the social and foraging behavior of fish.

The constant discharge of ECs into the aquatic environment increases in their concentration and given their polarity they are easily dissolved in water wherethey are conducted in the best-case scenario to wastewater treatment plants (Becerril, 2009). However, these compounds are not included in the monitoring wastewater treatment programs (Janet Gil et al., 2012). Thus, the absence of treatment causes wastewater to be widely discharged into surface waters, creating a risk to human health, flora and fauna (Acevedo, 2008). This suggests a major problem, especially, in Latin America and the Caribbean, as these areas lack policies for the care and management of wastewater treatment facilities, so they are considered the geographical areas most susceptible to higher levels of anthropogenic contamination in aquatic biota (Llorca et al., 2016). As a result, anthropogenic pollution has been shown to be one of the main drivers of surface water degradation, evidenced especially in South America specifically the Amazon. The Amazon is one of the most important watersheds in the world and is threatened because urban areas do not have wastewater treatment facilities (Lowe, Browne, Boudjelas, 2004; SNIS, 2020; Vélez, Esquivel-Hernández, Cipriani-Avila, Mora-Abril, Cisneros, 2019). Wastewater is discharged directly into the river network (Fabregat-Safont et al., 2021). Undoubtedly, this represents a problem as freshwater contamination spreads throughout the river basin, reaching protected areas of high ecological value (Saunders et al., 2002). Among the most affected areas are floodplains, key areas forsustaining fish reproduction and providing food resources to local populations(Begossi et al., 2019; Hurd et al., 2016).

According to reports by Rico et al. (2021) and Fabregat-Safont et al. (2021) the ECs that threaten freshwater ecosystems in the Amazon are a total of 51 pharmaceuticals, illicit drugs and metabolites. Fabregat-Safont et al. (2021) reported those compounds most common in the freshwaterecosystems of the Brazilian Amazon, among which are: diclofenac, ibuprofen, nicotine, triclosan, sulfadimidine, paracetamol, estradiol, among others. Most of these compounds were identified in areas with significant urban impact. In the context of the Ecuadorian Amazon, Capparelli et al. (2021) conducted the study on the occurrence of ECs in the northeastern Ecuadorian Amazonian; reporting the presence of caffeine (19.3 and 31.5μg L^{-1}) and trimethoprim (2 μg L^{-1}) in areas of urban incidence. Therefore, the protection of freshwater Amazonian aquatic ecosystems requires research on water quality that does not only focus on nutrients, microbial contaminants and heavy metals but also onanalysis of ECs emitted in the river network and them distribution.

The broad area of ecotoxicology has tools to identify the effects of ECs on aquatic ecosystems (Carriquiriborde, 2021). For this purpose, bioindicator organisms are used, which are a set of organisms that provide information on certain ecological, physicochemical, biological and functional characteristics of the ecosystem under analysis. The organisms selected must be feasible to be maintained under laboratory, have to be highly sensitive to toxicants (Lemos et al., 2005) (De Silva et al. 2007) Among the organisms used as bioindicators are fish fry that are in the developmental stage and are therefore more sensitive to ECs, and are ideal because they cover many links in the food chain, are capable of accumulating toxic substances and react easily to low concentrations of ECs (Colón 2009; Gustavino et al. 2001).

To know the toxic effects of chemical substances the freshwater organisms, toxicity tests have been used with, *Daphnia magna* and the fish *Danio rerio* (Pal et al., 2010). These toxicity tests can be either acute toxicity (LC50) or chronic toxicity (EC50) tests. The acute toxicity tests determine the lethal concentration (LC50) in a short exposure time of emerging compounds and allow documenting the presence or absence of apparent effects on aquatic organisms (Vásquez et al. 2005). Chronic toxicity tests use lower concentrations over longer exposure periods; effects can be assessed with the use of biomarkers, what are you any modification of biological/physiological responses that may become permanent and lead to population effects and community and ecosystem implications (García Vásquez et al., 2009). These damages can be analyzed through the ability of emerging compounds to cause damage to genetic material or also known as genotoxicity (Kayani & Parry, 2010). This is the so-called micronucleus (MN) assay, which detects the effect of mutagenic agents on chromosomes through the identification of acentric fragments and/or straggler chromosomes that form such structures when they remain outside the nucleus (Osman et al., 2010; Torres-Bugarín et al., 2007).

Findings on the effects of ECs are of fundamental importance to demonstrate the risks they could cause in ecosystems and aquatic species, as they exhibit toxicity to aquatic organisms even at low concentrations (Voloshenko-Rossin et al., 2015). For this study, tilapia fry of the species *Oreochromis niloticus*, an invasive species that is considered a good bioindicator of genotoxicants in water resources and for environmental monitoring (De Andrade et al., 2004; Masutti et al., 2006; R. Oliveira et al., 2009), were used. In addition, the choice of this species is due to the fact that they are easy to obtain

because they are cultivated in many regions. According to FAO (1998) tilapia is the most cultivated cichlid in the world. According to De Silva et al. (2007), these characteristics make them a good bioindicator, since they are in a developmental stage that makes them sensitive to exposure to ECs.

In this study we evaluated the toxicity produced by emerging contaminants: sulfamethoxazole, estradiol, triclosan and nicotine; in *Oreochromis niloticus* fry exposed to different concentrations. Taking into account that fry are more sensitive individuals in the developmental stage and, therefore, vulnerable to contamination (Dann & Hontela, 2011) lethal concentrations (LC50) were expected to be less than the concentrations reported in the literature for adult fish and that no observed effect concentrations (CENO) will be higher since the higher the CENO the lower the toxicity produced by the ECs. In addition, it was expected to know the concentrations to generate effects in *Oreochromis niloticus* fry and to identify the genotoxic effects through the frequency of anomalies and micronuclei presented by the fry. The present work aims to evaluate the lethal doses of ECs in *Oreochromis niloticus* fry and to observe that even at non-lethal doses the compounds cause genotoxicity. The results provide a warning about the presence of ECs in wastewater, as well as the importance of regulating these compounds in countries where there is a deficiency of wastewater treatment.

2. Methods

2.1 Maintenance and experimental design

The fry was acquired from culture pools certified for human consumption. Fry that was 15 days old at hatching were used, their maximum weight was 2 to 7g and a length of 5 to 7cm. The fry was kept in static aquaria with aeration. Water was changed every two days. The conditions of the aquarium systems, were: light for 14h; 10h dark photoperiod at 28 ± 0.5 C. Both control and test solutions were replaced every 24h. Each day measurements of the parameters: pH, conductivity(S/cm), temperature, O2, nitrite and ammonia (mg/L)were performed.

2.2 Experimental design

The fry was kept for 10 days in a phase of acclimatization to the laboratory conditions before starting the experiments. Four replicates were made for each of the concentrations used, between 5 to 6 fry per 5L fish tank (Figure 1). Every 48 h the experimental medium was replaced and the fish were checked for mortality and fed. The experiment lasted 10 days.

