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Acute toxicity on early life stages of *Pachychilon pictum* (Heckel & Knerr, 1878) from Lake Ohrid

Original Article

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Abstract:

This study investigates the use of the fish embryo toxicity test on early life stages of *Pachychilon pictum*, obtained from wild populations in Lake Ohrid. Various chemical compounds were assessed for mortality, and median lethal concentrations at 96^{h} post fertilization were determined. The findings demonstrated the successful application of the test requirements, confirming that embryos of this species are suitable for toxicity testing. Notably, this represents the first ever toxicological investigation conducted on a fish species from Lake Ohrid, highlighting the importance of the resulting data.

Key words:
fish embryo, Lake Ohrid, *Pachychilon pictum*, toxicity

Apstrakt:

Akutna toksičnost na ranim razvojnim stadijumima vrste *Pachychilon pictum* (Heckel & Knerr, 1878) iz Ohridskog jezera

U ovom istraživanju ispitivana je primena testa embrionalne toksičnosti riba na ranim razvojnim stadijumima vrste *Pachychilon pictum*, dobijenim iz divljih populacija u Ohridskom jezeru. Procenjivani su različiti hemijski spojevi u pogledu smrtnosti, a određene su srednje letalne koncentracije nakon 96 sati od oplođenja. Rezultati su pokazali uspešnu primenu metodoloških zahteva testa, potvrđujući da su embrioni ove vrste pogodni za ispitivanje toksičnosti. Važno je istaći da ovo predstavlja prvo toksiološko istraživanje sprovedeno na jednoj ribljoj vrsti iz Ohridskog jezera, što naglašava značaj dobijenih podataka.

Ključne reči:
embrion ribe, Ohridsko jezero, *Pachychilon pictum*, toksičnost

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Introduction

Many aquatic bodies are currently subjected to a variety of environmental stressors, which have the potential to negatively alter the conditions of water ecosystems and biodiversity. The growing awareness of the possible environmental effects of human activity has led to the development of numerous methodologies that attempt to predict how stress could impact fish populations (Power, 1997). These methodologies are based on determination of physiological, biochemical, genotoxic changes in fish (van der Oost et al., 2003), and occurrence of mortality (Wedekind et al., 2007). High amounts of anthropogenic contaminants entering the environment have caused the necessity for reliable and accurate toxicity tests (Fent, 2004). Acute toxicity tests are significant in environmental risk assessment as they provide an evaluation of the relative toxicity of substances in a variety of species (Wedekind et al., 2007).

Fish, as the most widespread aquatic vertebrate class, have been considered an essential part of toxicity assessment testing (Braunbeck & Lammer, 2005). Acute fish toxicity data are regarded as an essential requirement in the hazard and environmental risk evaluation of different types of chemicals (Cooney, 1995). Primarily, most of the acute toxicity tests are applied using adult fish, which requires the sacrifice of lots of specimens. Because the targeted endpoint of acute toxicity testing is mortality, fish are most likely to experience pain and suffering (Chandroo et al., 2004), which conflicts with current animal welfare legislations. The developing fish embryo and larvae are regarded as the most sensitive stages in the life cycle of a fish, with expressed sensitivity to environmental changes (von Westernhagen, 1988). With that in considera-

tion, and the fact that the early life stages of fish are not protected by the legislations like the European Union directive 2010/63/EU on the protection of animals used for scientific purposes, the early life stages of fish proved to be excellent candidates for an alternative test subject. The acute fish embryo toxicity test was designed as an alternative method and validated by the Organisation for Economic Co-operation and Development (OECD) (Busquet et al., 2014) as test guideline OECD 236 (OECD, 2013). It has been demonstrated that fish embryo tests can be used to measure and rank different types of toxic substances, confirming the embryo toxicity tests as a useful method for assessing environmental dangers and threats to the ecological status of waterbodies (Rundeva, 2014). Studies have confirmed that acute toxicity tests with fish embryos are good predictors of the results obtained with acute tests on adult fish, and therefore they are a good testing alternative (Belanger et al., 2013). Besides their sensitivity, these tests have practical advantages over tests with adult fish, in the sense that they require less test volume and space, which enables the use of greater numbers of test organisms and replicates (Wedekind et al., 2007).

