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The effects of algal-derived organic matters (AOMs) and chlorinated AOMs on the survival and behavior of zebrafish

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Abstract. Algal organic matters (AOMs) are challenging to remove using traditional water treatment methods. Additionally, they are recognized as disinfection by product (DBP) precursors during the chlorination process. These compounds have the potential to seriously harm aquatic creatures. Despite the fact that AOMs and DBPs formed from algae can harm aquatic species by impairing their cognitive function and causing behavioral problems, only a few studies on the effects of AOMs and associated DBPs have been conducted. To assess the impact of extracellular organic materials (EOMs) produced by three different hazardous algal species and the chlorinated EOMs on zebrafish, this study used fish acute embryo toxicity (FET) and cognitive function tests. With rising EOM concentrations, the embryo's survival rate and mental capacity both declined. Of the three algal species, the embryo exposed to Microcystis aeruginosa EOM exhibited the lowest survival rate. On the other hand, the embryo exposed to EOMs following chlorination demonstrated a drop in CT values in both the survival rate and cognitive ability. These findings imply that EOMs and EOMs treated with chlorine may have detrimental effects on aquatic life. Therefore, an effective EOM management is needed in aquatic environment.
Keywords: algal organic matter(AOM); aquatic ecosystem; FET test; toxicity

1. Introduction

Algal bloom commonly occurs due to climate change and excessive inputs of nutrients such as phosphorus and nitrogen, which has become a serious problem in drinking water treatment plants (Huang *et al*. 2015). Algae and algal organic matters (AOMs) can cause many problems in the water treatment process. AOMs can serve as precursors of disinfection by-products (DBPs) during chlorination process. Also, the sedimentation rate of algae cells are depending upon cell size, shape, and growth phase. Among them, the shape of algae is one of the most important factors which can cause a membrane fouling in water treatment process (Choi *et al*. 2006). In addition, mucilage produced by diatoms can cause a serious membrane fouling in drinking water treatment facility (Liu *et al*. 2017). Algae cells are known to produce various AOMs composed of intracellular organic matters (IOMs) and extracellular organic matters (EOMs). EOMs were reported to have more abundant low molecular (LMW) weight proteins than IOM (Rao *et al*. 2023). In case of *Microcystic aeruginosa* hydrophilic and hydrophobic properties of EOM were different from those of IOM (Li *et al*. 2012). The main components of AOMs are polysaccharides, nucleic acids, proteins, and glycolic acids. Among the main components it was reported that conventional flocculation process could not remove polysaccharides easily (Paralkar and Edzwald 1996, Golsan *et al*. 2017).

Chlorination of water containing AOM can cause formation of DBPs such as trihalomethanes (THMs) and haloacetic acids (HAAs) (Hong 2018). DBPs have been reported to have adverse effects on human health, including carcinogenesis and mutagenesis (Wang *et al*. 2007, Fawell *et al*. 2003). For the chlorination of AOMs produced by *Microcystis aeruginosa*, production of carbonaceous DBPs (C-DBPs) increased with increasing the reaction time, chlorine dosage, and temperature (Fang *et al*. 2003). Chen *et al* (2017) showed that the properties of AOMs extracted from *Microcystis aeruginosa* varied with the growth phases, and more formation of DBPs occurred during stationary growth phase than other growth phases such as the exponential and decline phases. On the other hand, some studies related to DBPs have been reported to pose ecological risks to the aquatic environments, such as cytotoxicity and genotoxicity of mammalian cells (Cui *et al*. 2021, Richardson *et al*. 2008). In addition, DBPs released during disinfection have a negative impact on zebrafish such as behavioral pattern disturbances and cognitive impairment (Yoon *et al*. 2020). *Microcystin-LR* are known to have allelopathic ability that inhibits oxygen production or growth through photosynthesis of aquatic plants (Pflugmacher 2002). Also, AOM can produce methyl-mercury (MeHg) in the lake, which poses risks to aquatic

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Table 1 E3 medium for zebrafish embryo

Chemical	Concentration
NaCl	5 mM
KCl	0.17 mM
$CaCl_2$	0.33 mM
$MgSO_4$	0.33 mM
Methylene Blue	10^{-5}%