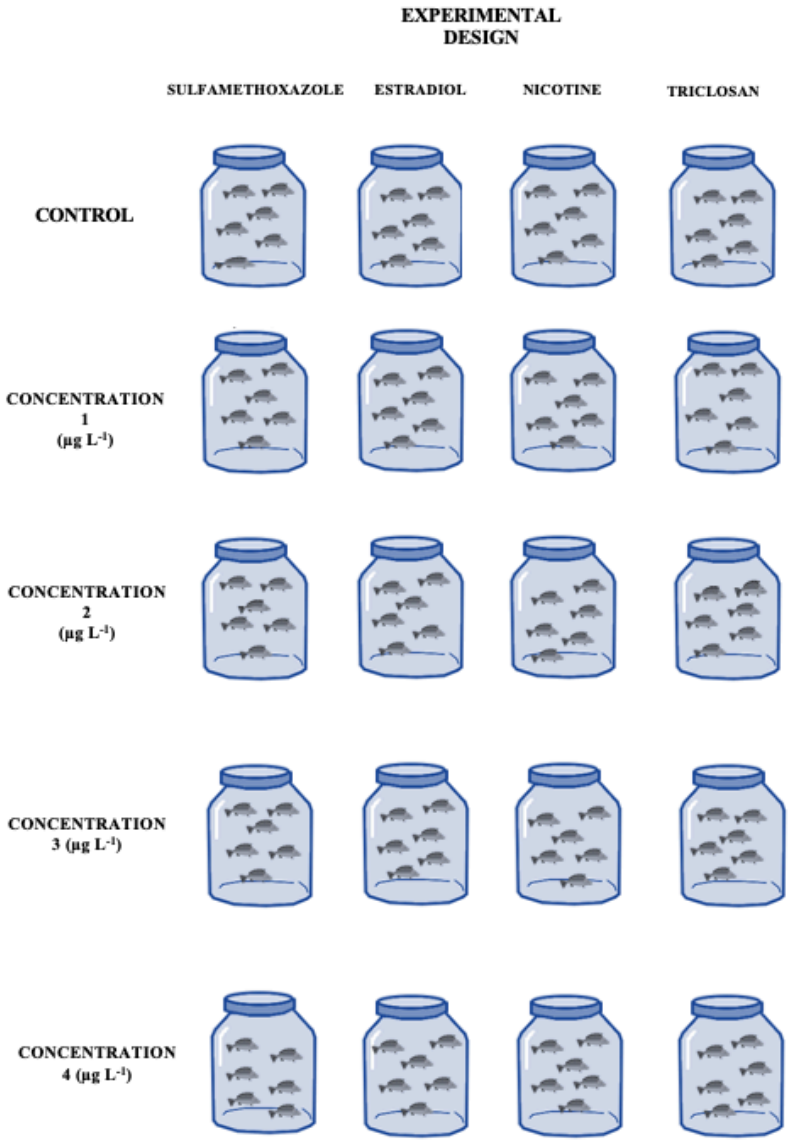


Figure *1:* Experimental design: sulfamethoxazole, triclosan, nicotine, estradiol fry was exposed to different concentrations of ECs for 10 days.
Done By: C.C. Jakeline, 2022.

2.3 Concentrations of ECs

To evaluate the lethal concentration and genotoxicity, water samples were prepared with different concentrations of emerging contaminants. For the stock solution, water was collected from the Colonso Chalupas Ecological Reserve, the water in this location was considered control, as it does not present toxicity and is within the allowed limits for water quality and contaminants, including emerging contaminants (Capparelli et al. 2020; Galarza et al. 2021) the focus of the current project. The concentrations of ECs used in the experiments were chosen based on Glassmeyer et al. (2017) which indicates that ECs are chosen based on whether they are present in environmental waters and whether they are found in concentrations that may cause human or ecological health problems.

The concentrations used for this experimentation were as follows.

- Sulfamethoxazole: 0, 0.3, 3, 30 μg L^{-1}
- Triclosan: 0, 0.03,0.3, 3, 30 μg L^{-1}
- Nicotine: 0, 1, 10, 100, 500 μg L^{-1}
- Estradiol (17β-estradiol): 0, 10, 100, 500 μg L^{-1}

2.4 Acute toxicity tests: LC50, CENO

To identify the toxicity of the ECs a simplified methodology of dose-effect experiment evaluation, developed by Litchfield & Wilcoxon (1948) and its 95% confidence limits, was used. These tests consist of: i) the effective concentration that causes the death of 50% of the organisms at 10 days (240h), and the concentration of no observed effect (CENO) at 10 days (240h).

2.5 Survival time

Survival analyses were performed by comparing treatment survival curves (lethal time at 10 days) with controls using a log-rank test, for each of the contaminants: triclosan, sulfamethoxazole, estradiol and nicotine. A significance level of p=0.05 was used for each of the procedures. In experimental biology, a significance level of 0.05 is common and accepted, as in Villegas et al. (2022).

2.6 Genotoxicity biomarker: micronucleus analysis with acridine orange (NA)

Genotoxicity analyses were performed based on the technique described in (Gusso-Choueri et al. (2016) with minor alterations. 20µl of peripheral blood was withdrawn from the caudal vein or operculum opening using insulin syringes for the micronucleus test and preserved in an eppendorf and the same amount of acridine orange was placed. It was then placed on a slide. With the help of a micropipette the total content was gently aspired and returned to the slide for better homogenization. The material was then rubbed with the help of a slide. The material was left to dry for 24 hours at room temperature. Afterwards, they were fixed with absolute ethanol for 10 minutes. Staining was performed immediately, as these slides cannot be stored for a long time due to loss of NA fluorescence. For the identification of micronuclei, 2000 erythrocytes were examined under the Leica® epifluorescence microscope of the Universidad Regional Amazónica Ikiam at 1000 magnification. The observation was performed following the methodology of Vignardi et al. (2015) where 1000 cells were calculated from each slide, noting the number of young erythrocytes (polychromatic) with and without micronuclei and the number of mature erythrocytes (monochromatic) with and without micronuclei.

2.7 Nuclear lesions

Five nuclear lesions were observed, blebbed nuclei are those with relatively small evagination of the nuclear envelope; lobed nuclei are those with two or more differentiated lobes; kidney-shaped and heart- shaped nuclei are those with appreciable depth in a nucleus containing no nuclear material; and irregularly shaped nuclei are those with an irregular shape (Osman et al. 2010).

2.8 Statistical analysis of genotoxicity assays

Statistical analyses for the genotoxicity assays were performed by one-way analysis of variance (*ANOVA) Test in R Programming - CORE Team* (2021) program. Differences were considered significant at $p<0.05$ with respect to the control group. Data are presented as the mean SD of ten animals per group.

