

# AEO-7 Surfactant is “super Toxic” and Induces Severe Cardiac, Liver and Locomotion Damage in Zebrafish Embryos

**Maha Al-Asmakh**

Qatar University

**Amin F. Majdalawieh**

American University of Sharjah

**Aboubakr M. Abdullah**

Qatar University

**Nadin Younes**

Qatar University

**Sahar I. Da'as**

Sidra Medicine

**A. Bahgat Radwan**

Qatar University

**Mostafa H. Sliem**

Qatar University

**Houria Ech-Cherif**

Qatar University

**Gianfranco Pintus**

University of Sharjah

**Gheyath K. Nasrallah** (✉ [gheyath.nasrallah@qu.edu.qa](mailto:gheyath.nasrallah@qu.edu.qa))

Qatar University <https://orcid.org/0000-0001-9252-1038>

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## Research

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# Abstract

## Background

Fatty alcohol polyoxyethylene ether-7 (AEO-7), a non-ionic surfactant, has recently been receiving extensive attention from the ocean pipeline industry for its ability to inhibit corrosion. However, the present lack of information concerning the potential environmental toxicity of AEO-7, especially towards aquatic organisms, is a major impediment to its wider application. Here, we assess potential adverse effects of AEO-7 on zebrafish embryos employing a variety of assays, including (i) a mortality/survival assay which allowed the median lethal concentration ( $LC_{50}$ ) to be calculated; (ii) a teratogenicity assay on the basis of which the no observed effect concentration (NOEC) was determined; and (iii) specific assays of cardiotoxicity, neurotoxicity (based on locomotion), hematopoietic toxicity (the level of hemoglobin as revealed by o-dianisidine staining) and hepatotoxicity (liver steatosis and yolk retention examined by staining with Oil Red O).

## Results

AEO-7 caused mortality with a calculated  $LC_{50}$  of 15.35  $\mu\text{g/L}$ , which, according to the U.S. Fish and Wildlife Service (USFWS) Acute Toxicity Rating scale, should be considered “super toxic”. Although at its NOEC (0.8  $\mu\text{g/L}$ ), there were no signs of significant teratogenicity, cardiotoxicity, or hemopoiesis toxicity, 3.2  $\mu\text{g/L}$  AEO-7 exerted dramatic detrimental effects on organ development.

## Conclusion

On the basis of these findings, we recommend that the industrial usage and environmental impact of AEO-7 be re-evaluated and strictly monitored by environmental and public health organizations.

## 1. Introduction

Surfactants have attracted wide attention due to their unique properties and their abilities to be tailor-made to suit various applications in comparison with conventional solvents [1-4]. Surfactants are widely used in the industry of detergents and other household products such as hair conditioners and personal care products. In addition, because of their unique physico-chemical properties, surfactants have attracted considerable commercial interest [2, 4], especially in connection with many fundamental industrial problems such as pollution of the aqueous environment with oil and, very importantly, inhibition of corrosion [4], as it remains challenging to develop “green”, environmentally friendly, organic corrosion inhibitors that are still cost-effective [5]. As a result, the annual production of surfactants is continuously growing reaching around 13 million metric tons in 2008 [6, 7]. Following the use of surfactants in industrial and household usage, residual components are dispensed into sewage systems or directly into surface waters, most of which end up dispersed into various environmental pools such as water, soil, or sediment therefore disrupting water cycles in the ecosystem. Additionally, surfactants can accumulate in living organisms leading to unknown potential toxic effects [6, 8].

The toxicity of surfactants has been characterized to some extent [9-12] and these compounds are often considered the best “green” inhibitors of corrosion [4, 13-19]. However, their potential toxic effects, towards aquatic organisms, remain a concern [6-8]. For instance, when zebrafish embryos were exposed to three commonly employed surfactants (sodium dodecyl sulfate, dodecyl dimethyl benzyl ammonium chloride, and fatty alcohol polyoxyethylene ether), two of these proved to be highly toxic at concentrations as low as 1 µg/mL [20]. Moreover, both anionic and, in particular, non-ionic surfactants are highly toxic to various aquatic fauna [21]. Therefore, the environmental friendliness of many of these widely used compounds needs to be reconsidered in order to be able to select the least toxic and most biodegradable surfactants for commercial use.

Fatty alcohol polyoxyethylene ethers (AEOs) are the largest and most rapidly expanding family of non-ionic surfactants commonly utilized in household detergents and for many eco-friendly applications. In addition, our collaborators have recently demonstrated that fatty alcohol polyoxyethylene ether-7 (AEO-7) acts as a highly efficient “potentially green” inhibitor of corrosion, even under extremely harsh conditions [16]. However, the presence of these compounds, even at concentrations as low as 1 mg/L, can result in the formation of a persistent foam on wastewater, which attenuates the exchange of gas and causes the water to stink [22]. Furthermore, high concentrations of AEOs in wastewater not only kill microorganisms and inhibit the degradation of other toxic substances, but also reduce the level of dissolved oxygen [22]. Although, comprehensive characterization of the potential toxicity of this surfactant towards a model aquatic animal is essential prior to its extensive utilization in various applications, no such study has yet been reported. Accordingly, the present study was designed to evaluate the potential toxic effects of AEO-7 using zebrafish embryos as a model for the marine fauna. Zebrafish (*Danio rerio*) is an ideal model system to study environmental toxicity and is accepted by the National environmental toxicity and by the National Institutes of Health (NIH, USA) as an alternative model for exploring human diseases [2, 23-25]. Since no toxicity studies have been performed on AEO-7, we investigated a wide range of concentrations (0.4, 0.8, 3.2, 6.4, 12.8, 25, µg/L) to assess the no observed effect concentration (NOEC) and the median lethal concentration (LC50). In this regard, the selected concentrations were consistent with previously published work using surfactants (SAPDMA paper and Wang et al., 2015) and within the toxicity rating scale provided by the U.S. Fish and Wildlife Service (USFWS) (El-Harbawi, 2014).