Although the design of this test is based on the early life stages of zebrafish (*Danio rerio*) as test organism, to encompass possible different species sensitivities, it was proposed to apply and adapt the protocol to other fish species (Braunbeck et al., 2005; Lammer et al., 2009; Belanger et al., 2013). Different fish species may vary in their sensitivity to environmental contaminants (Nagel, 2002), and according to Wedekind et al. (2007), in acute toxicity testing, fish species should be representative of the region where they occur.

Lake Ohrid is an oligotrophic water body located on the Balkan peninsula, and as the oldest natural lake in Europe, it has a high level of endemism and, as a part of that endemism, a unique fish fauna (Albrecht & Wilke, 2008). As for now, there are no ecotoxicological studies incorporating toxicity tests on any of the fish species from Lake Ohrid. With the increasing anthropogenic pressures on the lake, present from decades ago (Matzinger et al., 2006; Kostoski et al., 2010), and the industrialization and urbanization of the area in modern times, toxicity data could be of great importance for environmental and risk assessment.

Therefore, the aim of this study was to apply and adapt the embryo toxicity test to the early life stages of *Pachychilon pictum* (Heckel & Knerr, 1878), a representative of the cyprinid fish fauna from Lake Ohrid. This is the first study of this kind, applied to fish from Lake Ohrid, and for the fish species native to the freshwater bodies of North Macedonia in general.

Materials and Methods

The embryos for this study were provided through artificial reproduction of wild specimens of *Pachychilon pictum*. It is a cyprinid fish species, endemic to the freshwater bodies of the southwestern Balkan (Lakes Ohrid and Skadar, river Black Drin) (Kottelat & Freyhof, 2007; Talevski et al., 2025). Male and female specimens were sampled during the spawning period in early summer. The sampling took place in the littoral area of Lake Ohrid, using gill nets with various mesh sizes. Reproductive material (eggs and milt) was collected by stripping, after which the fish were released back into the lake. The artificial reproduction was carried out by using the "dry method" (Billard et al., 1996), and the acquired fertilized embryos were used for the acute toxicity test.

For the implementation of the acute toxicity test, eight chemical compounds were selected. The selected chemicals were of different types and had different levels of toxicity. They are commonly used in various industries as raw materials, precursors, active substances, and agricultural biocides. According to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (UN, 2023), these chemicals have different toxicity to fish, ranging from non-toxic to very toxic (OECD, 2011; OECD, 2012). The ranges of the test concentrations used for the toxicity test were selected based on data obtained from literature from zebrafish - based toxicity tests. This was done to compare the sensitivity of *P. pictum* embryos to zebrafish as the standard model for toxicity testing. The test concentrations were obtained from the OECD validation study for the acute fish embryo toxicity test (OECD, 2011; OECD, 2012; Busquet et al., 2014). The chemicals used for preparation of the solutions had a minimum purity grade of ≥ 98%, and all test solutions were freshly prepared using standardized dilution water (OECD, 2013). The tested chemical compounds, along with the test concentrations, are presented in **Tab. 1**.

The acute toxicity test was performed according to the OECD 236 test guidelines (OECD, 2013). Freshly fertilized embryos were inspected for fertilization success, and only embryos without any irregularities were used for the test. After selection, the embryos were transferred to 24-well plates filled with 2 ml of the test solution, with one embryo placed in each well. On one plate, 20 embryos were placed in the test solution, and 4 embryos in dilution water, which served as an internal control for the plate. The test uses positive control (4 mg/L

Table 1. List of the chemical compounds and the test concentrations

Chemical	Cas number	Test concentrations (mg/L)	Chemical type
Copper (II) sulfate pentahydrate	7758-99-8	0.15, 0.3, 0.6, 1.2, 2.4	biocide
Dibutyl maleate	105-76-0	0.25, 0.5, 1, 2, 4	industrial organic
3,4-Dichloroaniline	95-76-1	0.5, 1, 2, 3.7, 4, 8	industrial organic
4,6-Dinitro-o-cresol	534-52-1	0.18, 0.32, 0.58, 1.05, 1.89	biocide
Ethanol	64-17-5	5300, 8000, 12000, 18000, 27000	industrial organic
1-Octanol	111-87-5	2.5, 5, 10, 20, 40	industrial organic
Sodium chloride	7647-14-5	1000, 2000, 4000, 8000, 16000	industrial inorganic
Triethylene glycol	112-27-6	20, 30, 45, 67.5, 101.25	industrial organic

