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Impact of DBPs on the fate of zebrafish; Behavioral and lipid profile changes

Hyojik Yoon[1,2], Yunsu Lim[3], Sungkyu Maeng[4], Yongsuk Hong[1,2], Seokjong Byun[5], Hyun-Chul Kim[6], Byoungsoo Kim[*1,2,7] and Sungpyo Kim[**1,2]

[1]*Department of Environmental Engineering, Korea University, Sejong 30019, Republic of Korea*
[2]*Program in Environmental Technology and Policy, Korea University, Sejong 30019, Republic of Korea*
[3]*BKT Co. Ltd., 25 Yuseong-daero 1184 beon-gil, Yuseong-gu, Daejeon 34109, Republic of Korea*
[4]*Department of Civil and Environmental Engineering, Sejong University, Seoul 05006, Republic of Korea*
[5]*Department of Research and Development, Jeollanamdo Environmental Industries Promotion Institute, Jeollanam-do 59205, Republic of Korea*
[6]*Research Institute for Advanced Industrial Technology, Korea University, Sejong 30019, Republic of Korea*
[7]*Department of Clean Water Supply Facilities Maintenance, K-water, Daejeon 34350, Republic of Korea*

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Abstract. In recent years, the generation of disinfectant by-products (DBPs) in drinking water system has been highlighted for their potential negative impact on humans. A commonly used disinfectant, chlorine, produces a by-product which is highly hazardous and a known carcinogen. This study investigated the toxic effects of DBPs from several organic matter as a function of contact time with chlorine-based disinfectants were investigated using zebrafish. The results indicated that the generation of DBPs was dependent on the composition of dissolved organic matter (DOM) in water. Suwannee river natural organic matter and experimental site water sample (complex DOM) were almost 2.5 times higher than that of a single dissolved organic matter, such as microcystin-LR (MCLR) at 120 min. The behavior of zebrafish was significantly affected by complex composition DOM. In vivo biomarker analysis result from lipid profile analysis, reaction in vivo showed different depending on the composition of the DOM. Through this study, the effect of DBPs were observed via lipid metabolic and movement changes in aquatic organisms can be considered as a new biomarker for the drinking water risk assessment.

Keywords: dissolve organic matter(DOM); disinfection byproducts(DBPs); toxicity assessment

1. Introduction

The dissolved organic matter (DOM) normally found in aquatic environments has a wide range of properties (Montserrat, 2009; Jin *et al.* 2018), and can be classified as either allochthonous or autochthonous (Kornegay, B.H *et al.* 2000). Allochthonous organic matter originates from soil. It is hydrophobic due to the corrosion of its organic content, which consists mainly of plant and animal matter. Autochthonous organic matter refers to the natural organic matter (NOM) produced by the metabolic activity of microorganisms – typically bacterial and algae byproducts which are characterized by their polarity and hydrophilicity (Thurman 1985). The chemical composition, molecular weight, and structure of organic matter vary depending on regional and environmental characteristics, but typically has a complex molecular structure (Uyguner and Bekbolet 2011). The representative substance for allochthonous NOM is humic acid, which has several functional groups including alcohol, carboxyl, and ketone. These compounds are highly capable of combining with ionic molecules to form complexes (Thurman 1985). Generally, NOM that has high aromatic carbon content, especially that which is hydrophobic and has a high molecular weight, is known to be a precursor to disinfection by-products (DBPs) when reacting with chlorine (Bond *et al.* 2009, Trang *et al.* 2012). When algal blooms occur frequently in drinking water reservoirs, general water treatment operators and utility officers typically express concern regarding the role of aesthetic pollutants and toxic substances such as microcystin. Algal blooms are produced by freshwater cyanobacteria, primarily species of the microcystis genus. Microcystin is a toxic substance secreted by blue-green algae or cyanobacteria, such as microcystis, oscillatoria, and anabaena, the chemical structure of which contain a ring peptide (Park *et al.* 2008). The most common microcystin is microcystin-LR (MCLR), where the variable L-amino acids are leucine (L) and arginine (R) (Dawson 1998). Several researchers found that microcystin ingestion causes liver damage (Ned *et al.* 2009). For remediating these pollutants, water treatment systems generally use a disinfectant process such as chlorination, which successfully removes algal toxins (Yansen *et al.* 2016). Chlorine is the most widely used disinfectant in drinking water systems, even though its

*Co-Corresponding author, Ph.D.
E-mail: bosung@kwater.or.kr
**Co-Corresponding author, Professor
E-mail: ub1905ub@korea.ac.kr