## 2. Materials And Methods

### 2.1. Chemicals

Two compounds were employed as positive controls for general toxicity: diethylaminobenzaldehyde (DEAB, Sigma-Aldrich, Steinheim, Germany), an inhibitor of aldehyde dehydrogenases that causes significant pathologies and mortality in zebrafish embryos [25-27] and nanoparticles of zinc oxide (ZnO, diameter < 100 nm, catalog #721077-100G, Sigma-Aldrich, Steinheim, Germany) known to cause mortality and morphological deformities in zebrafish embryos and used previously as a positive control in toxicological studies [28-30]. In addition, in connection with the assays of neurotoxicity, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinehydrochloride (MPTP, Sigma-Aldrich, Steinheim, Germany), which causes

permanent symptoms of Parkinson's disease in these same embryos, was used as the positive control [31].

To facilitate visualization under the microscope, the zebrafish embryos were incubated in E3 egg water (Sigma-Aldrich, Steinheim, Germany) containing N-phenylthiourea (PTU, Sigma-Aldrich, Steinheim, Germany) which inhibits pigmentation with melanin. The AEO-7 surfactant was obtained from Shanghai Dejun Technology Co., Ltd, China. The chemical structure of the surfactant is shown in Supplementary Fig. S1. A stock solution of AEO-7 (2 mg/L) was prepared by adding 5  $\mu$ L of the viscous liquid to 4.995 mL PTU medium and overtaking until fully dissolved. Stock solutions of DEAB, ZnO, PTU, E3 egg water, and phosphate buffer saline (PBS) were prepared as described previously [2, 25].

## **2.2. Zebrafish embryo culture**

The three types of zebrafish (*Danio rerio*) embryos used were the wild-type AB strain, the naturally transparent Casper strain and Tg[fabp10: RFP] transgenic AB zebrafish, which express red fluorescent protein (RFP) in all liver cells [32]. The wild-type animals were maintained in an aquatic system at the Biomedical Research Center (BRC) of Qatar University (QU) and embryos generated by natural pairwise mating, as described in the Zebrafish Book [33]. Dead and unfertilized eggs were removed 4 hours post-fertilization (hpf). Prior to 7 days post-fertilization (dpf), the embryos receive their nourishment from the yolk sac and, thus, no feeding is required [34].

Since different organs of the zebrafish become fully functional at different stages [26], acute toxicity, cardiotoxicity, and hematopoiesis were assessed once every 24 h for three days after initiating exposure at 24 hpf, and central nervous system (CNS) toxicity and hepatotoxicity following treatment from 96-120 hpf, as illustrated in Fig 1 [24, 25, 27, 35-39]. During early embryogenesis the protective chorion envelope around the embryos might interfere with uptake of the compounds being tested. Therefore, at 24 hpf the zebrafish embryos were dechorionated using a solution of pronase (0.5 mg/mL, Sigma-Aldrich, Steinheim, Germany) [2, 26].

All procedures were conducted in compliance with the guidelines provided by Qatar University and the Department of Research at the Ministry of Public Health, Qatar.

## **2.3. Acute toxicity**

The acute toxicity of AEO-7 was assessed with an assay adapted from the guidelines for testing chemical toxicity formulated by the Organization for Economic Co-operation and Development (OECD) (N° 203, 210 and 236) [3, 40, 41]. Since we could find no previous reports on the toxicity of AEO-7 in the scientific literature, a wide range of concentrations was evaluated.

At 24 hpf, 20 healthy, dechorionated wild-type embryos were placed into each well of 12-well plates together with 3 mL of either E3 medium (NC) alone or this same medium with one of six different concentrations (0.4, 0.8, 3.2, 6.4, 12.8, and 25  $\mu$ g/L) of AEO-7 or 1  $\mu$ M DEAB. Thereafter, cumulative survival and morphological deformities were assessed under a standard stereomicroscope at 96 hpf.

Embryos where the fertilized egg had coagulated, no somites formed, no heartbeat was detectable and/or the tail-bud had not detached from the yolk sac were considered dead. Defects or variations in body length or the size of the eyes, heart or yolk were considered teratogenic effects.

The median lethal dose (LC<sub>50</sub>) with a 95% confidence interval was calculated by fitting a sigmoidal curve to the data on mortality using the GraphPad Prism 8 software (version 8.2.1, San Diego, CA, USA), as described elsewhere [25, 39, 42]. Variations in body length and the size of the eyes and yolk sac size were captured at 21-fold magnification with the HClmage software and then assessed with the ImageJ software (version 1.52a, NIH, Washington DC, USA) in combination with Java 1.8.0\_172 [25, 43].

Both the data on mortality and teratogenicity were utilized to calculate the no observed effect concentration (NOEC) of AEO-7, i.e., the highest concentration that does not cause a significant ( $p < 0.05$ ) effect relative to the negative control (PTU-E3 medium). If the cumulative mortality in the negative control was >20%, the experiment was repeated. As indicated above, n=20 in all cases.

#### **2.4. Hatching rate**

At 4-5 hpf, exposure of the embryos in the same manner as described above was initiated and hatching monitored once every 24 h for 4 days thereafter. The hatching rate was calculated as follows:

$$\text{Hatching rate} = \frac{\text{number of hatched embryos}}{\text{total number of living embryos}} \times 100$$

#### **2.5. Cardiotoxicity**

For assaying cardiotoxicity, the average peak blood flow, as well as pulse (based on the flow of red blood cells (RBC)) were monitored in the two major vessels in the trunk of the embryos, the dorsal aorta (DA) and posterior cardinal vein (PCV), as shown in Fig 4A. RBC tracking was accomplished by algorithms in the video analysis program MicroZebraLab blood flow (version 3.4.4, Viewpoint, Lyon, France). As described above, treatment with 20 µg/L ZnO were used as the positive control for cardiotoxicity [2, 28]. At 96 hpf, 10 embryos exposed to each treatment (section 2.3) were selected at random, anesthetized by immersion in 0.7 µM tricaine methane sulfonate (A4050, Sigma-Aldrich, St. Louis, MO, USA) in E3 medium, and imaged as described previously [2, 36, 39].

#### **2.6. Staining for hemoglobin**

To evaluate the effect of AEO-7 on hemoglobin synthesis, Casper embryos were stained with o-dianisidine stain (Catalog #D9143-5G, Sigma, USA) in accordance with a protocol described previously [44]. This compound oxidizes hemoglobin, producing a dark red stain in cells that contain this protein. At 24 hpf, healthy embryos were transferred to a 12-well plate and incubated for 96 h at 28 °C with PTU (negative control), 1 µM DEAB (positive control), or 0.8 or 3.2 µg/L AEO-7.