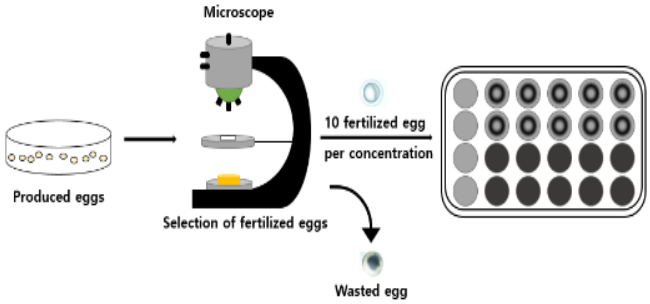

Fig. 1 The procedure of checking for egg fertilization and embryo development of zebrafish

species (Lei *et al*. 2021). Although it is clear that DBPs and AOM have a potential adverse effect on aquatic eco-systems, many studies have focused on the characteristics of AOM and their DBPs produced during chlorination.

For toxicity test of chemicals to aquatic organisms, various types of fish such as bluegill sunfish, fathead minnow and zebrafish were used (Embry *et al*. 2010). Among many toxicity studies, zebrafish has widely been used for toxicity testing of various types of hazardous chemicals (Berry *et al*. 2007, Pereira *et al*. 2019). Zebrafish embryos were reported to be vulnerable to exposure to contaminants (Kurobe *et al*. 2018). In addition, it is easy to observe deformities in zebrafish embryos because of the transparency (Ali *et al*. 2011). Based on these advantages, zebrafish can be a tractable model for the acute toxicity test.

The aim of this study is to investigate the harmful effects of algal-derived organic matters (AOMs) and chlorinated AOMs produced by three different algal species on zebrafish. In order to achieve the aim, fish acute embryo toxicity (FET) and color maze test were conducted to measure survival rate and cognitive ability of zebrafish.

2. Materials and method

2.1 Zebrafish breeding and production

Zebrafish breeding was carried out by *maintaining* the temperature range of 27-30℃ and relative *humidity* range of 30-70%, respectively. The breeding system was maintained in a *cycle* of 14 h of *light* and 10 h of *darkness* per day. The water temperature of the breeding system was kept at 28-29° and pH of 6.5-8.5, respectively. The amount of ammonia (NH_3) was kept below 0.02 mg/L, and the amount of dissolved oxygen (DO) was maintained at 8-11 mg/L. On the other hand, zebrafish embryos were obtained through once-a-week mating of zebrafish. The embryos were collected immediately after mating through a filter and stored in E3 egg water (Table 1). *Embryo* culture for *day 5* was *performed* in an incubator at 28.5°C with the 14:10 light-dark cycle. For zebrafish mating both male and female fishes of comparable length and age were selected. Eggs were collected by filtering them through a clean sieve and rinsing them by E3 egg water into a crystalline dish (Bai *et al*. 2010).

2.2 Fish acute embryo toxicity (FET) test

FET test, OECD test guideline TG 236 was performed within 3 hours after fertilization of the egg (Schulte *et al*. 1994). The light microscope (Olympus BX53M, Japan) was used for the fertilization check (Fig. 1). Fertilized eggs can be clearly distinguished from unfertilized eggs by their transparency. Eggs with obvious abnormalities (asymmetry, vesicle formation) or damaged membranes were not used. In order to investigate toxicity of algal-derived organic matters on zebrafish embryos, extracellular organic matters (EOMs) produced by three different algae species (*Oscillatoria sp*., *Anabaena sp*., and *Microcystis aeruginosa*) were collected.