3. Results and Discussion

3.1 LC50 and CENO of CEs on Oreochromis niloticus fry and perspective compared to literature results

According to several studies (Table 1, Table 2) the LC50 toxicity for triclosan varies between $4x10^4$ and 720 µg L^{-1} and the CENO value varies between 40 and 390 µg L^{-1}. Our results in *Oreochromis niloticus* fry (Table 1, Table 2) reveal an LC50 value = $3x10^1$ µg L^{-1} and a CENO value = $3x10^2$ µg L^{-1}, both values are within toxicity grade 1, which represents high toxicity (HSE, 2014).

Based on the literature, LC50 and CENO values have been reported for nicotine in different aquatic organisms, such as: *Acacia nilotica, Daphnia magna, Pseudokdirchneriella subcapitata*, *Oreochromis niloticus* (adults) and *Clarias gariapinus* (Table 1, Table 2), the ranges are between $1.56x10^6$ to 370 µg L^{-1} for LC50 and values between $1x10^3$ to $2x10^3$ µg L^{-1}. As for our study in *Oreochromis niloticus* fry, a value of 206 µg L^{-1} was observed for LC50, a value that is within toxicity grade 1 according to HSE (2014), meaning very toxic, and a value of 10 µg L^{-1} for CENO, a value that is also within toxicity grade 1 according to HSE (2014), meaning very toxic. The concentrations of 1 and 10 µg L^{-1} showed no significant differences with respect to the control. A similar study in adult fish *Oreochromis niloticus* reported an LC50 of 370µg L^{-1} (Table 1, Table 2), highly toxic doses represent 100% mortality for this species, either fry or adult fish.

In the case of sulfamethoxazole, numerous LC50 and CENO studies have been conducted on different species, including: *Daphnia magna, Ceriodaphnia dubia, Brachionus calyciflorus*. The LC50 ranges from 103- $1.56x10^6$ µg L^{-1}, values ranging from toxicity grade 1 to 4, from very toxic to non-toxic to aquatic life (Table 1). As for CENO values, they range from $1.71x10^4$ to $5.62x10^5$ µg L^{-1}, values that are in toxicity grade 3 and 4 (Table 2). As for our study, the value of 103 µg L^{-1} was observed for LC50, value of toxicity grade 1 (very toxic) and CENO value of $1.36x10^2$ belonging to toxicity grade 1 (very toxic) according to HSE (2014).

Table 1: Acute toxicity reported: nicotine, triclosan, estradiol and sulfamethoxazole, for different species reported in the literature.

Compound	Specie	Acute Category	CENO (µg L^{-1})	Exposure time (hours)	References
Estradiol	*Daphnia magna*	3	$1,20x10^6$	48	Parada, (2019)
	Rana pipiens	1	4,56	336	Hogan et al. (2006)
	Oophaga sylvatica	1	2,5	336	Hogan et al. (2006)
	Oreochromis niloticus (fry)*	1	5,7	240	Present study
Nicotine	*Acacia nilotica*	1	1,25	48	Bahmani et al. (2014)
	Oreochromis niloticus	1	370	12	Millan et al. (2013)
	Oreochromis niloticus (fry)*	1	206	240	Present study
	Clarias gariepinus	4	$1,56x10^6$	120	Musa et al. (2013)
Sulfamethoxazole	*Brachionus calyciflorus*	4	$2,62x10^5$	48	Santos et al. (2010)
		4	$7,50x10^5$	72	Kim et al. (2007)
	Daphnia magna	4	$1,89x10^5$	96	Kim et al. (2007)
		3	$2,52x10^4$	96	Kim et al. (2007)
		4	$1,48x10^6$	72	Isidori et al. (2005)

	Thamnocephalus platyurus	3	$3{,}53x10^4$	48	Isidori et al. (2005)
	Ceriodaphnia dubia	3	$1{,}55x10^4$	24	Isidori et al. (2005)
	Oreochromis niloticus (fry)*	1	103	240	Present study
Triclosan	*Oryzias latipes*	1	720	96	(Nassef et al., 2010)
	Artemia franciscana	1	180	72	(Neumegen et al., 2005)
	Daphnia magna	1	220	48	Chalew and Halden (2009)
		1	650	72	Thomaidi et al. (2015); Dussau et al. (2008)
	Danio rerio	1	340	96	Oliviera et al. (2009)
	Danio rerio (fry)	1	420	96	Oliviera et al. (2009)
	Oreochromis niloticus	2	$4x10^4$	96	Vijitha et al. (2017)
	Oreochromis niloticus (fry)*	1	$3x10^{-1}$	240	Present study

Done by: C.C. Jakeline, 2022

Table 2: Higher continuous concentration measured: nicotine, triclosan, estradiol and sulfamethoxazole for different species reported in literature.

Compound	Specie	Acute Category	CENO (µg L^{-1})	Exposure time (hours)	References
Estradiol	*Oryzias latipes*	3	< $2{,}93x10^5$	72	Kang et al. (2002)
	Oreochromis niloticus (fry)*	1	2	240	Present study

Nicotine	*Daphnia magna*	2	$1x10^3$	96	Rodríguez (2020)
	Raphidocelis subcapitata	2	$1x10^3$-$2x10^3$	72	Rodríguez (2020)
	Oreochromis niloticus (fry)*	1	10	240	Present study
Sulfamethoxazole	*Planorbis carinatus*	3	$1,71x10^4$	72	(Pounds et al., 2008)
	Daphnia magna	4	$1,77x10^5$	96	Kim et al. (2007)
		4	$1,75x10^5$	96	Kim et al. (2007)
		3	$1,34x10^5$	336	Heckmann (2007)
	Oryzias latipes	4	$5,62x10^5$	96	Kim et al. (2007)
	Oreochromis niloticus (fry)*	1	$1,36x10^2$	240	Present study
Triclosan	*Daphnia magna*	1	390	72	Orvos et al. (2002)
		1	240	168	Orvos et al. (2002)
	Daphnia similis	1	100	48	Lameira (2008)
	Ceriodaphnia dubia	1	90	48	Lameira (2008)
	Oreochromis niloticus (fry)*	1	$3x10^{-2}$	240	Present study

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3.2 Survival time (ST) Oreochromis niloticus fry