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disinfectant by-products (DBPs) are known to be toxic (Steve 2009). The formation of DBPs depends on several factors, such as the pH, temperature, presence of organic matter, contact time, and the concentration of the disinfectant agent in the water (Krishna *et al.* 2007). In particular, the type of by-product that is generated during the disinfection process is typically dependent on the concentration of OM in the water, the sterilizing agent used, and the contact time (Guanghui and David 2008, Hu *et al.* 1999). Organic matter in water affects many aspects of the formation of potentially harmful DBPs during chlorination (Taha and Doanh 2000). Discharged disinfection by-products have been found to have undesirable and harmful effects (e.g., genotoxic, mutagenic, and carcinogenic) on aquatic animals (Susan *et al.* 2008, Sun *et al.* 2009). However, limited information is available regarding the sub-lethal toxic effects of DBPs originating from different types of dissolved organic matter. For example, the difference in the generation of DBPs and its toxic effect to humans via drinking water containing different OM such as algal organic matter (AOM) or DOM has not been studied might be different. Fish behavior analysis has been recognized as a sub-lethal methodology, which can estimate the toxicity of external substances, or an environmental stress in the environment (Mingzhe *et al.* 2018, Careau *et al.* 2008, Rupia *et al.* 2016). Lipids play essential roles in the normal functioning of organisms; almost all fish diseases are associated with abnormal lipid metabolism. Lipid profiling analysis produces optical clarity that enables the monitoring of biological processes (Shiu *et al.* 2004). Behavior changes of zebrafish could be related to changes in metabolic profiles, such as lipid profiles in the body, and have been used to determine toxic effects in living organisms. This study conducted behavior change analysis and sequentially analyzed the lipid profile changes in zebrafish to investigate the toxic effect of DBPs. It was assumed that the toxic effect of each DOM disinfection byproduct would differ as a function of exposure time. *Danio rerio* AB strain(Zebrafish) were used, as a model organism, to quantify the toxic effects of DBPs resulting from the inflow of DOM to chlorine-based disinfection processes. For investigating the toxicity of the DBPs of DOM, two types of DOM, microcystin and Suwannee river NOM, were selected. The potential toxicity of the DBPs of each target compound was evaluated based on changes in cognitive function, zebrafish behavior and lipid profiles after exposure to disinfected DOM-containing water. Observation of cognitive and behavior changes (external changes) were visually identified, and the lipid profiles of exposed zebrafish were analyzed to assess internal changes.

2. Material and method

2.1 Model DOM

In this study, microcystin-LR (CAS 101043-37-2, Enzo Life Sciences, USA) was considered as one of model DOMs from algae. The other DOM models are Suwannee River NOM (SRN) was purchased in the International Humic Substances Society (IHSS) and humic acid (CAS 1415-93-6, WAKO pure chemical corporation, Japan). Chuso is a region in the Geum river basin, and the water in this region is frequently deteriorated in summer. It was used as a natural water DOM model in this study. Based on the previous studies, the dissolved organic carbon of DOM used in the study was identified as MCLR 3mg C/L (Andrew J.F *et al.* 1999), humic acid 4.5 mg C/mL (Luiz F.Z *et al.* 2006), SNR 1.9 to 4.02 mg C/L (Yanan Xing 2010), respectively. Chuso was analyzed average of 3 mg C/L.

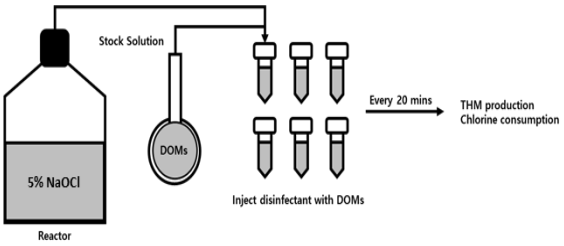

Fig. 1 Schematic of lab-scale chlorine consumption and DBPs production test

2.2 Determination of residual chlorine

For the disinfection experiment used a 5% sodium hypochlorite solution (CAS 7681-52-9) was used (Fig. 1). Initial concentration of sodium hypochlorite was 1.5 mg Cl_2/L. The pH level of each sample was adjusted to 7 using a phosphate buffer. The buffer was prepared by dissolving 87.09 g (1M) dipotassium phosphate, K_2HPO_4 (99.0%), and 68.045 g (1M) potassium dihydrogen phosphate, KH_2PO_4 (99.5%) in 500 mL of distilled water. Since MCLR was detected at 100 ppb in Geum river, one of South Korea's four major rivers at 2016, the concentration of each DOM, except the Chuso sample, was set to 100 ppb. The residual chlorine was measured by DPD method, one of the standard methods (Danial 2002), as it rapidly reacts with the DOM in the sample. For measuring DBPs, the THM Plus kit (Hach #2790800, USA), which detects the total amount of THM and Haloacetic acids (HAAs), was detected by a DR6000 UV/VIS spectrophotometer (Hach, USA). Through determination of residual chlorine experiment and DBPs production test with DOM, highly realistic materials among the DOM, SNR (complex compound) and MCLR (single compound) were selected and applied to further zebrafish experiments.