In addition, hemoglobin in embryos treated at 96 hpf as described above in section 2.3 was determined with o-dianisidine (Sigma-Aldrich, Steinheim, Germany) in accordance with protocols described previously by Paffett-Lugassy and Zon [2, 37, 45]. In brief, the embryos were stained with 0-dianisidine in the dark for 30 min as described elsewhere [2], positioned horizontally on microscope slides and embedded in 3.0% (w/v) methylcellulose for bright field microscopic imaging (Stemi 508 Zeiss) at 50X in combination with a Zeiss AxioCam ERc 5s camera. The average surface area of erythrocytes stained dark red in 10 embryos in each group was determined using the ImageJ software for comparison to the negative control.

### **2.7. Locomotion (*neuromuscular toxicity*)**

To assay locomotion, embryos were collected in a Petri dish containing E3 medium, abnormal and unfertilized embryos discarded, and healthy embryos incubated at 28.5 °C. At 96 hpf, 15 embryos were transferred to each well of a 12-well plate and incubated for 24 h at 28 °C with E3 medium (negative control), 100 µM MPTP (positive control), or (iii) 0.8 or 3.2 µg/L AEO-7. Thereafter, each embryo was placed separately in a well on a 96-well plate for evaluation of locomotion utilizing the DanioVision device (Noldus Information Technologies, Wageningen, Netherlands) as described previously [27, 46].

In brief, the 96-well plates were placed in a chamber at 28.5 °C and irradiated for 20 min with white light to allow the embryos to adapt to this environment. Then, their movement was monitored during 10-min periods, the first in darkness, followed by two in bright light, another in darkness, and two final periods in bright light. The total average distance moved during these 60 min by the treated embryos was compared to the negative and positive controls.

### **2.8. Hepatotoxicity**

Hepatotoxicity was assessed in Tg[fabp10: RFP] transgenic AB zebrafish, which express red fluorescent protein (RFP) in their hepatocytes, allowing good-quality staining of the liver. At 96 hpf, the embryos were incubated for 24 h at 28 °C with E3 medium (negative control), 1% ethanol (positive control) or 0.8 or 3.2 µg/L AEO-7, following which liver size (as an indication of necrosis and hepatomegaly) and yolk retention (as a reflection of hepatic lipid metabolism) were evaluated as described previously [26, 27]. At 120 hpf, the liver of zebrafish embryos is fully developed [47].

For determination of liver size, this fluorescent organ was examined in 10 embryos exposed to each treatment with a fluorescence stereomicroscope (Olympus MVX10) and a digital camera (Olympus DP71). The images were filtered with RFP and liver size analyzed utilizing the DanioScope software (Noldus, Wageningen, Netherlands) [35].

Yolk retention was assessed by treatment with Oil Red O (ORO) (Catalog #1320-06-5, Sigma-Aldrich, USA), a lysochrome, fat-soluble stain for neutral triglycerides and lipids, as described by Yoganantharajah and colleagues (2017) [48]. In brief, 0.035 g ORO powder was added to 7 mL 100% isopropanol and dissolving by stirring overnight with a magnetic stirrer at room temperature. To obtain the staining

solution utilized, an aliquot of this stock solution was mixed with an equal volume of 10% isopropanol in Milli-Q water.

Following treatment as described above, the PTU-E3 medium was removed from the embryos by washing with 60% isopropanol and they were then placed in 1 mL of the staining solution for 75 min. Thereafter, the embryos were washed for 30 s with 60% isopropanol and then rinsed again for 3 min in 60% isopropanol, followed by a 30-s wash in 1% PBS. Next, the ORO stain was extracted from the embryos for quantification.

For this purpose, 5 embryos were pooled in an Eppendorf tube, with 5 or 6 such tubes for each treatment. Following removal of the PBS, 250 mL 4% ethanol in isopropanol was added to each tube and the samples vortexed briefly and then incubated overnight at room temperature to ensure complete extraction of the ORO stain. Finally, 200 mL of the solution was pipetted into each well of a 96-well plate and the OD (absorbance) at 495 nm determined with a Tecan GENios Pro 200 spectrophotometer.

## **2.9. Statistical analysis**

In most cases, the values for the treated and negative control were compared statistically with one-way ANOVA followed by the Dunnett test and paired two-tailed student t-test. In the case of the hatching assay, the Chi-square test was utilized for this purpose. Statistical significance is indicated as \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; or \*\*\* =  $p < 0.001$ . All statistical analyses were performed with the GraphPad Prism 8 software (version 8.2.1).

# **3. Results And Discussion**

## **3.1. AEO-7 is extremely toxic towards zebrafish embryos**

Zebrafish embryos are most sensitive to xenobiotic from 24-96 hpf [26, 49-51]. At 96 hpf, mortality following exposure to 100  $\mu\text{M}$  DEAB (the positive control) was 100%, with severe teratogenicity, including abnormalities in the heart and yolk sac edema (Fig. 2A). The  $\text{LC}_{50}$  calculated for DEAB was 24.1  $\mu\text{M}$  (Fig. 2C).

In the case of exposure to AEO-7, no significant mortality was observed at 96 hpf with 0.4-12.8  $\mu\text{g}/\text{L}$ , whereas mortality was 100% at 25  $\mu\text{g}/\text{L}$  (Fig. 2B), with an  $\text{LC}_{50}$  of 18.3  $\mu\text{g}/\text{L}$  (Fig. 2C). Thus, on the Fish and Wildlife Service Acute Toxicity Rating Scale [52], the AEO-7 surfactant would be classified as “super toxic”.

Moreover, 6.4  $\mu\text{g}/\text{L}$  AEO-7 reduced the size of the embryo's eyes (Fig. 2D-F). At the same time, this surfactant decreased body length and the size of the yolk sac only at a concentration of 12.8  $\mu\text{g}/\text{L}$ , which caused a wide range of other embryopathies as well.

## **3.2. AEO-7 did not affect the hatching rate (HR)**

The rate of hatching, which normally occurs with zebrafish embryo from 48-96 hpf, is a critical indicator of the developmental state of these embryos [53, 54]. At a concentration of 25 µg/L, AEO-7 eliminated hatching (Fig. 3). The explanation for the pronounced difference from lower concentrations, which had no impact on this process, is unknown, but it is possible that the chorion protects against lower concentrations of this compound [55, 56]. The reduction in hatching with higher concentrations of AEO-7 may reflect structural and functional disturbances [57, 58] and/or the inability of the embryo to break out of its eggshell due to developmental delay [59].

