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CENTRO DE ECOLOGIA

LABORATÓRIO DE ECOTOXICOLOGIA

Influência da Idade Larval na Sensibilidade do Zebrafish (*Danio rerio*)  
em Ensaios de Toxicidade Aguda

Trabalho de Conclusão de Curso de graduação como requisito parcial para obtenção do grau de bacharel em Ciências Biológicas, formatado conforme as normas de submissão da revista Environmental Toxicology and Chemistry

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## RESUMO

Ensaio de Toxicidade Aguda com Peixes (AFT – Acute Fish Toxicity) são amplamente utilizados em avaliação de risco com efluentes e no monitoramento ecotoxicológico. Em vários países a execução de ensaios AFT utilizando *Danio rerio* como organismo modelo é regulada por protocolos como a OECD 203 ou a brasileira NBR ISO 15088, que determinam a utilização do peixe em sua fase juvenil ou adulta. Considerando que estudos trazem a informação de fases larvais de *D. rerio* exibirem uma sensibilidade muito mais elevada, o objetivo principal deste estudo é a avaliação das diferentes idades larvais em exposição a uma solução de referência (KCl), com o intuito de que uma ótima idade ou faixa etária possa ser estabelecida para seu uso em ensaios AFT. Peixes com idades de 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 e 24 dpe (dias pós-eclosão) foram expostos a cinco diferentes concentrações de KCl (mais grupo controle) em ensaios AFT de 48 horas. Ademais, um ensaio de toxicidade com um efluente real foi realizado para observar se os resultados se encaixariam na tendência dos de KCl. Complementarmente, medidas de comprimento de peixes nas diferentes idades avaliadas foram coletadas. Como resultado, a sensibilidade ao KCl evidenciou uma faixa ótima para o uso de *D. rerio* em ensaios AFT do 6° dpe até o 14° dpe. O ensaio com efluente apresentou uma tendência de curva semelhante ao que foi encontrado nos ensaios com KCl. Considerando a maior sensibilidade de *D. rerio* na fase larval, ressaltamos a necessidade de revisão dos atuais protocolos de ensaio de toxicidade aguda com peixes, em função da idade dos organismos utilizados.

**Palavras-chave:** Ensaio Larval, *Danio rerio*, Variação de Sensibilidade, Ensaio de Sensibilidade, Toxicologia de Água Doce

**INFLUENCE OF POST HATCHING AGE ON THE SENSITIVITY OF  
ZEBRAFISH (*DANIO RERIO*) IN ACUTE TOXICITY TEST**

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## ABSTRACT

Acute Fish Toxicity (AFT) tests are largely used for effluent risk assessment and/or ecotoxicological monitoring. In many countries, AFT tests execution using *Danio rerio* as a model organism are regulated by protocols like OECD 203 or the Brazilian NBR ISO 15088, which determine the use of juvenile or adult fish. Considering that studies bring the information of larval stages of *D. rerio* exhibiting a much-increased sensitivity, the main objective of this study is the evaluation of different larval ages in exposure to a reference solution (KCl), in order that an optimum age or range can be established for its use in AFT tests. Fish aging 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 dph (days post-hatching) were exposed to five different concentrations of KCl (plus control) in 48 hours AFT tests. The LC50 results were inserted in a sensitivity curve. Moreover, an effluent toxicity test was performed to observe if the results fit the tendency of the KCl ones. Complementary, length measurements from the fish ages were taken. As results, the KCl sensitivity evidenced an optimum range for the use of *D. rerio* in AFT tests going from the 6<sup>th</sup> dph until the 14<sup>th</sup> dph. The effluent testing showed similar curve pattern to what was found in KCl testing. Considering the increased sensitivity of *D. rerio* during the larval stage, we emphasize the need for a review of the actual fish toxicity test protocols, due to used organism age.