2.3 Ecological toxicity assessment

2.3.1 Zebrafish

Danio rerio AB strain(Zebrafish) was purchased from the aquarium. The fish were placed in a 10 L tank with air diffusers for proper oxygen transfer and maintained on a cycle of 12 h of light followed by 12 h of darkness. The water temperature was maintained at 27 °C, and the fish were fed twice daily with Artemia (brine shrimp; Lawrence, 2007). Adult fish were bred once a week to get larvae. Since zebrafish spawning begins with hormonal stimulation by light, the health condition of the fertilized egg was checked

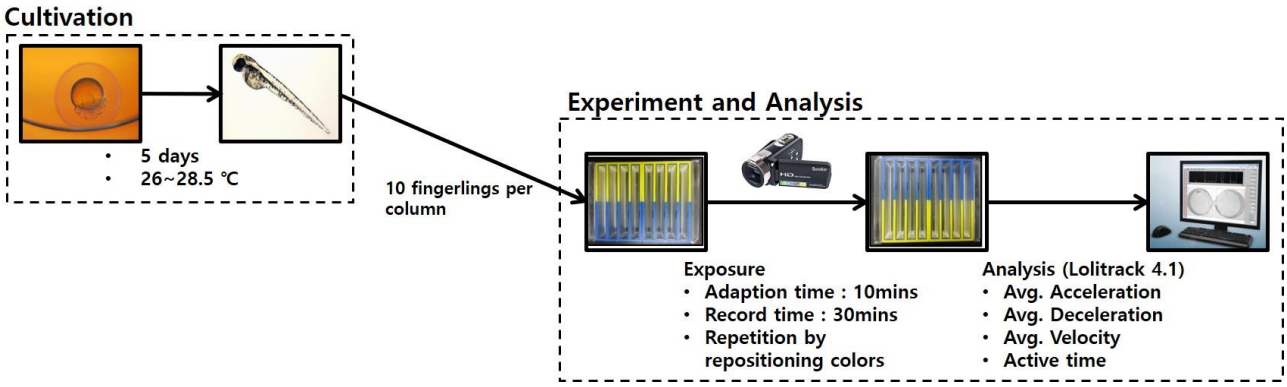

Fig. 2 Zebrafish behavior test model

two hours after light exposure. The fertilized eggs were collected and washed with methylene blue to prevent contamination. The eggs were then kept in 60 ppm egg water and were incubated for 5 days. Health status of fertilized eggs was checked in accordance with OECD guideline 236 and only healthy eggs were continued to be incubated.

2.3.2 Zebrafish behavior test

The 5 days post fertilization (dpf) zebrafish larvae can swim independently and distinguish visible rays. Zebrafishes are known to prefer a short wavelength of light but the presence of toxins in the body can affect the expression of this preference. A behavior-based test was conducted using a color maze kit (Genomic Design TM, Korea), in order to analyze the wavelength preference of zebrafish larvae exposed to chlorine-treated DOM solutions. Larvae which are good movement were selected and transferred to the color maze kit. To reduce the reactivity errors due to the characteristics of zebrafish, 70 larvae (n = 10 for each experiment) were used and all assays were conducted at least 7 times to minimize the statistical errors. The behavior of a control group not exposed to chlorine-treated DOM solutions was also analyzed. The color maze kit consists of a transparent acrylic plastic sheet, one half of which is blue (the preferred color) and the other is yellow (the non-preferred color; Fig. 2). Normal zebrafish (control fish) are expected to swim within the area with the preferred wavelength (i.e. the blue area, since zebrafish prefer short wavelengths; Avdesh *et al.* 2012). The movements of the zebrafish larvae were recorded using a camera and then analyzed using LoliTrack 4.1 (Loligo®systems, Denmark), which populates the mean acceleration, deceleration, speed, and active time of the zebrafish. The active time is calculated as a percentage of the length of time spent on the activity compared to the control.

2.3.3 Lipid profile test

The lipid profiles of the zebrafish were investigated to observe the changes in their lipid metabolite profile after exposure to chlorine-treated DOM solutions. This test was performed in four individual experiments. Five adult zebrafish per each experiment used in this test and exposed to each chlorine-treated DOM solution for 24 h. During this time, feeding was stopped, and microbial propagation was prevented by injecting a methylene blue solution of 1 mL solution per 1 L tank. The residual chlorine was neutralized with sodium thiosulfate ($Na_2S_2O_3$) to prevent toxic effects on the fish. After exposure, the zebrafish were frozen at below -20℃. The frozen fish were first separated into six parts, namely the head, upper part, middle part, body part, lower part, and tail. Each body part was then homogenized by grinding. The homogenized samples were then analyzed using GC-FID (Varian 450 high-resolution gas chromatography). The columns used in GC-FID were DB-Wax (Agilent J&W GC Column, 30m × 0.25 mm × 0.25 μm).