### ***3.3. Exposure to AEO-7 induces cardiac dysfunction in zebrafish embryos***

Zebrafish have proven to be an excellent model for studying the cardiotoxic effects of xenobiotics [27, 38], with an overall success rate for predicting cardiotoxic and non-cardiotoxic drugs of 100% according to some investigators [60, 61] and a rating of excellent (>85%) for identifying cardiovascular toxins based on the criteria proposed by the European Center for the Validation of Alternative Methods [61]. The dorsal aorta (DA), the major axial artery in the trunk, is one of the vessels that forms first during the early development of all vertebrates. This aorta forms immediately below the notochord and above the posterior cardinal vein (PCV), which is the major axial vein in the zebrafish trunk (Fig. 4A) [62].

At a concentration of 3.2 µg/L, AEO-7 elevated the heart rate of zebrafish embryos (Fig. 4B), which is indicative of cardiovascular dysfunction. The NOEC for this effect was calculated to be 0.8 µg/L, which is why we used this concentration and the higher concentration of 3.2 µg/L in subsequent toxicity assays.

In disagreement with previous reports [63, 64] (Fig. 4B-C), ZnO did not decrease heart rate significantly in our animals (Fig. 4B-C), a discrepancy that remains to be explained.

### ***3.4. AEO-7 alters the locomotion of zebrafish embryos***

MPTP (PC), which has been identified as a neurotoxin in humans and zebrafish [65], attenuated the total distance that our embryos moved (Fig. 5A-B). In addition, exposure to this compound altered their locomotive behavior. These findings are consistent with the report by Wang and colleagues [20] that AEO-7 reduces the number of periods of rest, as well as the total and waking activity of zebrafish embryos in a concentration-dependent manner. Thus, we confirm here that AEO-7 has a toxic influence on the locomotor activity of zebrafish embryos.

### ***3.5. At its NOEC, AEO-7 does not cause hematopoietic toxicity in zebrafish embryos***

As shown in Fig. 6A and B, at its NOEC (0.8 µg/L), AEO-7 did not affect the level of hemoglobin in our zebrafish embryos, whereas at a concentration of 3.2 µg/L, the number of hemoglobin-positive cells was reduced. This reduction could reflect decreased production of red blood cells in the bone marrow and/or lowered hemoglobin synthesis in erythrocytes due to blockage of heme synthesis [66, 67].

### ***3.6. At its NOEC, AEO-7 exerts no adverse effect on hepatic function in zebrafish embryos***

Zebrafish have been shown to be a good model for predicting hepatotoxicity [68-70], probably because the enzymes and pathways involved in xenobiotic metabolism (e.g., the aryl hydrocarbon receptor and isozymes of cytochrome P-450 and aldehyde dehydrogenase) are all evolutionarily conserved and all functional from the early stages of the development of zebrafish embryos, including our experimental window [63, 71, 72].

By 120 hpf, zebrafish embryos have normally consumed their entire yolk, which is 70% lipid, and begin to seek exogenous sources of food [73]. Since the yolk is metabolized primarily by the liver [74], retention of the yolk is an indirect indication of impaired liver function [75-77].

Hence, we assessed hepatotoxicity in two different ways, e.g., on the basis of liver size and yolk retention. As expected, 1% ethanol decreased the size of the liver, indicating necrosis [27, 36, 78]. At its NOEC (0.8 µg/L), AEO-7 had no influence on liver size, while hepatomegaly was observed at 3.2 µg/L (Fig.7 A-B). Secondly, as shown in Fig. 7, exposure of embryos to 1% ethanol (positive control) elevated their content of lipid, in agreement with previous studies [27, 36, 78]. At its NOEC, AEO-7 resulted in a minor, but statistically insignificant increase in lipid retention, whereas the higher concentration of this compound enhanced this retention significantly (Fig. 7C-D).

## 4. Conclusions

The results of all of the toxicity assays– including calculation of the LC<sub>50</sub> and NOEC values, as well as evaluation of potential toxic effects on the heart, hematopoiesis, locomotion and the liver- indicate strongly that at least at the concentrations tested the non-ionic surfactant AEO-7 exerts potent concentration-dependent toxic effects on organ development in zebrafish embryos. To our knowledge, ours is the first investigation of the toxicity of AEO-7 and certainly the first complete assessment of the toxicity of this surfactant towards a model freshwater organism.

Indeed, the mortality of 96-hpf embryos following exposure to AEO-7 rose with increasing concentration, becoming 100% at 25 µg/L. The LC<sub>50</sub> and NOEC values were 15.35 and 0.8 µg/L, respectively. On the basis of these values alone, we conclude that according to the USFWS Acute Toxicity Rating Scale (Table S1) [79], AEO-7 should be classified as “super toxic”. Therefore, industrial use of AEO-7 and its presence in the aquatic environment should be re-evaluated and monitored carefully by different environmental and public health organizations.

## 5. Declarations

### **Ethics approval and consent to participate**

Not applicable

### **Consent for publication**

Not applicable

## Availability of data and materials

Not applicable

## Competing interests

None of the authors has any competing interests to declare.

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## Author Contributions

Maha Al-Asmakh: writing, data analysis, visualization, provision of resources, project administration, acquisition of funding. Gianfranco Pintus: writing - review and editing, acquisition of funding. Amin F. Majdalawieh: visualization, validation, writing. Nadin Younes: development of methodology, use of software, formal analysis, performance of experiments, writing, visualization. Sahar I. Da'as: use of software, formal analysis, validation. Aboubakr M. Abdullah: supervision, performance of experiments. A. Bahgat Radwan: development of methodology, performance of experiments. Mostafa H. Sliem: development of methodology, performance of experiments. Houria Ech-Cherif: development of methodology, performance of experiments. Gheyath K. Nasrallah: conceptualization, validation, formal analysis, provision of resources, storage of data, visualization, supervision, project administration, acquisition of funding.