In order to collect EOM solution, around 50 mL of algae cultivation solution was centrifuged at 5000 rpm for 10 minutes using a centrifuge (FLETA 5, Korea). After centrifugation, the supernatant were filtered using a glass fiber membrane of 0.45 μm (Whatman GF/C Glass Microfiber Filter, UK). Each algal EOM solution with different three concentrations (0.3 mg/L, 3 mg/L, and 30 mg/L) was prepared. In addition, chlorine-treated EOM solution was prepared to examine impacts of organic compounds in EOM solution after chlorine disinfection on zebrafish. The chlorine reaction experiments with EOM solutions were performed under various CT (concentration of free chlorine × contact time) values (52, 59, and 68 mg·min/L). On the other hand, E3 egg water and 3,4-dichloroaniline (3.7 mg/L) were used as negative control (>90% embryo survival) and positive control, respectively. Ten fertilized eggs were selected using a stereomicroscope and transferred to the 24-well plate. Each well contained 2 mL of freshly prepared test solution and control solution. The developmental changes in zebrafish embryos were observed using the microscope at 24, 48, 72, and 96hours past fertilization (hpf). Development of zebrafish embryos was observed over 96 hpf. In addition, developmental alterations such as coagulated eggs, somite formation, lack of tail detachment, and absence of heart-beat are used as core endpoints of acute lethality (OECD, 2013) (Fig. 2).

The cumulative hatching rate and mortality rate of zebrafish embryos in each experimental group at 24, 48, 72 and 96 hpf were also counted.

2.3 Zebrafish cognitive function test

Zebrafish have been reported to have a preference for a certain wavelength (Park *et al*. 2016). Zebrafish are fully hatched within 2-3 days after spawning, and after 5 days the visual organs are complete. Generally, zebrafish prefer the short wavelength region (blue) over the long wavelength region (yellow) (Yoon *et al*. 2019, 2020). Cognitive

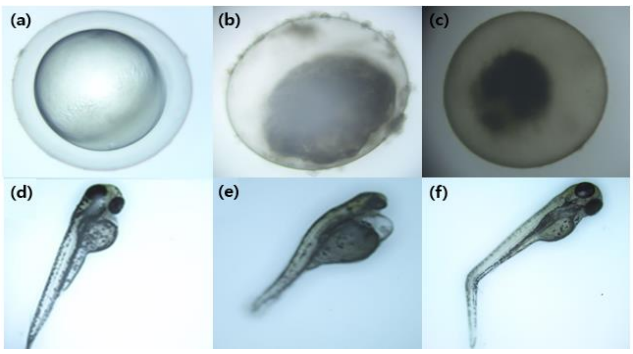

Fig. 2 Lethal and teratogenic phenomena observed in the zebrafish embryo experimental (a: normal embryo, b: roe dissolution, c: roe coagulation, d: normal larvae, e: pericardial cyst, f: spine curvature)

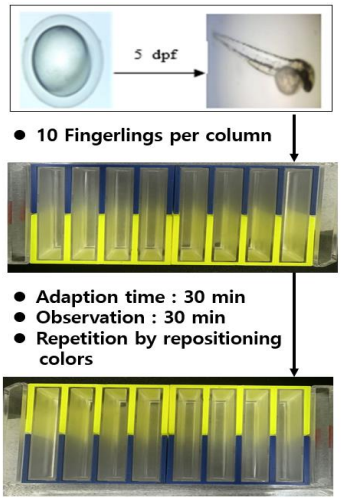

Fig. 3 An experimental procedure for zebrafish cognitive function test

intelligence experiments for 5- day-old zebrafish fry were conducted using a color maze kit consisting of a total of 8 columns (Genomic Design TM, Korea). Ten zebrafish fry were placed in each column filled with 4 mL of E3 egg water and the tested solution. The light intensity was set to 20,000-25,000 Lux using lighting equipment, and zebrafish behavior in color maze was recorded using a video recorder and then analyzed using LoliTrack 4.1 (Loligo®systems, Denmark). The cognitive intelligence of zebrafish was assessed by counting the number of fry placed in the preferred wavelength area (blue area column) using the recorded images (Fig. 3). The variation ratio in cognitive ability was calculated by the following Eq. (1).

$$Ratio = \frac{Number\ of\ exposed\ zebrafish\ in\ blue}{Number\ of\ control\ zebrafish\ in\ blue} \quad (1)$$

3. Results and discussion

3.1 Toxicity impact of EOM on zebrafish

The survival rates of negative and positive controls were examined to the accuracy and precision of each test (OECD, 2013). In this study, the survival rates of negative and positive controls were 100% and 50%, respectively (Table 2).