3,4 – dichloroaniline) and negative control (dilution water), and is considered valid if the mortality rate in the positive control is minimum 30% and the maximum mortality rate in the negative control is 10%. The well plates were placed in an incubator at 26±1 °C, under semi-static exposure, and the tests were performed in duplicate. Embryos were observed daily (24, 48, 72, 96 hpf - hours post fertilization) for the occurrence of mortality, determined by the endpoints according to OECD 236 (OECD, 2013). The endpoints specified in the guidelines are: embryo coagulation, lack of somite formation, lack of tail detachment, and lack of heart beat (OECD, 2013). The embryos were observed and analyzed under an inverted microscope Zeiss PrimoVert (Carl Zeiss GmbH, Oberkochen, Germany), and pictures were taken using the Zeiss ZEN Software.

Lethal concentrations corresponding to effect levels of 10% (LC_{10}) and 50% (LC_{50}) were estimated at 96 hours post fertilization. These values, along with the 95% confidence limits, were calculated using probit analysis performed with statistical software (SPSS 23.0).

Results and discussion

The mortality of *Pachychilon pictum* embryos was recorded according to the endpoints of the test guideline OECD 236. The values for LC_{10} and LC_{50} at 96 hpf for the tested chemical compounds are shown in **Tab. 2**. The highest LC_{50} value was observed for triethylene glycol, and the lowest for 4,6-dinitro-o-cresol. There was no observed mortality in the control group or in the internal plate controls. The embryos of *P. pictum* have transparent chorion, allowing direct observation of the embryonic development (**Fig. 1**). During the test, we documented the occurrence of two of the required endpoints: coagulation of the embryo and lack of heartbeat (**Fig. 1**). In the control group, all embryos were hatched at 72 hpf which is slightly faster compared to zebrafish, with a hatching period at 72 to 96 hpf (Braunbeck & Lammer, 2005; Braunbeck et al., 2005) in the same water temperature.

Although the fish embryo acute toxicity test is designed on the basis of zebrafish embryos, this study showed that it can be successfully applied to different fish species. In that regard, *P. pictum* joins the group of fish species subjected to this test protocol (Lammer et al., 2009; Fiorino et al., 2018). The test validity was verified by confirming that the mortality observed in both the negative and positive control plates fell within the

Table 2. Acute toxicity data from the fish embryo test with *P. pictum* embryos

Chemical	LC_{10}	95% confidence limits	LC_{50}	95% confidence limits
Copper (II) sulfate pentahydrate	0.708	0.468–0.891	1.342	1.095–1.69
Dibutyl maleate	0.792	0.512–1.02	1.657	1.337–2.094
3,4-Dichloroaniline	1.056	0.413– 1.532	2.242	1.519–3.174
4,6-Dinitro-o-cresol	0.217	0.152–0.267	0.383	0.320–0.459
Ethanol	6214	4903–7081	8706	7763–9779
1-Octanol	7.142	4.450–9.432	17.038	13.506–22.270
Sodium chloride	2712	1795–3451	5380	4369–6699
Triethylene glycol	34852	28124–38917	44597	39798–49124