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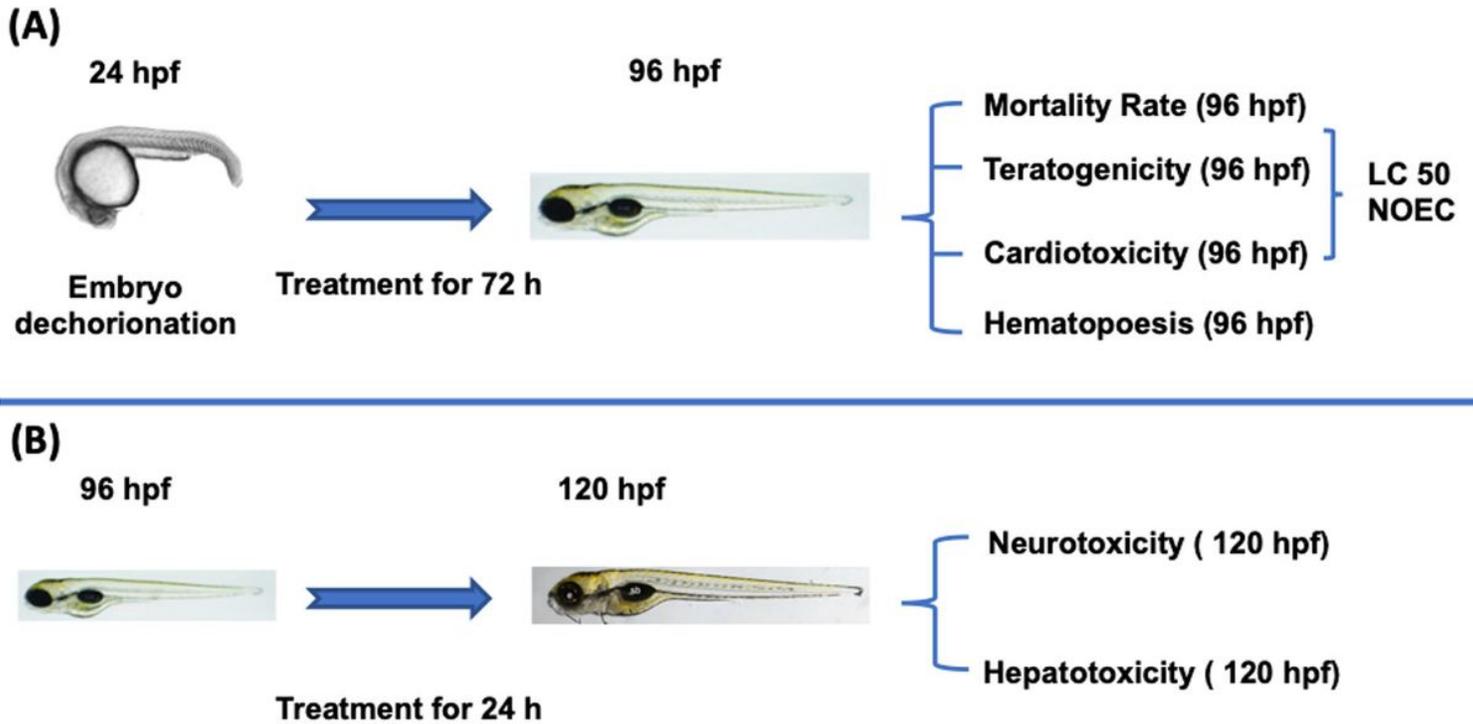
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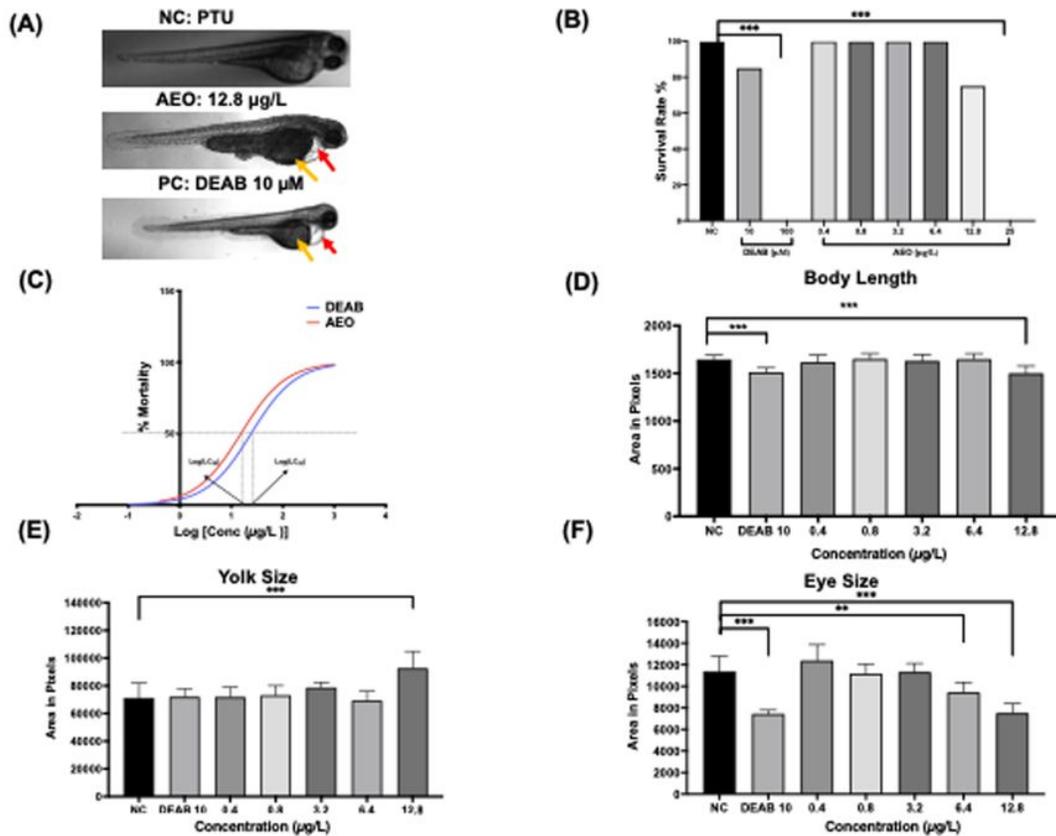
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## Figures



**Figure 1**

A summary of the experimental procedures utilized to assess different types of toxicity. (A) At 24 hpf, the embryos were dechorionated and exposed to different concentrations of AEO-7, Positive control (PC), and Negative control (NC) until 96 hpf, when acute toxicity was assessed. (B) Starting at 96 hpf, the embryos were exposed to different concentrations of AEO-7, PC, and NC for 24 hr and organ toxicity then evaluated.