**Key words:** Larval Test, *Danio rerio*, Range Sensitivity, Sensitivity Test, Fresh Water Toxicity Test.

## INTRODUCTION

The usage of Acute Fish Toxicity (AFT) tests for effluent risk assessment and/or ecotoxicological monitoring is largely spread worldwide. Fish represent the main secondary consumers in the aquatic habitat (Costa et al. 2008), and its application in ecotoxicological tests may differ according to the used species or the methodology addressed. Countries such as Brazil adopted other countries standard protocols but with some slight modifications, so is the Brazilian NBR ISO 15088, originated from the previously existent OECD 203. Both guidelines, original and modified, determine the execution of AFT tests with *Danio rerio* (Zebrafish), in juvenile or adult stage using the size of the fish as selection parameter for the test, that must be around the specific length of  $2 \pm 1$  cm. Occurs that previous studies have already shown a considerably higher sensitivity of larval stages of *D. rerio* when compared to the post-larval stages required by these actual protocols (Freiry et al. 2014). Besides this, the literature bring us that body length is not suitable as a requirement in the selection of organisms to be used in toxicity tests (U.S. Environmental Protection Agency (USEPA) 2002), and moreover cannot be used as parameter to stipulate the age of fish, once growth is directly affected by culture conditions (Sipáuba et al. 1995).

According to USEPA (2002), it is recommended that ecotoxicological tests should be performed with test-organisms in early stages of development, making sure that the most sensitive organisms will be tested and furthermore be protected in the natural environment. Freiry et al. (2014), evaluated both larval ( $10 \pm 2$  dph (days post-hatching)) and juvenile ( $60 \pm 4$  dph) stages of *D. rerio*, exposed to three different chemicals (CuSO<sub>4</sub>, KCl and NaCl) in 48 hours AFT tests. For all the tested chemicals the response of the larval stage was much more sensitive, evidencing the better efficiency of this alternative method for toxicity detection when compared to the actual NBR ISO 15088 or OECD 203 protocols. Also, Stelzer et al (2018), testing untreated hospital effluent for the base methods OECD 203 (juvenile), USEPA 2000.0 (larvae) and OECD 236 (embryo) evidenced the LC50 four to five-fold higher for the larvae of this same species, only comparing different life stages. This 2018's study highlighted as well, some biological key points that may be associated with the decrease of the sensitivity, such as differences in the relation of body volume and surface of exposure, conferring more resistance to juveniles and bigger organisms. Added to the dependence of the vitelline yolk sack in the first days post-hatching, due to the incomplete development of the digestive tract in just-hatched fish, that does not allow ingestion and foraging to be considered as a possible contamination route.

Considering already existent data in the literature, referring to possible alterations in the organism sensitivity due to its age, and the relevance of substituting *Danio rerio* juvenile stage for the larval stage in AFT tests. It makes necessary a deeper knowledge about how the sensitivity behaves in the different ages during the early life stages of the fish. This data is crucial for the establishment of the most suitable life stage for the usage of the Zebrafish (*D. rerio*) in acute toxicity tests. Having this in mind, the aim of this work was to evaluate the response of different larval ages (2,4,6,8,10,12,14,16,18,20 and 24 dph) when exposed to a reference solution (KCl), in order that an optimum age or range could be established for the use of *Danio rerio* as a model in acute toxicity tests.

## METHODOLOGY

### *CULTIVATION*

All the fish used in the tests were cultivated in the Ecotoxicology Laboratory, located in the Ecology Center facilities of the Federal University of Rio Grande do Sul (UFRGS) under a high-quality control. These fish were maintained in constant temperature of  $27 \pm 2$  °C, photoperiod (16/8 h light/dark cycle), pH ( $7.4 \pm 0.2$ ), non-toxic ammonia levels, and sufficient food supply system. A high-quality control is essential once culture conditions have a direct impact on organism health. Deficient living conditions may lead to possible pre-selection of the strongest individuals to be used in the tests, and thus, to the output of fake results. Divergent food necessities of *D. rerio* life stages were respected. Therefore, newborn, juveniles and adults diets were composed of *Paramecium sp.*, freshly hatched Artemia nauplii and frozen Artemia, respectively, being fed five days a week. For reproduction, the proportion followed was of two males for each female in the breeding tanks.