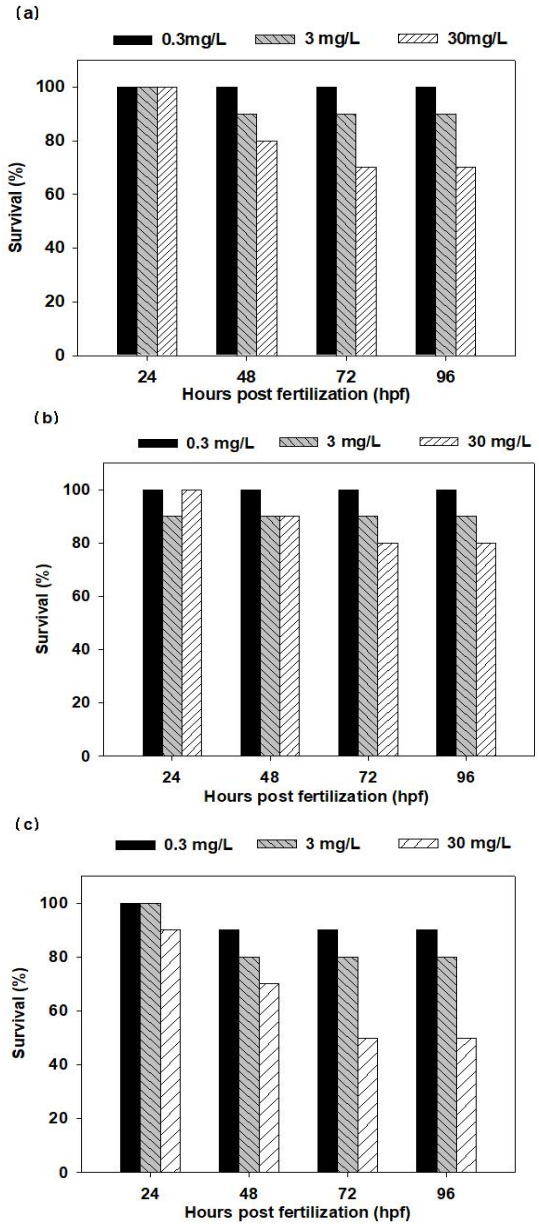

Fig. 4 Survival rate of zebrafish embryos after exposure to EOMs produced from three different algal species (a: *Oscillatoria sp*., b: *Anabaena sp*., c: *Microcystis aeruginosa*)

Table 2 Survival of zebrafish egg by control solution

Control	Solution	Survival (%)
Negative	E3 egg water	100
Positive	3,4-dichloroaniline (3.7 mg/L)	50

Fig. 4 shows the survival rate of fertilized zebrafish embryos after 96hr exposure to different algal EOM concentrations produced from three different algal species at 28.5 ℃.

For the exposure to EOM produced by *Oscillatoria sp*., all the zebrafish embryos survived after 24hpf under all