3.2.1 Triclosan

Survival analysis (Figure 2) shows significant differences between each of the concentrations used in the experiment with the control ($p < 0.0001$). It can be observed that at lower concentrations there is a lower survival of the fry although over the 10

days there was 100% mortality at all concentrations. For example, the 0.03 μgL^{-1} concentration of triclosan shows that the probability of survival decreases from 0.75 to 0.50 on the fifth day of experimentation and from 0.30 to 0.10 on the seventh day of experimentation. In contrast, for the 0.3 $\mu g\ L^{-1}$ concentration, a less marked decrease is observed as the days of experimentation pass, the most significant point being the seventh day, going from a survival probability of approximately 0.50 to 0.30 survival. Finally, for the 30 $\mu g\ L^{-1}$ concentration, the most significant days are the seventh day, with a decrease in survival from 0.60% to 0.35%, and the ninth day, from 0.25% to 0% survival probability. These results show that the level of triclosan toxicity of triclosan is very high from very low doses. Based on the literature Geronoglu et al (2018) reported EC50 of 7 $\mu g\ L^{-1}$ in microalga *Raphidocelis subcapitata*, Ding et al (2018) reported 145 μgL^{-1} in diatom *Navicula* ; evidencing a hormesis phenomenon. In experiments conducted by Eriksson et al (2015) on microalgae they observed that at low concentrations of triclosan the amount of pigment decreased, but with increasing concentrations the amount of pigment increased. This was interpreted as a tolerance mechanism of the microalga. Elevated levels would capture more light energy which in turn would maintain the pH gradient over the thylakoid membrane and mitigate the effect of triclosan. Peng et al (2013) reported that at doses 1-16 $\mu g\ L^{-1}$ the growth rate was increased at low concentrations and at high concentrations (64-128$\mu g\ L^{-1}$) of triclosan it was significantly reduced. These effects also occurred in *Oryzias latines* and juvenile rainbow trout (Ishibashi et al. 2004; Orvos et al. 2012). Increased fecundity is an example of a phenomenon known as overcompensation hormesis, an adaptive response to low levels of stress that results in improved fitness over a finite period (Flaherty and Dodson 2005). On the other hand, triclosan at high doses may act as an endocrine disruptor (Foran et al. 2000; Ishibashi et al. 2004). Regarding our results, further studies are needed to better understand what happens in *Oreochromis niloticus* fry.

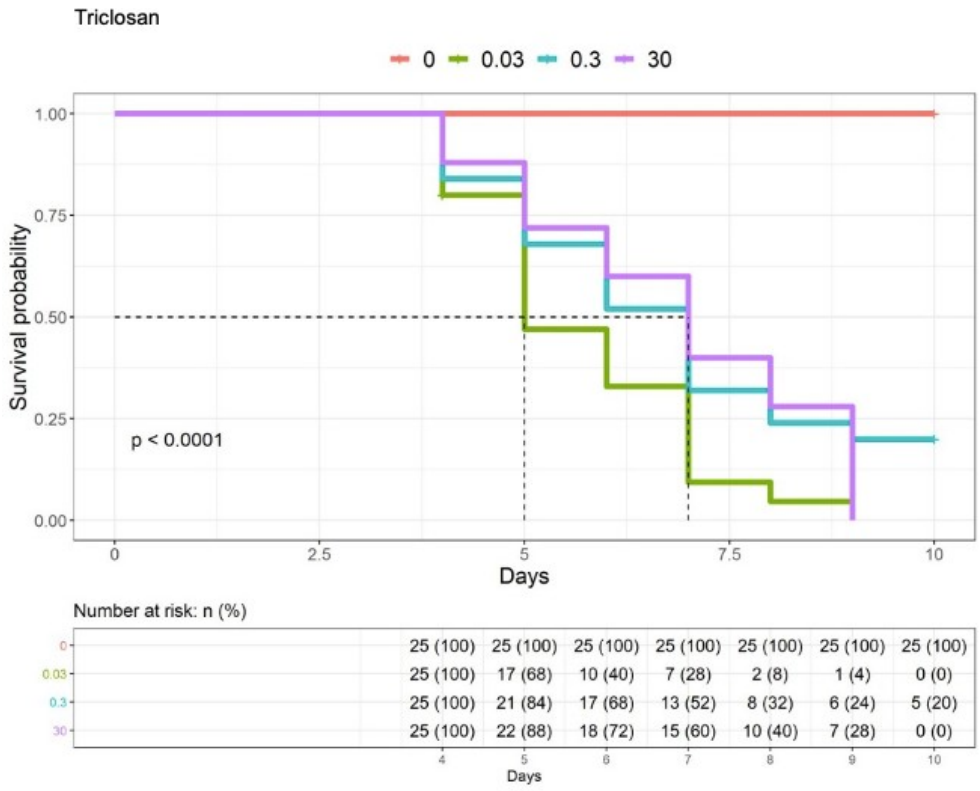


Figure 2: Survival probability. Table shows the number of individuals, in parentheses the predicted survival percentage.
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3.2.2 Nicotine

The assays identified a marked decrease in survival in exposed individuals from a concentration of 100 µg L^{-1} nicotine. Survival analysis (Figure 3) shows significant differences between the 100 and 500 µg L^{-1} treatments and the control ($p < 0.0001$). In addition, it also predicts a high mortality risk fororganisms exposed to 500 µg L^{-1} (100% mortality).

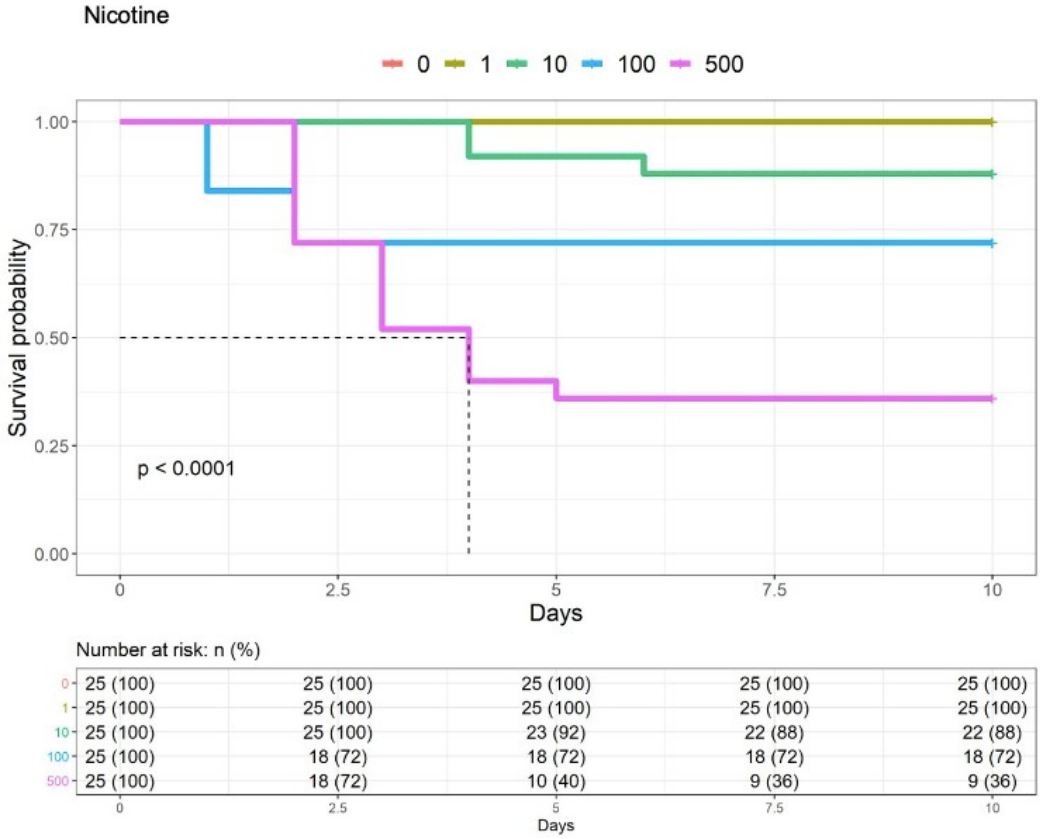


Figure 3: Survival probability curve for Nicotine exposure. Table shows the number of individuals and in parentheses the predicted survival percentage.
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3.2.3 Estradiol