### *DILUTION Water*

The dilution water that was used in the tests was deionized and reconstituted to the final hardness of 40-47 mg. L<sup>-1</sup> CaCO<sub>3</sub> and pH 7.4 - 7.5, and was prepared according to ISO 12890 (ISO, 1999) protocol.

### *ECOTOXICOLOGICAL Tests and Statistical Evaluation*

All the ecotoxicological tests were performed in the Ecotoxicology Laboratory of UFRGS. The life stages evaluated were given from the 2<sup>nd</sup> dph (days post-hatching), until the 24<sup>th</sup> dph, being these tests performed every two days and having a duration of 48 hours. Thus, the ages tested were 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 dph. Different larvae batches were used and a minimum of three tests per age was accomplished, willing to reduce the influence of variable results.

The protocol used is an adaptation of the 48 hours acute toxicity test with *P. promelas* established by the "US EPA 2000.0". The main changes consist basically in the modification of the used species to *D. rerio* and the expansion of the tested ages until 24days post-hatching, while the original standard establishes the use only from the 1<sup>st</sup> dph to 14<sup>th</sup> dph. All the larvae were nurtured with *Paramecium sp.* Ad libitum until 2 hours prior testing.

The reference solution used for the sensitivity evaluation was Potassium Chloride (KCl). A total of five concentrations of KCl were tested (0.1, 0.2, 0.5, 1.0 and 1.5g/L) plus control, this one containing dilution water only. All concentrations and control group had two replicates of 250ml, containing five fish each. The test was maintained in static renewal system at the temperature of  $25 \pm 2$  °C and photoperiod of 16/8 h light/dark cycle. After 48 hours of exposure, it was determined the LC50 (lethal concentration required to kill 50% of the population) and its confidence intervals using Trimmed Spearman-Kärber 1.5. The difference in the LC50 results was verified based on the method of overlapping the confidence intervals (Payton et al. 2003).

### *EFFLUENT Toxicity*

To ensure that the larval sensitivity behaves in the same pattern when exposed either to KCl or to a real effluent, three different larval ages of 2, 7 and 22 dph were exposed to a domestic effluent diluted in different concentrations during a 48 hours AFT test. After that, the LC50 results obtained were plotted into a sensitivity graph that could be used to make a comparison between the results of effluent and KCl toxicity. The effluent used in the test is descendent from the Federal University of Rio Grande do Sul (UFRGS) facilities and in past studies have exhibited toxicity.

### *FISH Length*

With the aim to analyze the growing pattern it was measured the length of all the ages evaluated in the experiments. In addition, the length at the hatching day (day zero) was also taken. A minimum of ten individuals per age was selected randomly to avoid biased results. The

measures were taken using a stereo microscope with a coupled scaled eyepiece and the collected data was plotted on a graph.

## RESULTS

### *KCl Toxicity*

The average LC50 results for each tested age during the forty-three validated KCl toxicity tests performed in this study are shown in FIGURE 1. It is exhibited in the LC50 curve a non-continuous distribution pattern that can be distinguished in three main phases, increase, stabilization following by a decrease of the sensitivity. At the age of 2 dph it is presented an average LC50 = 0.523g.L-1, which tends to decrease until the 6<sup>th</sup> dph, increasing sensitivity. From the 6<sup>th</sup> dph until the 14<sup>th</sup> dph the sensitivity tends to stabilize, with LC50 values around 0.305 g.L-1. On the subsequent ages, the sensitivity keeps decreasing and reaching at the 24<sup>th</sup> dph a LC50 = 0.653g.L-1, presenting the highest LC50 value and lowest sensitivity to KCl of all period tested. Among all tested ages, the maximum coefficient of variation (CV) presented was 24.5% and agrees with the requirements of a maximum value of 30% (Environment Canada 1990; Busquet et al. 2014). Beyond this, the R<sup>2</sup> (determination coefficient) was equal to 0.9445. A maximum R<sup>2</sup> value of 1 means that the dependent variable can be predicted without error from the independent variable, so on an R<sup>2</sup> equal to 0 represents the complete opposite. Considering that, the obtained determination coefficient reinforces the validity of the obtained data.