**Figure 2**

Teratogenic effects of the AEO-7 surfactant on zebrafish embryos. (A) Representative photographs of 96-hpf embryos exposed to DEAB nanoparticles (positive control), PTU medium (negative control), or AEO-7. Note the edema in the yolk sac (yellow arrow) and heart (red arrow) after exposure to 10  $\mu\text{M}$  DEAB. These images were captured with a ZeissStemi2000-C stereomicroscope (21 $\times$ ). (B) The survival rate of embryos exposed to different concentrations of AEO-7 and the positive and negative controls. (C) Dose-response curves used to calculate the LC50 for DEAB and AEO-7. (D) Average body length, (E) size of the yolk, and (F) eye size following treatment, as captured using the HClmage software and analyzed with version 1.52a of the ImageJ software version. One-way analysis of variance (ANOVA) followed by the Dunnett test was used to compare the groups, \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ,  $n = 20$ .

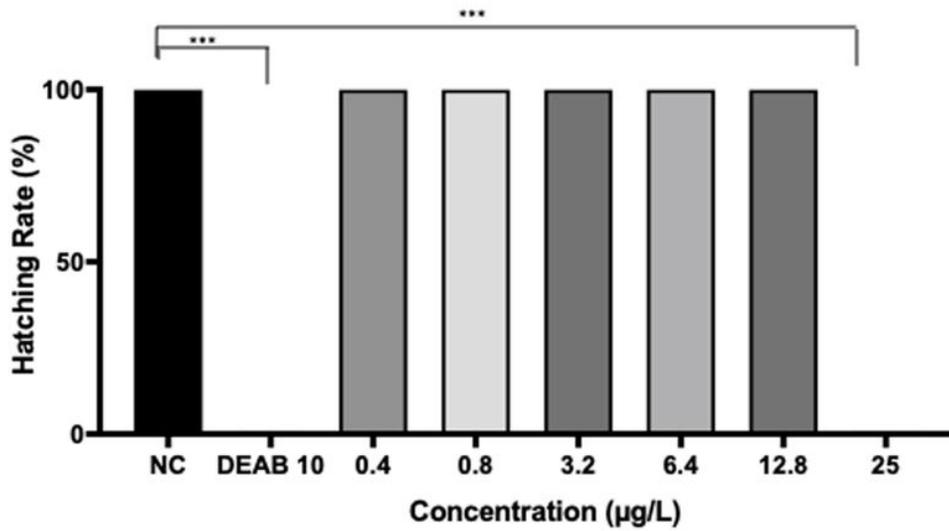
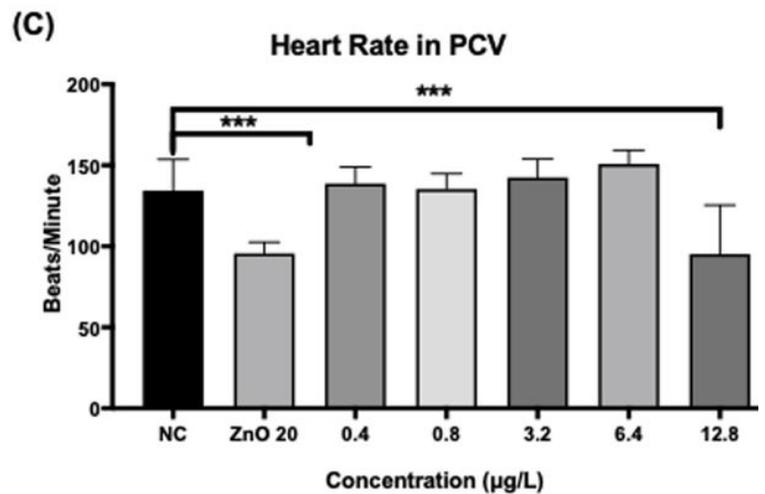
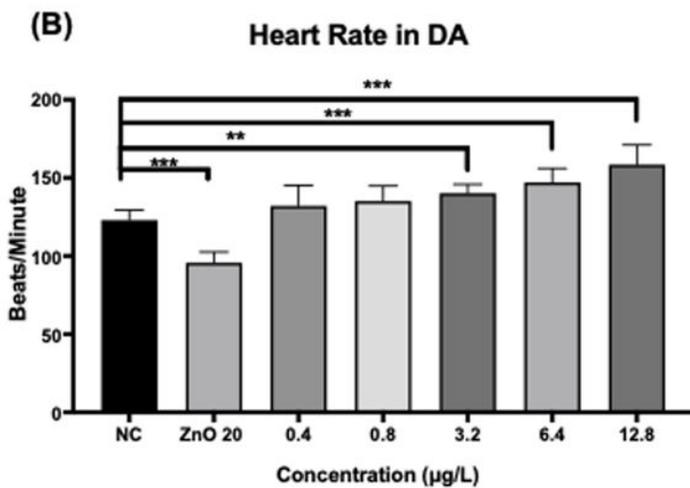
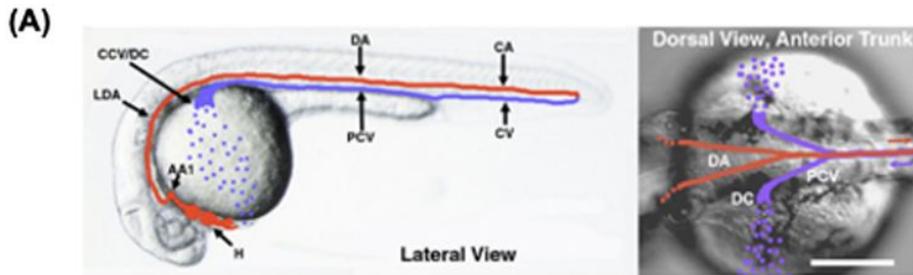