The contaminant exposure tests showed a marked decrease in the survival of individuals exposed to different concentrations of estradiol. For the concentration of 1 µg L^{-1}, a survival probability of 1.00% was evidenced during the whole experimental time. On the other hand, for the concentration of 10 µg L^{-1}, there was a decrease from 1.00% of the survival probability to 0.90% in the first four days of experimentation and from 0.90% to 0.80%, which was maintained until the last day of experimentation. The most important difference occurred with the concentration of 100 µg L^{-1}, where it was observed that the survival probability was maintained at 0.75% from the third day onwards. On the other hand, for the 500 µg L^{-1} concentration, different variations occurred, the most significant being on the second day of experimentation, dropping from 1.00% survival probability to 0.75% and on the third day from 0.75% to 0.50%. These variations show significant differences with the control ($p<0.0001$). Furthermore,

these results predict the high mortality risk of organisms exposed to 500 µg L^{-1} (100% mortality).

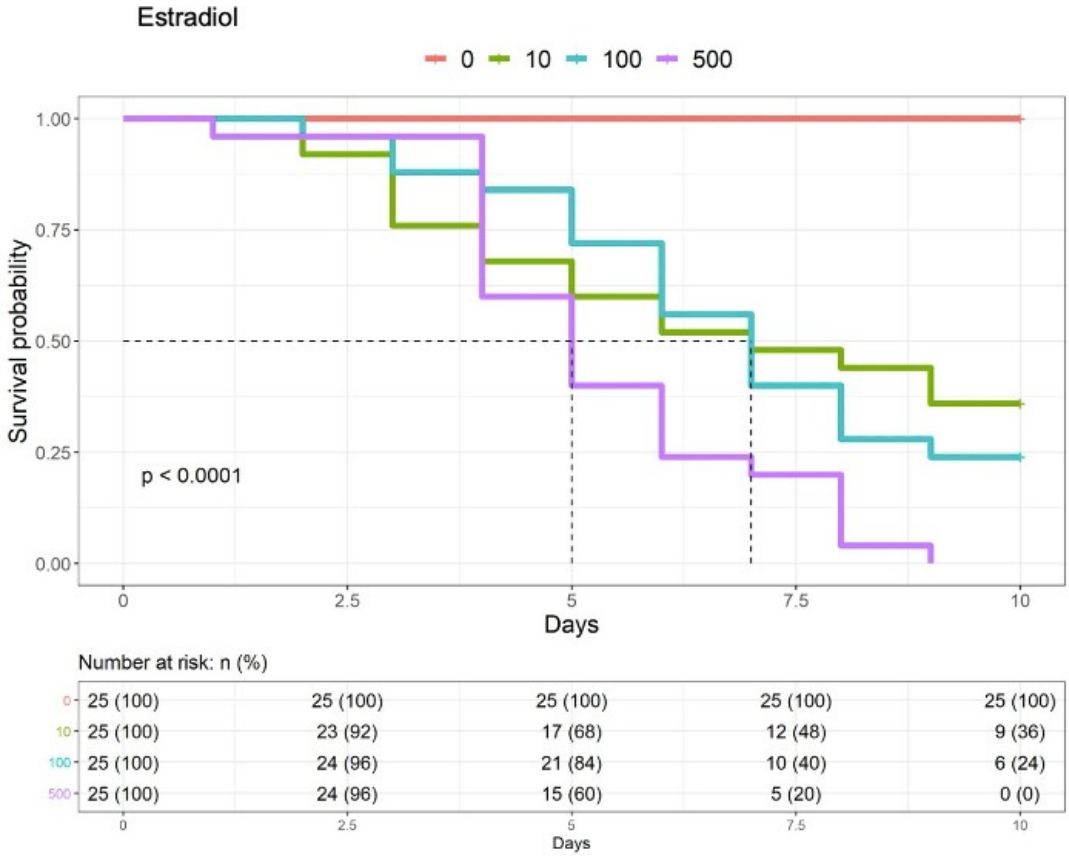


Figure 4: Survival probability curve for estradiol exposure. Can see the number of individuals and in parentheses the percentage of predicted survival
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3.2.4 Sulfamethoxazole

As for the survival assays, a decrease was seen in the exposed individuals from the concentration of 0.3 µg L^{-1} of sulfamethoxazole. The survival analysis (Figure 5) showed no significant differences between the treatments and the control ($p > 0.0023$). This shows that sulfamethoxazole does not predict a high mortality risk for organisms exposed to the different concentrations