The KCl sensitivity graph displays a consistent tendency of a lower sensitivity in both extremities of the tested ages, while in intermediary ages a higher sensitivity is presented. Even though it has been shown the highest sensitivity at the 10<sup>th</sup> dph, it has been concluded by analysis that the difference in sensitivity from the 6<sup>th</sup> dph until the 14<sup>th</sup> dph has no statistical relevance, being this period considered the optimum range for the use of *Danio rerio* in ecotoxicological testing.

### *EFFLUENT testing*

The Effluent sensitivity graph is shown in FIGURE 2. With the aim to verify the behavior of sensibility in a real effluent toxicity analysis, three distinct larval ages (2, 7 and 22 dph) were exposed in 48 hours acute toxicity tests to different concentrations of domestic effluent. Turn out that the results of the toxicity tests went in agreement with what has been concluded with KCl



exposure. The LC50 values for 2, 7, and 22 dph were 16.49%, 4.42% and 12.04%, respectively, following the same pattern of an optimum range in intermediary ages.

### *FISH Length*

FIGURE 3 shows the length values from 0 to 24 dph. The smallest organism measured at 0 dph had a length of 0.28 cm and at 24 dph the largest one had 0.73cm. The average growth percentage from the 0 dph until the 24<sup>th</sup> dph was equal to 68%. The growth pattern presented shows the measurement ranges with an overlap in the length of each day evaluated, conferring equal sizes to fish with different ages. Related to CV, there is an apparent tendency of an increased length variance according to fish aging.

## **DISCUSSION**

The tendency evidenced in the LC50 results of a decreased sensitivity of *Danio rerio* at advanced ages was already expected, once several literatures have already presented that earlier life stages of an organism would be the most sensitive ones (Dave and Xiu, 1991; McKim et al. 1975, 1977; USEPA 2000.0; Freiry et al. 2014,), thus being the most proper period for toxic evaluation and the performing of toxicity tests. McKim (1977), in a review study of a total 56 life-cycle toxicity tests, including 34 organic and inorganic chemicals and four different species of fish concluded that the embryo-larval and early juvenile stages presented a higher sensitivity. However, further studies show that within the early life stage of the fish there is still a relevant distinct sensitivity range according to the development stage approached. In this manner, the larval period would display a higher sensitivity in toxicity tests when compared to the embryo in numerous fish species (Eaton et al. 1978; Bansal S et al. 1980; Stelzer et al. 2018).

In the KCL sensitivity graph derived from the AFT tests developed in this study, it is shown a pattern where higher LC50 values are evidenced in both edges of the tested larval ages, whereas lower LC50 values behave in between intermediate ages, indicating a more sensitive stage of the organism. The differences in the sensitivity within the larval stage presented in the LC50 curve may be influenced directly or indirectly by some physiological key points. Having this in mind, we elucidated some relevant biological considerations that may interfere in larvae sensitivity and be a possible explanation for LC50 behaviour in the early stages post-hatching of the fish.

### *EXTERNAL feeding*

The digestive tract of *D. rerio* from the first to the second day after hatching is a functional open tube in contact with the environment and all the toxins present on it (Wallace & Pack 2003; Ng. et al. 2005). Nevertheless, after hatching the Zebrafish larvae gets into what is called “eleutheroembryo period”, which consists in the period between hatching and the beginning of foraging. During this period, the larvae feed exclusively from the yolk sack until the 2<sup>nd</sup> dph or 3<sup>rd</sup> dph. After that, the fish starts external nutrition even the yolk sack is not completely depleted (Holmberg et al. 2004; OECD 2006). These considerations may explain why just hatched larvae presented in the toxicity tests a higher tolerance which tended to diminish after the period equivalent to the onset of external feeding, possibly due to the increase of the water flux throughout the organism or the increased activity of general absorption mechanisms stimulated by foraging.