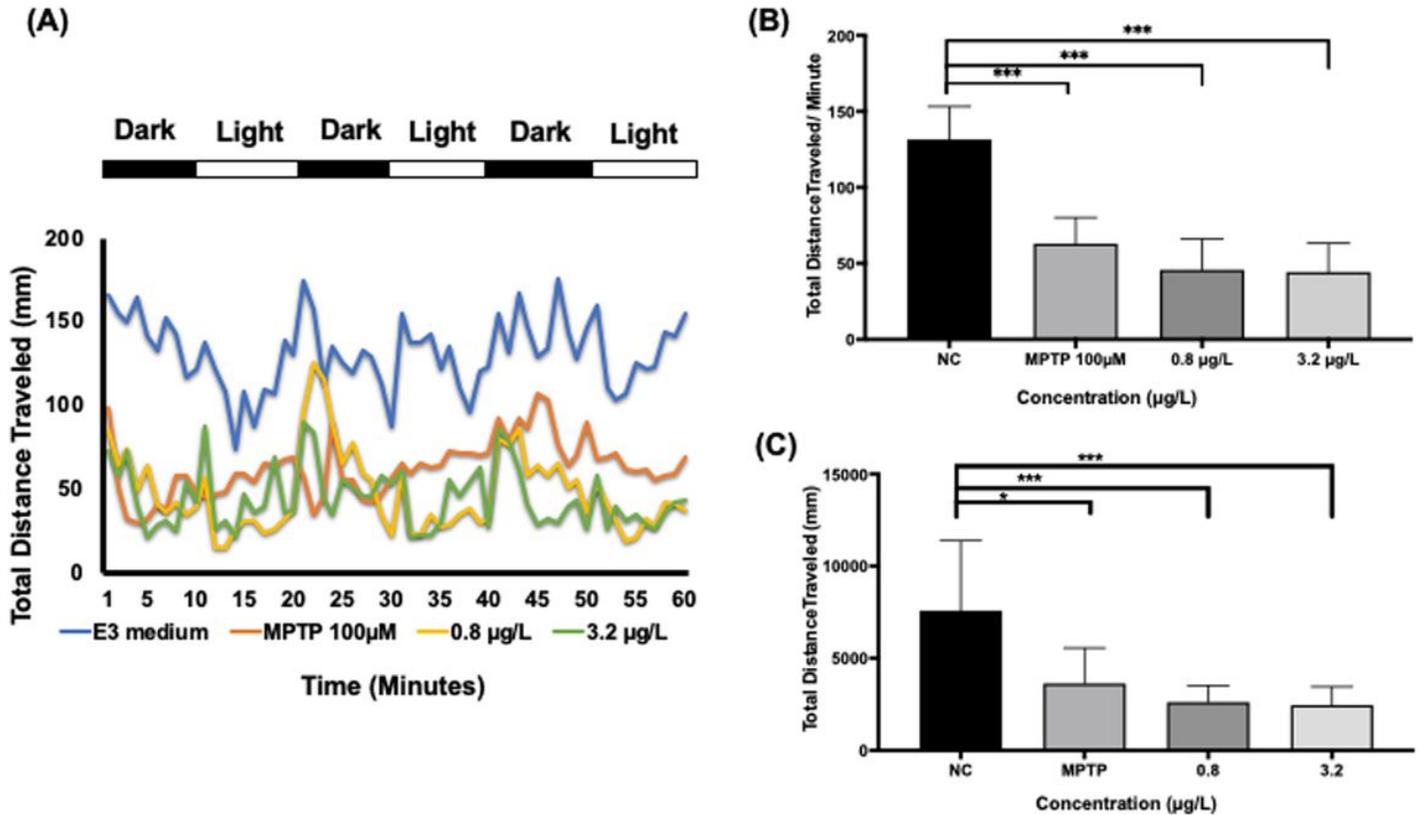
Figure 3

The effect of AOE-7 on the hatching of zebrafish embryos. The Chi-square test was used for comparison to the negative and positive controls, \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ ,  $n=20$ .



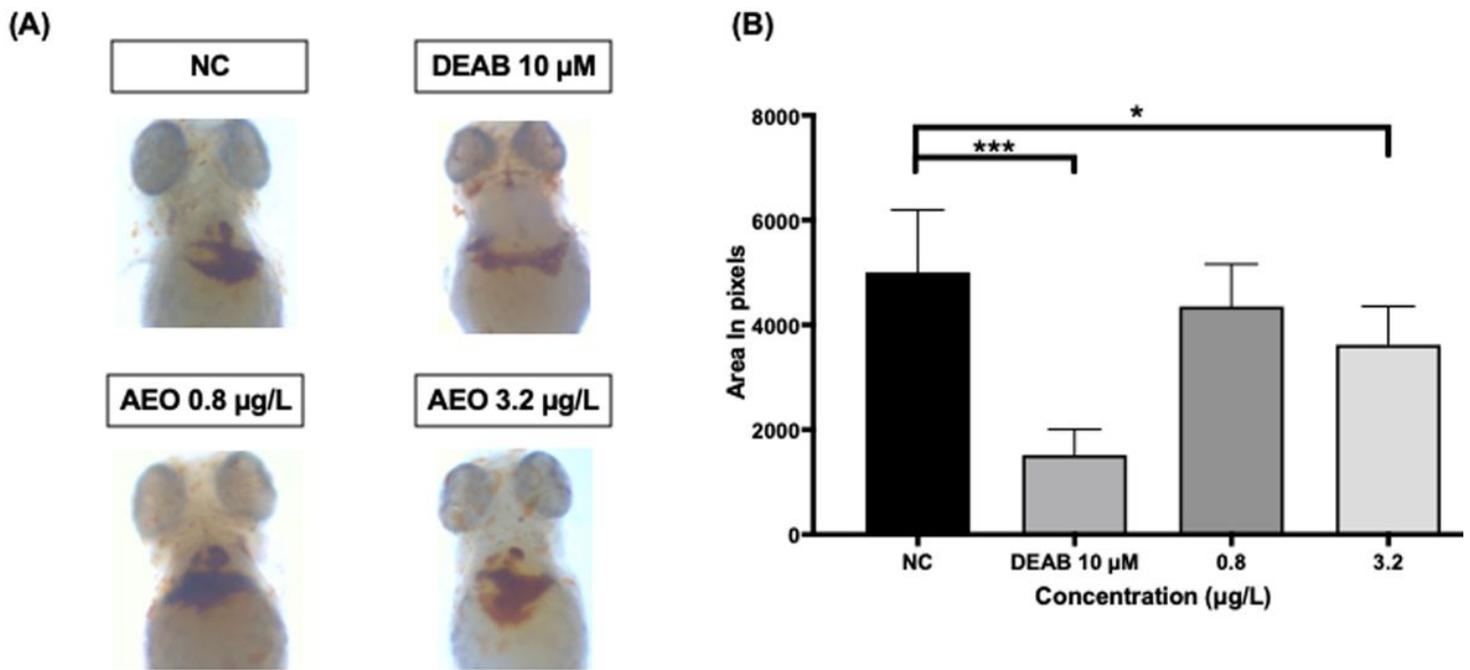
**Figure 4**

Exposure to AEO-7 increases the heart rate of zebrafish embryos. (A) Location of posterior cardinal vein (PCV) and dorsal artery (DA). (B) and (C) The heart rate monitored in these two vessels following the concentration indicated. One-way ANOVA was used to compare the groups. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ ,  $n = 10$ .