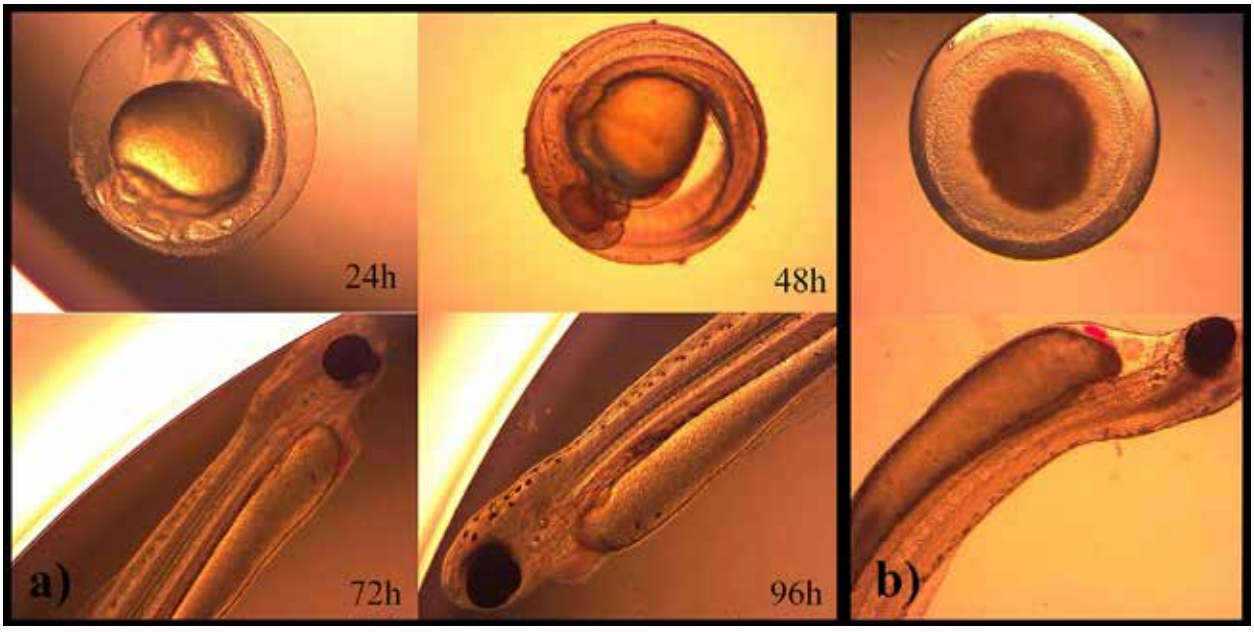

Fig. 1. Normal embryo development in the control plate (**a**); coagulated embryo and mortality observed by lack of heart beat in deformed hatched larva (**b**)

range specified by the test guideline. The rest of the validity requirements were also met, such as: high fertilization rate for the batch of eggs, maintained temperature at 26±1 °C for the duration of the test, and hatching rate of more than 80% in the negative control (OECD, 2013). The notion that we only detected two out of four mortality endpoints could be due to species specific differences in embryo development. All designated endpoints in *P. pictum* embryos were visible even at 24 hpf, which is not the case for zebrafish embryos (OECD, 2013). According to Braunbeck & Lammer (2005), in the zebrafish based test, all of the endpoints are observed until 48^{h} post fertilization. Based on our observations, *P. pictum* embryos had a slightly faster development rate than zebrafish embryos in the same water temperature. This could possibly be adjusted by changing the temperature during the test.

As expected, the embryos of *P. pictum* manifested different sensitivity to different types of chemical compounds (**Tab. 2**). 4,6-dinitro-o-cresol exhibited the highest level of toxicity, which correlates with other studies (Busquet et al., 2014; von Hellfeld et al., 2020). This compound is part of a group of herbicides that have a unique mechanism of toxicity, by uncoupling the oxidative phosphorylation and interfering with the production of ATP. This leads to energy not being available for essential functions in the cells (Solomon et al., 2014). The results for copper (II) sulfate and dibutyl maleate gave different values for LC_{50} compared to zebrafish values from the validation study (OECD 2011, OECD 2012, Busquet et al., 2014). In that study, these compounds were placed in the category ≤ 1 mg/L, which according to the GHS system of classification of chemicals (UN, 2023) is very toxic to fish. This means that *P. pictum* embryos are less sensitive regarding these two chemicals than zebrafish embryos (**Tab. 1**). Ethanol, sodium chloride, and triethylene glycol, according to the LC_{50} values, can be designated as non-toxic to *P. pictum* embryos, which is confirmed by other research based on zebrafish (Busquet et al., 2014; von Hellfeld et al., 2020). While LC_{50} values for most of the tested chemicals from the *P. pictum* test were generally comparable in their toxicity with those observed using zebrafish embryos as test subjects (Busquet et al., 2014), variations were observed, possibly reflecting species specific sensitivity to certain chemicals. These findings support the recommendations from previous studies that a greater variety of fish species should be involved and adapted to embryo toxicity testing (Nagel, 2002; Braunbeck et al., 2005; Wedekind et al., 2007). This could be particularly important when conducting toxicity tests on wild populations. The resulting data would be valuable for assessing environmental risks and potential negative effects on the fish populations and the ecosystems in general. According to Wedekind et al. (2007), a broader range of species should be tested, with embryos collected from natural populations of various species, including most fish that occur in the habitats for which the test substance is potentially relevant.