### *INTESTINE*

The literature brings us that intestine maturation continuous long after fish leaves the corium, even though being functional by the time of feeding (Holmberg et al. 2004) *Danio rerio* intestine morphogenesis is not yet completed. In Ng. et al. (2005), it is presented a complex speciation process after the anus opening at 2 dph, this process involving remodelling, compartmentalization and differentiation of the intestinal epithelium. The study relates a continuous villus-like folding of the intestinal epithelium until 11 dph. Villus propagation could influence sensitivity considerably, once these formations increase intestine absorption surface. Considering this approach, sensitivity should increase progressively, reaching a higher value at later ages. Which did not happen in our experiments, where the sensitivity wasn't always progressive and the optimum range behaved in an intermediate age of the larval stage.

Occurs, that this same study also relates a massive goblet cell differentiation in the mid-intestine until near the same 11<sup>th</sup> dph. Goblet cells have secretory rolls and are involved in cell protection, are related to the innate defence barrier, among other functions. A massive differentiation of goblet cells should have an opposite impact of the villus propagation, decreasing sensitivity gradually. As the results do not fit perfectly in any of these explanations it is still possible that both factors have mutual action, what could explain the intermediary optimum range and the fluctuation of the decrease and increase of the sensitivity. It is important to emphasize that while In Ng. et al. (2005) affirm the presence of goblet cells at 11 dph in all three regions of the intestinal tract, other literature as Wallace et al. (2005) bring us that goblet cells are present only in the mid-intestine during the larval stages and its presence in adult's posterior and anterior intestine would be result of cell migration during intestinal maturation.

Being the digestive tract considered by some authors (Wallace et al. 2005) essential not only in nutrient digestion and absorption but also as a barrier to environmental toxins. In a broad manner, it would be feasible the assumption that larval stages of Zebrafish are more vulnerable to toxicants once their digestive system remains immature.

### *OXYGEN Uptake*

As it happens with several different fish species, freshly hatched *Danio rerio* do not have functional gills, in fact, their gills take more than 30 days after hatching to reach the complete maturity. The lack of functional gills in the early life stages of the fish is supplied by a superficial layer of red muscle fibres which perform most of the gas exchange along with the body surface. During gill maturation this red layer shrinks while the gill surface increases progressively, assuming the role of oxygen uptake (El-Fiky et al. 1987; Rombough 2002). What occurs is that due to the early cutaneous breathing, young small fish larvae possess a much larger exchange surface, reaching until 10 times wider when compared to bigger and older individuals, dependent of gills for oxygen uptake. Meanwhile, the mass-specific metabolic rate, relative to fish metabolic demand for O<sub>2</sub> remains the same during larval and adult stages (Rombough 1999), conferring to early fish larvae a four to five-fold exchange capacity compared to their metabolic needs (Rombough & Moroz 1997). Increased exchange capacity and exchange surface also increase the exposure of the animal to the chemicals present in the environment, possibly leading the fish to higher vulnerability and higher sensitivity during early larval stages.

Rombough 2002, indicates that until the 11<sup>th</sup> dph the O<sub>2</sub> uptake of Zebrafish larvae would still be greatly supported by cutaneous breathing. Yet, by the age of 18 dph larvae would be already critically dependent on the gills for gas exchange, even it not being completely matured. At this stage, cutaneous breathing has a low influence on fish metabolism. The analysis of the toxicity tests performed with 18 dph organism exhibit a much higher LC<sub>50</sub> when compared to the values of the tests with earlier stages, going in agreement with what has been discussed above.

### *INCREASED energy demands*

It is related that certain stages of development may be more susceptible to toxin exposure by virtue of increased energy demands (Marty et al. 1990; Gray & Metcalfe 1999). Considering that during the larval stage occurs a threefold acceleration in the organism development (Augustine et al. 2011), it is reasonable to conclude that a higher development rate would

increase energy consumption and like that expose the larvae to a more vulnerable condition. Additionally, stress conditions are well known to increase the energy demands of the organism (Puvanewari et al. 2006). In this scenario, a young fish larvae exposed to environmental chemicals could be led to hazard and higher mortality rates, due to the combination of physiological factors as development necessities dysfunction and metabolic stress (Levitan & Taylor 1979).