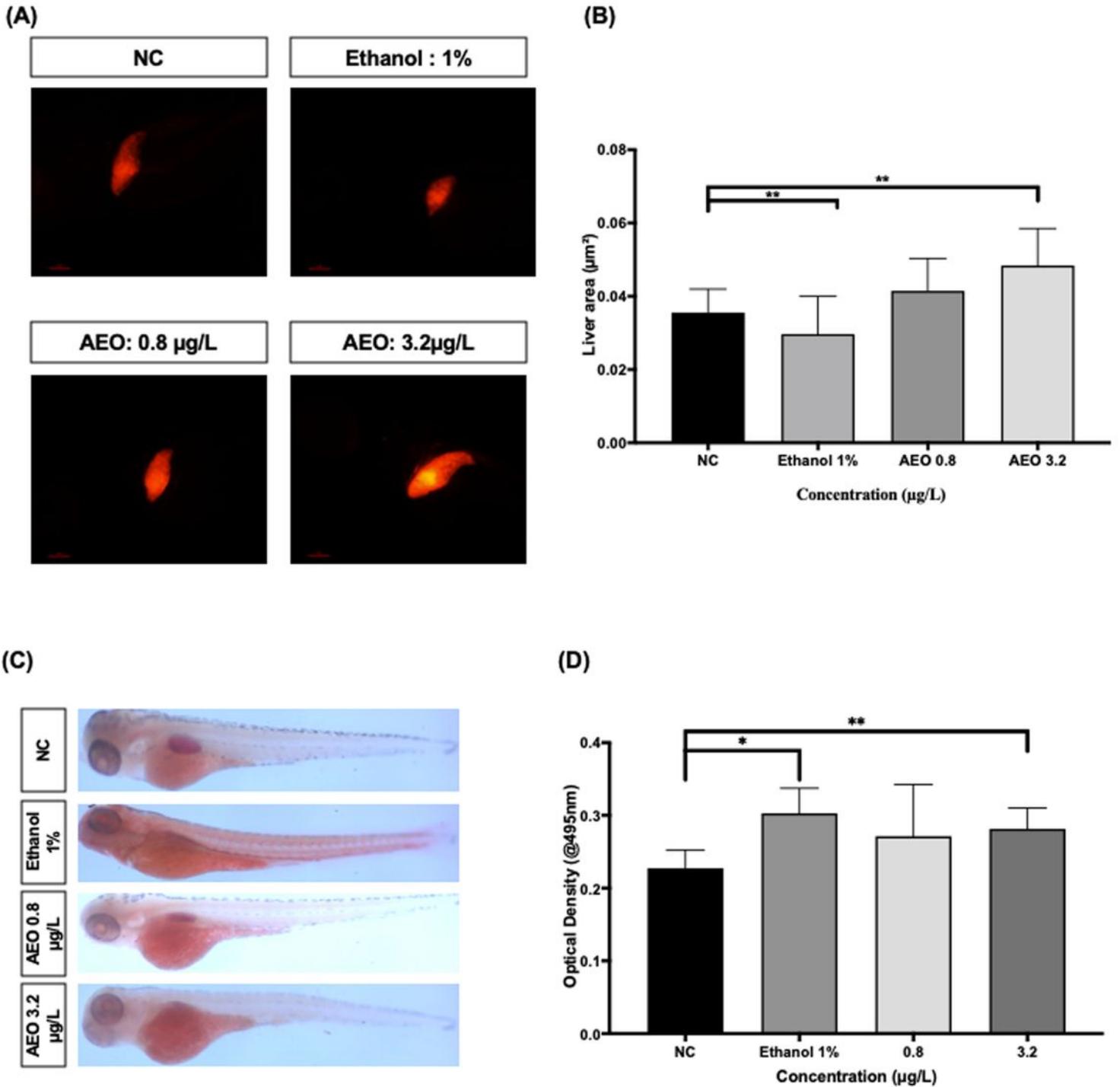
**Figure 5**

Assessment of the locomotion of zebrafish embryos following a 24-h exposure to different concentrations of AEO-7. (A) The average total distance moved (determined using the ViewPoint Microlab system) during every 5-min period by the 120 hpf-old embryos following exposure to E3 medium (negative control), MPTP (positive control), or 0.8 or 3.2 µg/L AEO-7. (B) The average distance (mm) moved per minutes. (C) The total distance moved in mm. One-way ANOVA was used to compare the differences between the treated groups and negative control. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ ,  $n = 10$ .



**Figure 6**

The influence of AEO-7 on the level of hemoglobin in zebrafish embryos. (A) Representative images depicting o-dianisidine staining of the yolk sac of 72-hpf zebrafish embryos exposed to the negative control, DEAB, or 0.8 or 3.2 μg/L AEO-7. (B) The number of erythrocytes stained by o-dianisidine in the embryos described in (A). One-way ANOVA followed by the Dunnett test was employed for comparison of the different groups. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ ,  $n = 10$ .



**Figure 7**

The effect of AEO-7 on liver size and yolk retention in zebrafish embryos (A) Representative images depicting the size of the liver following the different treatments (note the hepatomegaly or necrosis). (B) The RFP area of the liver (µm<sup>2</sup>) following the different treatments. (C) Representative images of yolk retention following the different treatments. (D) The levels of neutral triglycerides and total lipids in the

entire embryos following the different treatments. Paired two-tailed student t-test was employed to compare the treated groups with the negative control. \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001, n = 10.

## Supplementary Files

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