Conclusion

In conclusion, our results showed that embryos from *Pachychilon pictum* are suitable to be tested using the acute embryo toxicity test, and the guidelines of the OECD 236 protocol were successfully applied. Given that this is the first ecotoxicological study conducted on fish from Lake Ohrid, the generated toxicological data hold significant value. With the increasing anthropological pressure on the ecosystems, this data could be used for future evaluations of environmental threats and potential negative impacts. Our study contributes to the embryo toxicity research by adding another fish species to the increasing group of tested species and gives an insight into the applicability of this test to wild fish populations, and the possibility of their use in ecotoxicological evaluations.

References

Albrecht, C., Wilke, T. (2008). Ancient Lake Ohrid: biodiversity and evolution. *Hydrobiologia*, *615*, 103-140. https://doi.org/10.1007/s10750-008-9558-y

Busquet, F., Strecker, R., Rawlings, J.M., Belanger, S.E., Braunbeck, T., Carr, G.J., Cenjin, P., Fochtman, P., Gourmelon, A., Hubler, N., Kleensang, A., Knobel, M., Kussatz, C., Legler, J., Lillicrap, A., Martinez-Jeronimo, F., Polleichter, C., Rzodezcko, H., Salinas, E., Shneider, K.E., Scholz, S., van den Brandhof, E., van der Ven, L.T.M., Walter-Rhode, S., Weight S., Witters, H., & Halder, M. (2014). OECD validation study to assess intra and inter laboratory reproducibility of the zebrafish embryo toxicity test for acute aquatic toxicity testing. *Regulatory Toxicology and Pharmacology*, *69*(3), 496-511. https://doi.org/0.1016/j.yrtph.2014.05.018

Belanger, S.E., Rawlings, J.M., & Carr, G.J. (2013). Use of fish embryo toxicity for the prediction of acute fish toxicity to chemicals. *Environmental Toxicology and Chemistry*, *32*(8), 1768-1783. https://doi.org/10.1002/etc.2244

Billard, R., Cosson, J., Perchec, G., & Linhart, O. (1996). Biology of sperm and artificial reproduction in carp. *Aquaculture*, *129*, 95-112. https://doi.org/10.1016/0044-8486(94)00231-C

Braunbeck, T., Boettcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M., & Seitz, N. (2005). Towards an alternative for the acute fish LC(50) test in chemical assessment: the fish embryo toxicity test goes multi-species—an update. *Altex*, *22*(2), 87–102. https://altex.org/index.php/altex/article/view/911

Braunbeck, T., & Lammer E. (2005). Draft detailed review paper on fish embryo toxicity assays. German Federal Environment agency.

Chandroo, K.P., Duncan, I.J.H., & Moccia R.D. (2004). Can fish suffer?: perspectives on sentience, pain, fear and stress. *Applied Animal Behaviour Science*, *86*(3-4), 225-250. https://doi.org/10.1016/j.applanim.2004.02.004

Cooney, J.D. (1995). Freshwater tests. In: Rand, G.M. (Ed), Fundamentals of aquatic toxicology (2nd ed). Boca Raton, US: Taylor & Francis Group, CRC Press. https://doi.org/10.1201/9781003075363

European Union (2010). Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes. http://data.europa.eu/eli/dir/2010/63/oj

Fent, K. (2004). Ecotoxicological problems associated with contaminated sites. *Toxicology Letters*, *205*(3), 223-240. https://doi.org/10.1016/j.tox.2004.06.060

Fiorino, E., Sehonova, P., Plhalova, L., Blahoba, J., Svobodova, Z., Faggio, C. (2018). Effects of glyphosate on early life stages: comparison between *Cyprinus carpio* and *Danio rerio*. *Environmental Science and Pollution Research*, *25*, 8542-8549. https://doi.org /10.1007/s11356-017-1141-5