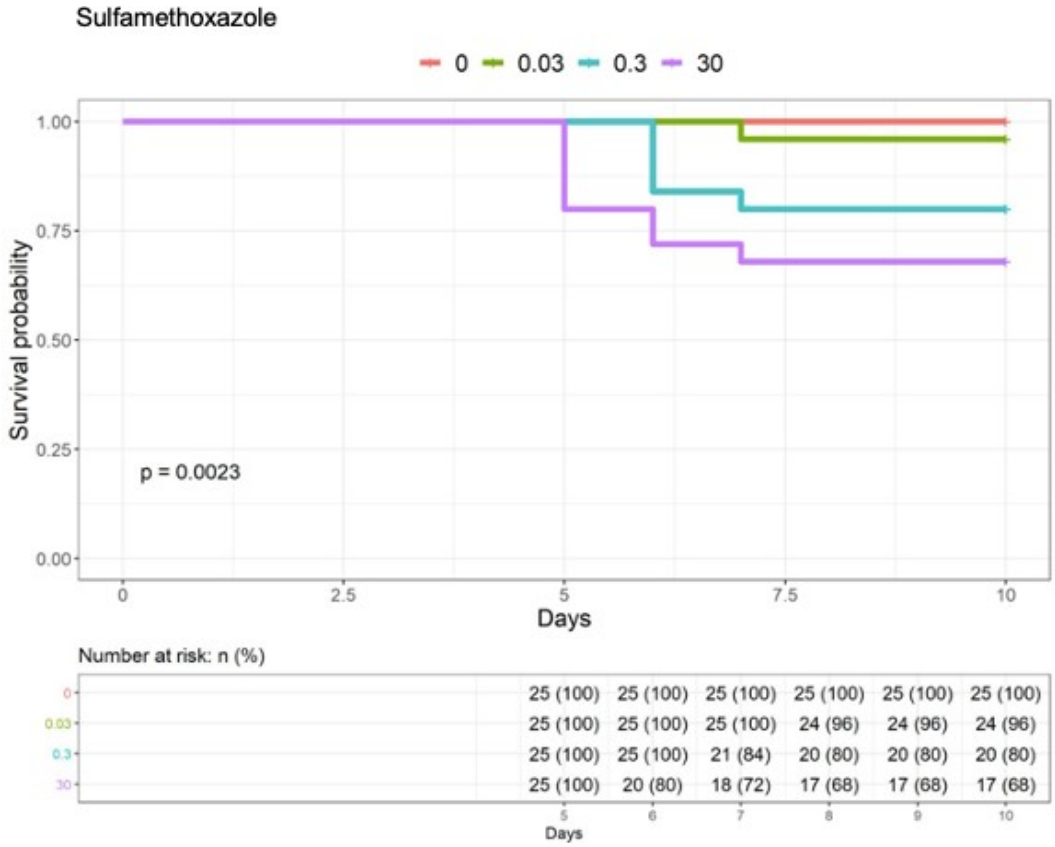


Figure 5: Survival probability curve for exposure to Sulfamethoxazole. Table shows the number of individuals, in parentheses predicted survival percentage
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3.3 Effects of different doses of ECs on Oreochromis niloticus fry

Based on the literature, toxicity has been reported forthe four ECs: estradiol, sulfamethoxazole, nicotine and estradiol. As can be seen in (Table 1), LC50 values, carried out on different vertebrate and invertebrate species, show that the pollutants estradiol and triclosan kill at lower concentrations and in a shorter exposure time. For example, for estradiol (Table 1), Hogan (2007) reported values of 336h LC50 4.57 µg L^{-1} and 336h LC50 2.5 µg L^{-1} for two species of toads *Rana pipiens and Oophaga sylvatica*. As for our results, carried out on tilapia fry *Oreochromis niloticus,* it was reported that 240h LC50 5.7 µg L^{-1}. In the case of triclosan, values between 180-720 µg L^{-1} (Mohamed et al. 2010; Neumegen 2005) for short periods between 72-96 hours of exposure in different species are also evident. As for our study, 240h LC50 $3x10^{-1}$µg L^{-1} is evidenced. This according to (HSE, 2014) estradiol and triclosan is within toxicity grade 1 for different vertebrate and invertebrate species. Something different occurs with the ECs nicotine and sulfamethoxazole (Table 1), as can be seen,

LC50 values are higher, this means that higher concentrations of ECs are needed to kill individuals in less time.

Similarly, it can be evidenced (Table 2) the CENO values of estradiol 72h CENO $2.93x10^5$µg L^{-1} (Kang et al. 2002) and triclosan values ranging between 90-390 µg L^{-1} between 48-168 hours of exposure (Musa et al. 2013; Orvos et al. 2002). Our study, on the other hand, showed for estradiol 240h CENO 2µg L^{-1} and for triclosan 240h LC50 $3x10^{-2}$µg L^{-1}; this is supported by (Figure 4) where it can be seen that at doses of 10 µg L^{-1} the probability of survival was already reduced from 1.00 to 0.90 and as the concentration increased the probability of survival decreased. Similarly, for triclosan (Figure 2), it can be seen that continuous low doses of 0.03 µg L^{-1} reduced the probability of survival from 0.75 to 0.50. These values demonstrate that at continued low doses of these ECs over a relatively short period of time, a biological response of the species is already evident. In contrast, for sulfamethoxazole (Table 2) the values to show no observed effects range from $1.32x10^2$ - 5. $62x10^5$ µg L^{-1} in times between 72-336 hours of exposure (Heckmann et al., 2007; Kim et al., 2007; Pounds et al., 2008) and in our study it is 240h CENO $1.36x10^2$µg L^{-1}; this is supported by the results of (Figure 5) where it is evident that there were no significant differences between the treatments and the control, which means that sulfamethoxazole does not present a high risk of mortality for *Oreochromis niloticus* fry.

As for nicotine, the values (Table 2) are: 10-$2x10^3$ µg L^{-1} in exposure periods between 72-96 hours (Rodriguez-Narvaez et al., 2017), while in our study they were 240h CENO 10µg L^{-1}; this is supported by the results obtained (Figure 3) where it is evident that the organisms exposed to the highest concentration of the treatment 500µg L^{-1} do present a high risk of mortality. Finally, this shows that high doses of sulfamethoxazole and relatively high doses of nicotine in a continuous period of time would be necessary to demonstrate biological samples in the analyzed species.

3.4 Genotoxicity: micronucleus assay

In the case of the contaminants triclosan and estradiol, genotoxicity tests were not performed because all the specimens died in the toxicity tests with the contaminants. On the other hand, in the case of sulfamethoxazole and nicotine, the specimens did survive, toxicity tests were performed and it could be evidenced that the frequency of normal nuclei decreased, since the incidence of nuclear abnormalities in erythrocytes

increased, evidenced by the formation of micronuclei (MN) together with lobulated (LO), blebbed (BLE) and segmented (SE) nuclei (Figure 8).

In the case of sulfamethoxazole, the frequency of micronuclei did not show significant differences in relation to the control. However, it was possible to observe an increase in the frequency of the anomalies called blebbed in relation to the control, in the two highest concentrations of sulfamethoxazole. In addition, it was possible to observe an increase in dose response in relation to this abnormality. At the highest concentration of Sulfamethoxazole 30 µg L^{-1}, there was a 4-fold increase in the frequency of blebbed cells. As for lobbed cells, no significant difference was observed relative to the control. However, the binucleus abnormality was significantly reduced relative to the control at the lowest concentration of Sulfamethoxazole 0.3µg L^{-1}. As for erythrocytes with vacuoles, exposure to a higher concentration of Sulfamethoxazole resulted in a higher frequency of this abnormality (Figure 6).

The formation of nuclear abnormalities is believed to be a defense mechanism that allows the expulsion of toxic chemicals that expand the genetic material of the cell nucleus (Sponchiado et al., 2011), among other things, the induction of micronucleus (MN) induced genotoxic damage and other nuclear abnormalities reveals the genotoxic effects of sulfamethoxazole in *Oreochromis niloticus* fry. Yokote et al. (1982) and Çavaş & Ergene-Gözükara (2003) reported that the presence of blebbed and fragmented nuclei in fish erythrocytes may be a consequence of folic acid deficiency. This is based on Shimizu et al. (2000) and Fenech & Crott (2002) who have demonstrated the induction of nuclear abnormalities such as nuclear budding and nucleoplasmic bridges in human lymphocytes under conditions of folic acid deficiency, since folic acid deficiency is known to cause gene amplification and DNA double strand breaks.