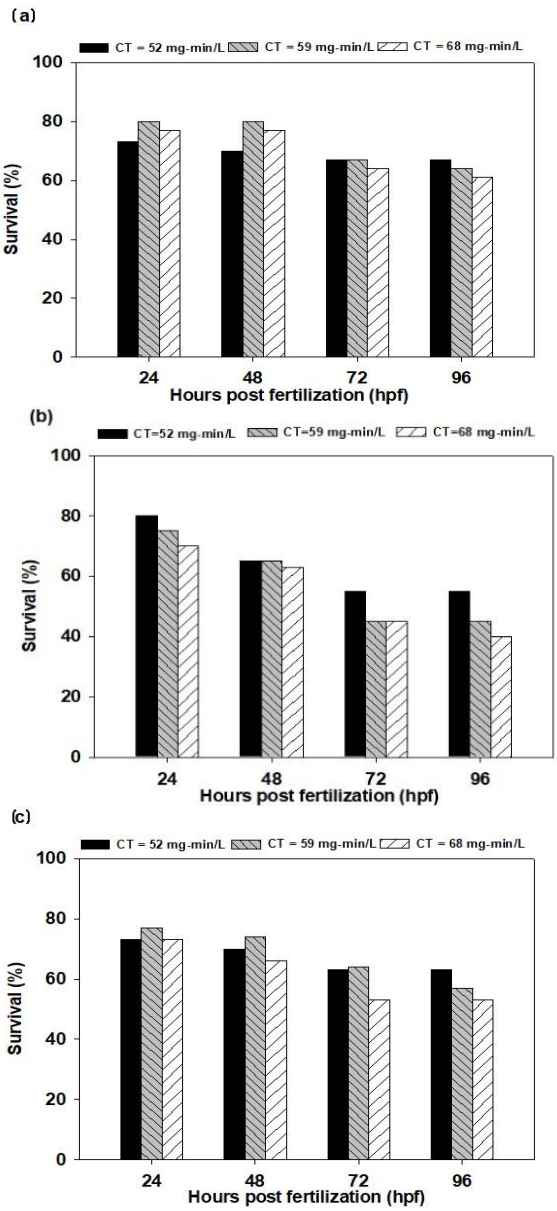

Fig. 5 Survival rate of zebrafish embryos after exposure to chlorine-treated EOMs produced from three different algal species (a: *Oscillatoria sp*., b: *Anabaena sp*., c: *Microcystis aeruginosa*)

EOM concentrations (Fig. 4a). After 96hpf exposure, the survival rates at 0.3 mg/L, 3 mg/L and 30 mg/L were 100%, 90%, and 70%, respectively. For the exposure to EOM produced by *Anabaena sp*., the survival rates at 0.3 mg/L, 3 mg/L and 30 mg/L after 24hpf exposure were 100%, 90%, and 100%, respectively. On the other hand, the survival rates at 0.3 mg/L, 3 mg/L and 30 mg/L after 96hpf exposure were 100%, 90%, and 80%, respectively (Fig. 4b). For the exposure to EOM produced by *Microcystis aeruginosa*, the survival rates at 0.3 mg/L, 3 mg/L and 30 mg/L after 24hpf exposure were 100%, 100%, and 90%, respectively. Especially after 96 hpf exposure, the survival rates at 0.3 mg/L, 3 mg/L and 30 mg/L were 90%, 80%, and 50%, respectively. Compared to the *Oscillatoria sp*. and *Anabaena sp*., EOM produced by *Microcystis aeruginosa*

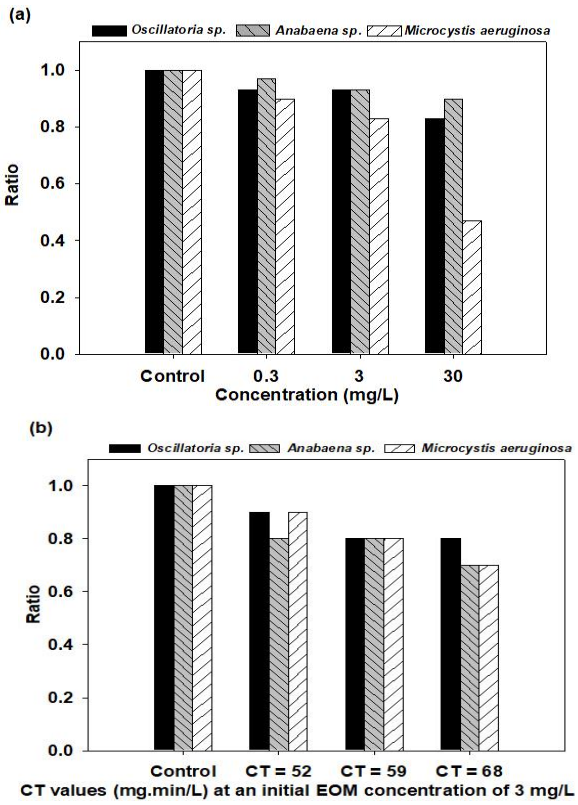

Fig. 6 Ratios of the number of zebrafish larvae in blue to yellow region during the exposure test (a: exposure test using EOMs, b: exposure test using chlorine-treated EOMs)

had a greater impact on the survival of the zebrafish embryos. This result suggests that the more toxic and harmful substances are present in EOM produced *Microcystis aeruginosa* than the other algal species.