Jezierska, B., Ługowska, K., & Witeska, M. (2008). The effects of heavy metals on embryonic development of fish (a review). *Fish Phisiology and Biochemistry*, *35*, 625-640. https://doi.org/10.1007/s10695-008-9284-4

Kostoski, G., Albrecht, C., Trajanovski, S., & Wilke, T. (2010). A freshwater biodiversity hotspot under pressure - assessing threats and identifying conservation needs for ancient Lake Ohrid. *Biogeosciences*, *7*, 3999-4015. https://doi.org/10.5194/bg-7-3999-2010

Kottelat. M. & Freyhof, J. (2007). Handbook of Europen Freshwater Fishes. Switzerland, Cornol: Publications Kottelat.

Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., & Braunbeck, T. (2009). Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? *Comparative Biochemistry and Physiology Part C*, *149*, 196–209 https://doi.org/10.1016/j.cbpc.2008.11.006

Matzinger, A., Spirkovki, Z., Patceva, S., & Wuest, A. (2006). Sensitivity of ancient Lake Ohrid

to local anthropogenic impacts and global warming. *Journal of great lakes research*, *32*(1), 158-179. https://doi.org/10.3394/0380-1330(2006)32[158:SOALOT]2.0.CO;2

Nagel, R. (2002). DarT: the embryo test with the Zebrafish *Danio rerio* – a general model in ecotoxicology and toxicology. *Altex*, *19*, 38–48. https://altex.org/index.php/altex/article/view/2125

OECD (2011). Validation report (Phase 1) for the zebrafish embryo toxicity test part 2. No. 157.

OECD (2012). Annexes to the validation report (Phase 2) for the zebrafish embryo toxicity test. No. 179.

OECD (2013). Guideline for Testing of Chemicals, Test No. 236: Fish Embryo Acute Toxicity (FET) Test. OECD Publishing. https://doi.org/10.1787/9789264203709-en

Power, M. (1997). Assesing the effects of environmental stressors on fish populations. *Aquatic Toxicology*, *39*, 151-169 https://doi.org/10.1016/S0166-445X(97)00020-9

Rundeva, I. (2014). Biomarkers for stress in fish embryos and larvae. Boca Raton, US: Taylor & Francis Group, CRC Press. https://doi.org/10.1201/b15378

Solomon, K.R., Dalhoff, K., Volz, D., & Van Der Kraak, G. (2014). Effects of Herbicides on Fish, In: Tierney, K.B., Farrell, A.P., & Brauner, C.J. (Ed). Organic chemical toxicology of fishes. USA: Academic Press. https://doi.org/10.1016/B978-0-12-398254-4.00007-8

Talevski, T., Talevska, M., & Talevska-Leshoska, A. (2025). Endemic fish species in the Macedonian fresh water. In: Cano Ortiz, A., & Pena-Martinez J. (Ed): Endemic species from around the world: Teaching for sustainability. IntechOpen. doi: 10.5772/intechopen.1003342

United Nations (2023). Globally harmonized system of classification and labelling of chemicals (GHS) (10th ed.). USA, New York: United Nations Publications.

van der Oost, R., Beyer, J., & Vermulen, N.P. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Enironmental toxicology and pharmacology*, *13*(2), 57-149. https://doi.org/10.1016/S1382-6689(02)00126-6

von Hellfeld, R., Brotzmann, K., Baumann, L., Strecker, R., & Braunbeck, T. (2020). Adverse effects in the fish embryo acute toxicity (FET) test: a catalogue of unspecific morphological changes versus more specific effects in zebrafish (*Danio rerio*) embryos. *Environmental Sciences Europe*, *32*, 122. https://doi.org/10.1186/s12302-020-00398-3

von Westernhagen, H. (1988). Sublethal effects of pollutants on fish eggs and larvae. In: Noar, W.S., & Randall, D.J. (Ed). Physiology of developing eggs, Part A: Eggs and larvae. USA: Academic Press. https://doi.org/10.1016/S1546-5098(08)60201-0

Wedekind, C., von Siebnethal, B., & Gingold, R. (2007). The weaker points of fish acute toxicity test and how test on embryos can solve some issues. *Environmental Pollution*, *148*, 385-389. https://doi.org/10.1016/j.envpol.2006.11.022