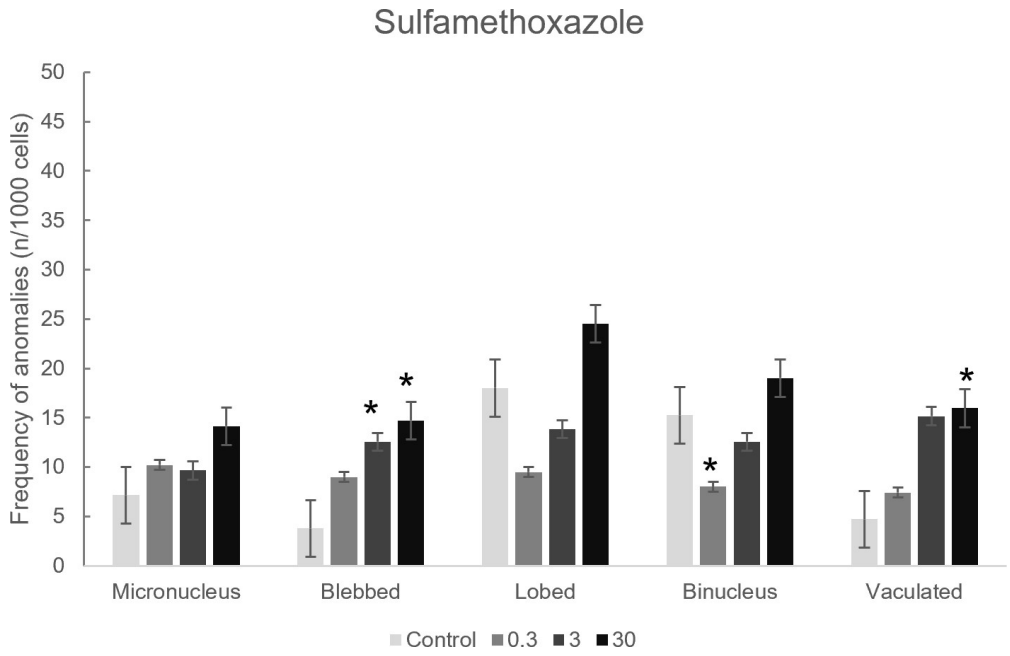


Figure 6: Frequency of anomalies in erythrocytes from Oreochromis niloticus fry exposed to Sulfamethoxazole (control,0.3; 3; 30 µg L^{-1}) for 10 days.
Done By: Moulatlet G, 2022

Regarding the frequency of micronuclei, no significant differences were observed in relation to the control. However, an increase in the frequency of the so-called lobulated abnormalities could be observed in relation to the control, at the highest concentrations of Nicotine (100 µg L^{-1}). As for lobulated cells, at the highest concentration, with a reduction in the frequency of this anomaly. The binuclear abnormality was significantly increased in relation to the control, at the highest concentration of nicotine 100 µg L^{-1}. As for erythrocytes with vacuoles, exposure to a higher concentration of nicotine caused a higher frequency of this abnormality (Figure 7). According to (Al-Sabti & Metcalfe (1995); Ayllon & Garcia-Vazquez (2000); Pacheco & Santos (1998); Sanchez-Galan et al (2001), nuclear lesions are well-established indicators of cytotoxicity and an association between the frequency of such lesions and exposure to genotoxic agents has been reported. According to Iturburu et al. (2017) an(M. Oliveira et al., 2010), the presence of lobular abnormalities is related to oxidative stress, a mechanism involved in oxidative DNA lesions. Consequently, this can lead to an alteration in reproduction or in the mortality rate, causing a reduction of genetic variability in populations (Bickham et al. 2000).

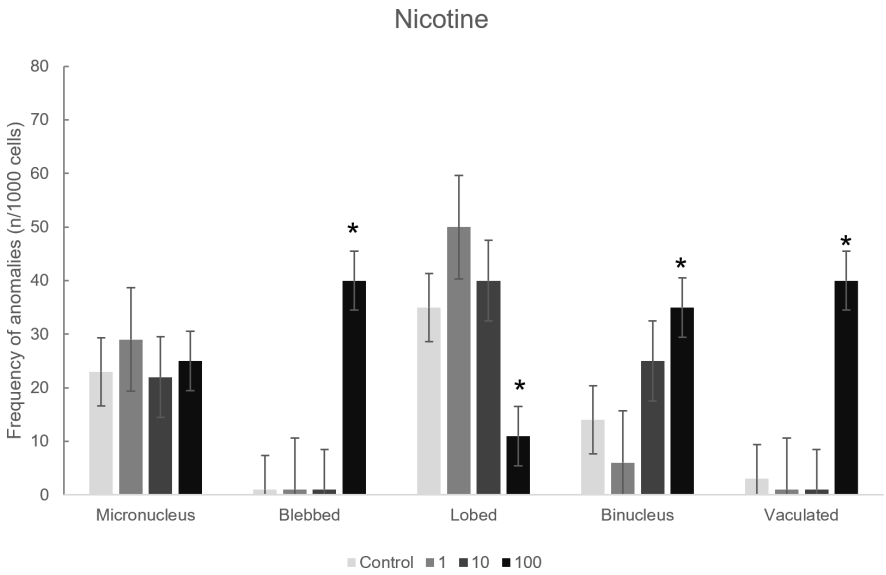


Figure 7:Frequency of anomalies in erythrocytes from Oreochromis niloticus fry exposed to Nicotine (Control, 1; 10; 100 µg L^{-1}) for 10 days.
Done By: Moulatlet G, 2022