3.2 Toxicity impact of chlorine-pretreated EOM on zebrafish

To investigate the effect of chlorine-pretreated EOM on zebrafish embryos, toxicity tests were conducted after different algal EOMs produced from three algal species were treated with chlorine. The chlorine-pretreated EOMs were prepared with chlorine treatment with an initial EOM of 3 mg/L under various CT values (52, 59, and 68 mg·min/L). The survival rates of zebrafish embryos after 96hr exposure to chlorine-pretreated EOM produced from three different algal species were shown in Fig. 5. For the exposure to chlorine-pretreated EOM produced by *Oscillatoria sp*., embryo survival rate gradually decreased with increasing CT values. This result indicates that DBPs produced by chlorination of EOM, some of which were more harmful than to embryos than EOM themselves. For both *Anabaena sp*. and *Microcystis aeruginosa*, embryo survival rate gradually decreased with increasing CT values. The minimum survival rates of embryo after exposure to chlorine-pretreated EOM for *Oscillatoria sp*., *Anabaena sp*. and *Microcystis aeruginosa* were 61%, 40% and 53%, respectively.

3.3 Zebrafish cognitive ability test

To estimate the effect of EOM produced by algae on cognitive abilities of living organisms, zebrafish cognitive function test was conducted using the color maze kit. Zebrafish was reported to prefer shorter wavelength colors such as blue and green to longer wavelength colors like yellow (Colwill *et al*. 2005, Avdesh *et al*. 2012). Fig. 6a shows the cognitive abilities of zebrafish after exposure to different EOM concentrations (0.3, 3, and 30 mg/L) compared to the control (E3 egg water). For exposure to EOM produced by *Oscillatoria sp*., the ratio of cognitive ability gradually decrease as EOM concentration increases. The ratios at 0.3 mg/L, 3 mg/L and 30 mg/L were 93%, 93% and 83% respectively. Like *Oscillatoria sp*. for exposure to EOM produced by *Anabaena sp*., the increase of ratios were in the following order: EOM concentration of 0.3mg/L > 3mg/L > 30mg/L. In case of *Microcystis aeruginosa*, the trend of cognitive ability was similar to the others. Interestingly, the ratio of cognitive ability was approximately 53% at exposure to 30 mg/L of EOM. This result indicates EOM produced *Microcystis aeruginosa* has a greater negative impact on zebrafish cognitive function compared to the other algal species.

On the other hand, Fig. 6b shows the variation rates in cognitive abilities of zebrafish after exposure to chlorine-t reated EOM prepared under various CT values (52, 59, and 68 mg·min/L) with an initial EOM of 3 mg/L. For all three algal species, the cognitive ability of zebrafish decreased as CT value increased. At CT value of 68 mg·min/L the minimum variation rates of cognitive ability for zebrafish exposed to chlorine-treated EOM produced by *Oscillatoria sp*., *Anabaena sp*. and *Microcystis aeruginosa* were 80%, 70% and 70%, respectively.

4. Conclusions

Impacts of EOMs produced by three different algae species and their DBPs on zebrafish were investigated. Based on the results of FET, the survival rates of zebrafish embryos decreased with increasing the exposure concentrations of EOMs. The order of the survival rates of zebrafish embryos exposed to the three different EOMs was *Anabaena sp*. (~80%) > *Oscillatoria sp*. (~70%) > *Microcystis aeruginosa* (~50%). The higher toxicity of EOM produced by *Microcystis aeruginosa* may be related to the released microcystins. On the other hand, the embryo survival rates decreased with increasing CT values. The result implies DBPs formation increased with CT values. The cognitive ability test for zebrafish showed their cognitive abilities decreased with the increase of EOM concentration. These results obtained in this study provide valuable information about the toxicity assessment of EOMs and DBPs formed during chlorination of EOMs on aquatic organisms.

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