### *FISH size*

Some literature as Rombough (1999) and Stelzer et al. (2018), relate an alteration on the relation of body volume and surface of exposure according to fish aging. The main argument would be that the fish growing larger its surface to volume ratio declines. In this manner, bigger bodies would possess a smaller surface of exposure. Taking into consideration the average larvae growth of 68% from the 0 dph to the 24<sup>th</sup> dph, we could say that it may lead older individuals to present a higher resistance in exposure to chemicals in comparison with younger ones. Furthermore, the measurement values exhibit a tendency of overlap in the length ranges for the different ages evaluated and an increase in the length dispersal in later ages. Turns out that both factors can become an issue if we take into consideration that the actual Brazilian protocol NBR ISO 15088 and OECD 203 use the size of the fish as an organism selection parameter for toxicity tests. Even though some organizations like the U.S. Environmental Protection Agency (USEPA 2002) have already affirmed that length is not suitable as a requirement in this aspect. Both considerations end up turning more susceptible to the selection of organisms with distinct sensitivities for ecotoxicological testing, turning the results of the toxicity test doubtful. Thus, it would be more consistent the use of younger life stages of *D. rerio* whereupon there is a higher length uniformity in comparison to posterior ages, summed to the fact that smaller organisms possess higher surface vs. volume ratios.

## CONCLUSION

When we talk about toxicity, a more conservative approach means protection, and simply testing the appropriate life stage of an organism could reduce impacts enormously. On behalf of this scoop, the efforts of this study made possible the establishment of an optimum range for the use of *Danio rerio* in acute toxicity testing. The execution of AFT tests using Zebrafish from the 6<sup>th</sup> day post-hatching until the 14<sup>th</sup> day post-hatching would generate more reliable data, and so, more accurate screening of the toxicity. Getting to know sensitivity behaviour, influences, and tracing an optimum range for the use of *Danio rerio* or any other model organism in toxicity testing is for sure of extreme relevance. Ecotoxicological tests were developed with the aim of reproducing in a simplified way the impact of toxicants in the environment. Predicting and analyzing in laboratory possible adverse effects and like that making sure that in the environment the organisms will be protected. In this perspective, the use of individuals with higher resistance for toxicity evaluation would only mask toxic effects, culminating in false negative results that may end up being harmful to higher sensitivity organisms. Based on these findings it is suggested a review on the actual protocols NBR ISO 15088 and OECD 203, where it is determined the execution of AFT tests with *Danio rerio* at juvenile and adult stages, already proved to exhibit higher resistance.

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FIGURE 1. Graph showing *Danio rerio* larvae sensitivity to KCl in 48 hours AFT tests, generated with the average LC50 of the distinct tested ages.