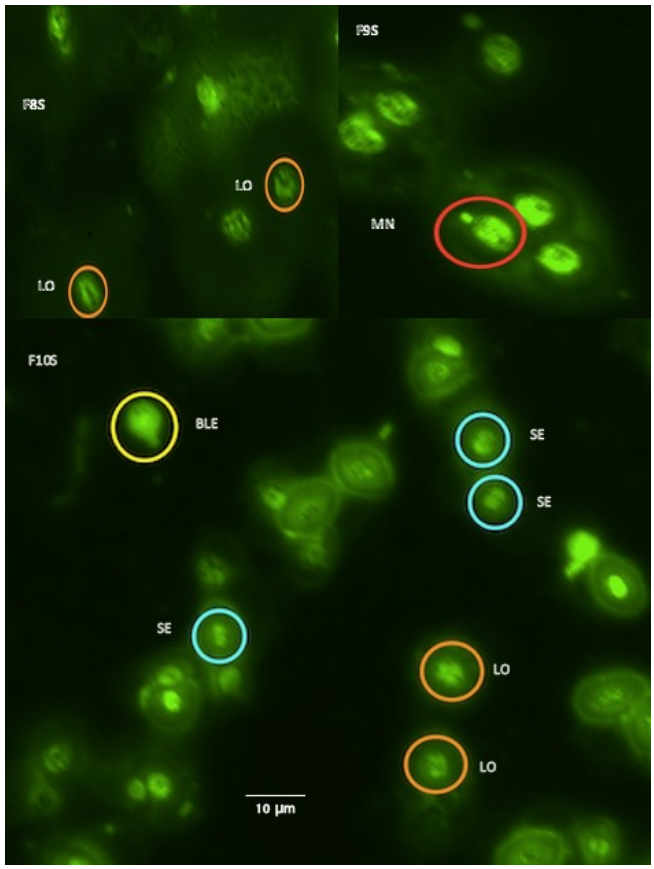


Figure 8: nuclear abnormalities: MN (micronucleus), LO (lobed), BLE (blebbed), SE (segment).
Done By: C.C.Jakeline,2022

Our findings are in agreement with previous studies that reported the simultaneous presence of nuclear abnormalities and micronuclei in erythrocytes of tilapia *Oreochromis niloticus* fry after genotoxic exposure. Our results suggest that the nuclear lesions found here should be considered indicators of genotoxicity, in addition to the typical forms of micronuclei, and should be included in routine testing when fish are used for toxicological experiments.

In summary, the most toxic ECs were estradiol and triclosan. Triclosan was the most toxic EC, since at very low concentrations of 0.03 µg L^{-1} it already reduced the probability of survival by 0.75 to 0.50 on the fourth day of exposure; while estradiol reduced the probability of survival from 1.00 to 0.90 on the fourth day at the lowest concentration of 1µg L^{-1}. Similarly, the genotoxicity results for the two ECs: estradiol and triclosan, were not carried out because all *Oreochromis niloticus* fry died during the LC50 and CENO tests, evidencing their high level of toxicity.

On the other hand, the genotoxicity analyses carried out on the ECs: sulfamethoxazole and nicotine, showed an increase in nuclear anomalies in erythrocytes, evidencing the formation of: micronuclei (MN), lobulated nuclei (LO), blebbed (BLE) and segmented (SE). At high sulfamethoxazole concentrations there was an increase in blebbed, lobulated and binuclear abnormalities. The most significant anomaly was the lobulated nuclei, since with increasing doses of sulfamethoxazole this increased four times in frequency, this is related to the LC50 and CENO values for sulfamethoxazole, which are 103 µg L^{-1} and 1.36 µg L^{-1}, respectively. On the other hand, at higher nicotine concentrations, increased lobular, binuclear, blebbed abnormalities were observed; this is related to the LC50 values of 206 µg L^{-1} and CENO values are 10 µg L^{-1}. This means that there is an important difference between the ECs sulfamethoxazole and nicotine because the CENO values show that at very low doses of sulfamethoxazole there is already a strong frequency of lobulated abnormalities, increasing this dose increases four times the frequency of lobulated abnormalities. In contrast, the CENO value of nicotine is relatively higher than that of sulfamethoxazole and although there are anomalies, these are not increased in greater quantity as with sulfamethoxazole.

In the case of triclosan Delfrate et al (2021); evidenced concentrations ranging from 0.05 to 0. 2 μg L^{-1} in all water samples collected in Rio Verde, Brazil, while Torres Méndez & Echeverría Barillas (2017), reported mean concentrations ranging from <18 to 520 μg L^{-1} in wastewater in Guatemala and Melendez-Marmolejo et al. (2020); reported in surface water samples in Costa Rica 0. 011-0.263 μg L-1; in Mexico in surface water 0.016-0.019 μg L^{-1} and in treated water 0.0015 μg L^{-1}. If we compare these concentrations with those used in this study (0.03, 0.3 and 30 μg L^{-1}), where all fry died at all relatively low concentrations. This means that the doses found in the environmental media are alarming, representing an important risk for aquatic species. In another study conducted in Uruguay, Pérez-Parada et al (2012); evaluated the presence of CEs in wastewater, among this nicotine where they reported concentrations of 0.02-73 μg L^{-1}, while Olivares, Guedes & Montesdeoca (2015) reported 0.0065 μg L^{-1} in wastewater. If we compare these data with the concentrations used in this study (1, 10 100 and 500 μg L^{-1}) it could be observed that the doses of 100 and 500 decreased the survival capacity of fry to 0.75, 73 μg L^{-1} is a relatively low concentration that would not represent an immediate risk, but it is a warning that should be considered. In the case of estradiol Madera-Parra et al (2018) reported doses of 8.7 μg L^{-1} in surface waters of Colombia and (Lopes et al., 2010), reported doses of 13-100 μg L^{-1} in natural waters of Brazil . This compares with our data where a decrease in fry survival was evidenced at 10 μg L^{-1} and above. This means that the data reported in environmental media would represent a risk to aquatic life. Regarding sulfamethoxazole Madera-Parra et al (2018), in studies conducted in Brazil, found doses ranging from 0.012-0.44 μg L^{-1} in surface water, while in wastewater they found doses of 0.58 μg L^{-1}. On the other hand, Melendez-Marmolejo et al (2020), reported doses ranging from 0.6-610 μg L^{-1} in surface water in Ecuador and in wastewater in Mexico doses of 0.0052- 6.57 μg L^{-1}. This compares with our study where it was observed that from 0.3-30 μg L^{-1} there was a decrease in the survival of the fry. This means that the reported doses are high, which represents a risk for aquatic species and is a warning that should be considered.

4. Conclusion

The most toxic ECs were triclosan (LC50 =0.03 µg L^{-1}; CENO= 0.003 µg L^{-1}) and estradiol (LC50 =5.7 µg L^{-1}; CENO= 0.2 µg L^{-1}), in contrast to the less toxic compounds sulfamethoxazole (LC50 =103 µg L^{-1}; CENO= 1.3 µg L^{-1}) and nicotine (LC50 = 206 µg L^{-1}; CENO= 10 µg L^{-1}). The ST for estradiol and triclosan was less than 10 days of exposure, while sulfamethoxazole was the only compound that did not cause mortality. Regarding the genotoxicity assays, it was not possible to perform assays with triclosan and estradiol due to the high mortality. Sulfamethoxazole and nicotine showed an increase in the frequency of nuclear anomalies in relation to the control. The present work evaluated in an unprecedented way the lethal doses of ECs in fry, in addition to observing that even at non-lethal doses, the compounds cause genotoxicity. Our findings highlight the importance of regulating ECs because they can produce lethal effects at very low doses and therefore represent a hazard to aquatic organisms.

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