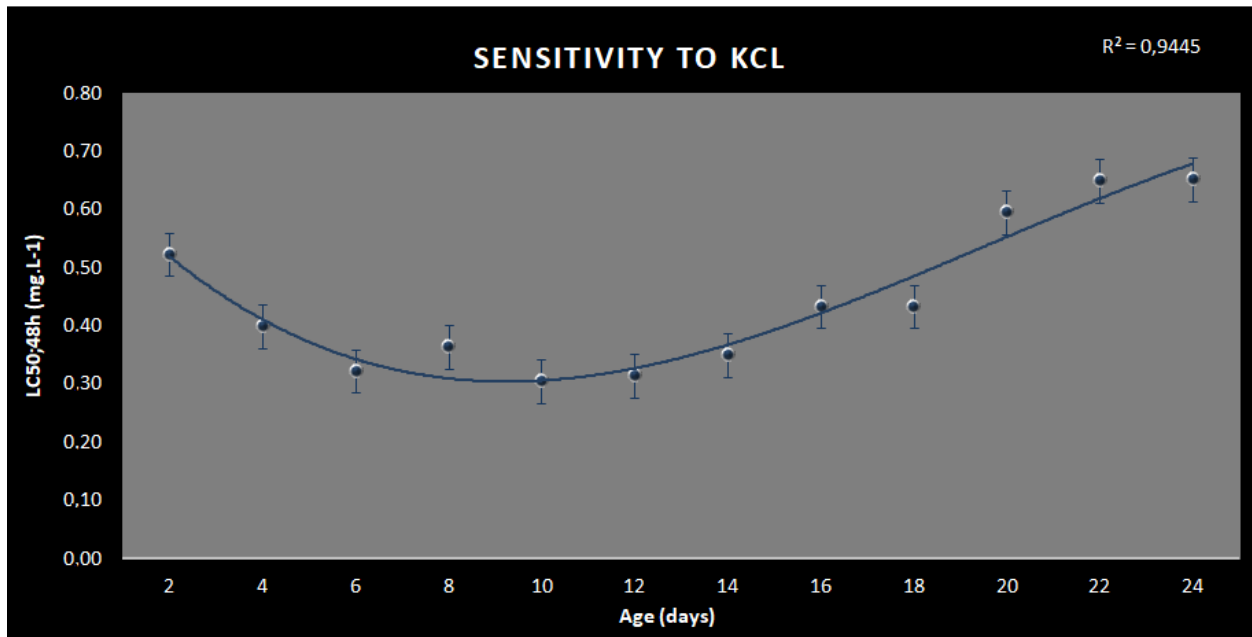


FIGURE 2. Effluent toxicity curve, presenting the LC50 results of the 48 hours AFT tests with *Danio rerio* at the larval ages of 2, 7 and 22 days post-hatching. Showing similar sensitivity pattern when compared to the 48 hours AFT tests with KCl.

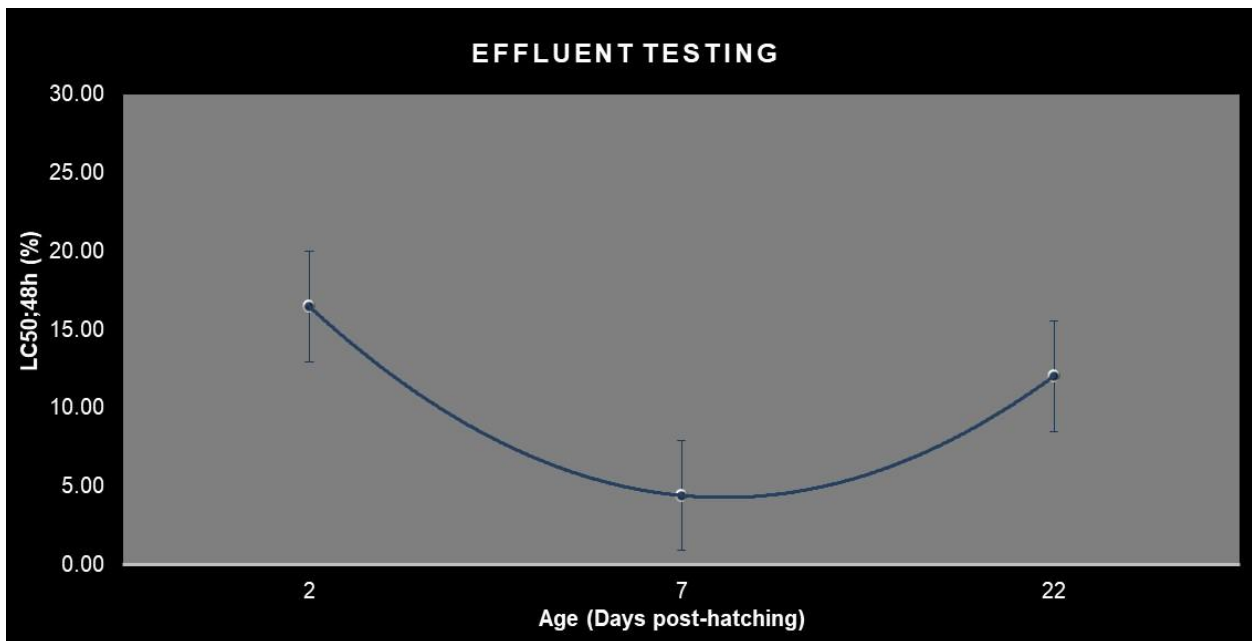


FIGURE 3. Length dispersal of *Danio rerio* larvae, presenting the length results for each individual measured showing the overlap on the age sizes.