The fatty acids detected in the lipid profile analysis are shown in Table 1.

2.4 Measure of Microcystin-LR

To measure the microcystin-LR in the sample, it was measured using a microcystin plate kit (ZEULAB, Spain). The absorbance is measured with a wavelength of 405nm and the microcystin is quantified by comparing absorbance of standard concentration and sample.

3. Results and discussion

3.1 Chlorination test (Consumption of chlorine and formation of DBPs)

It is known fact that the production of DBPs are affected by several factors such as DOC, nitrogen organic matter, disinfection time, chlorine concentration during disinfection process (Jinfeng *et al.* 2009, Jingyun *et al.* 2010). Fig. 3 shows the relationship between the concentration of residual chlorine and DBPs in various types of DOM solutions in order to confirm the effects of disinfection time. As the disinfection time increased, the residual chlorine concentration was decreased. To compare the decreasing amount of residual chlorine, the k value was calculated through this experiment assuming a first order reaction. Although the k value (0.35 to 0.49 hr^{-1}) did not vary much by types of DOM (as shown in Table 2), however, there was a significant difference in the formation of DBPs of each

Table 1 The fatty acids identified in the lipid profile analysis

Fatty acid Formula	Chemical Name	Molecular Formula	M.W (g/mol)	Fatty Acid Formula	Chemical Name	Molecular Formula	M.W (g/mol)
C 14:0	Methyl myristate	$C_{15}H_{30}O_2$	242.40	C 20:0	Methyl arachidate	$C_{21}H_{42}O_2$	326.57
C 16:0	Methyl palmitate	$C_{17}H_{34}O_2$	270.46	C 20:1	Methyl cis-11-eicosenoate	$C_{21}H_{40}O_2$	324.55
C 16:1	Methyl palmitoleate	$C_{17}H_{32}O_2$	268.44	C 20:2	Methyl cis 11,14-elcosadienoic acid methyl ester	$C_{21}H_{38}O_2$	322.53
C 17:0	Methyl heptadecanoate	$C_{18}H_{36}O_2$	284.48	C 20:3	Methyl cis 8,11,14-eicosatrienoic acid methyl ester	$C_{21}H_{36}O_2$	320.52
C 18:0	Methyl stearate	$C_{19}H_{38}O_2$	298.51	C 20:4	Methyl arachidonate	$C_{21}H_{34}O_2$	318.50
C 18:1	Methyl oleate	$C_{19}H_{36}O_2$	296.50	C 20:5	Eicosapentaenoic acid ethyl ester	$C_{22}H_{34}O_2$	330.5
				C 22:0	Methyl behenate	$C_{23}H_{46}O_2$	354.62
C 18:2 C 18:3	Methyl linoleate	$C_{23}H_{46}O_2$	294.48	C 22:6	All cis-4,7,10,13,16,19-docosahexaenoic acid methyl ester	$C_{23}H_{34}O_2$	342.52

Table 2 k-value of the four dissolved organic matter solutions

Organic matter or Source (Location)	Reaction constant (hr^{-1})	Ref
Microcystin-LR	0.40	
Suwannee river NOM (SRN)	0.49	
Humic Acid	0.38	
Chuso (Lake in Republic of Korea)	0.35	
Municipal water supplies	0.09 ~ 0.19	Fang Hua *et al.* 1999
Ohio and eagle river (USA)	0.25 ~ 0.49	Lewis A. Rossman *et al.* 2001
Queshan reservoir (China)	0.45 ~ 0.72	Xiao Zhan *et al.* 2010

DOM. The formation of DBPs were high at SRN and Chuso (Fig. 3b and 3d).

Since SRN and Chuso water samples composed of several substances more complex than others (MCLR and humic acid), it was expected that this has an effect to the degree of formation of DBPs. Accordingly, the health risk of DBPs from single type DOM such as MCLR or humic substances by chlorination might not be significant but that of DBPs from complex. Since the production pattern of DBPs from at MCLR and humic substances are similar (Fig. 3 (a) and (c)), the SNR (a complex compound), a substance to represent real water conditions, and MCLR (a simple compound) which are chosen for the further study.

3.2 Zebrafish behavior test

3.2.1 Cognitive function

For estimating the effect on DBPs from DOM through chlorination, zebrafish cognition test was conducted.

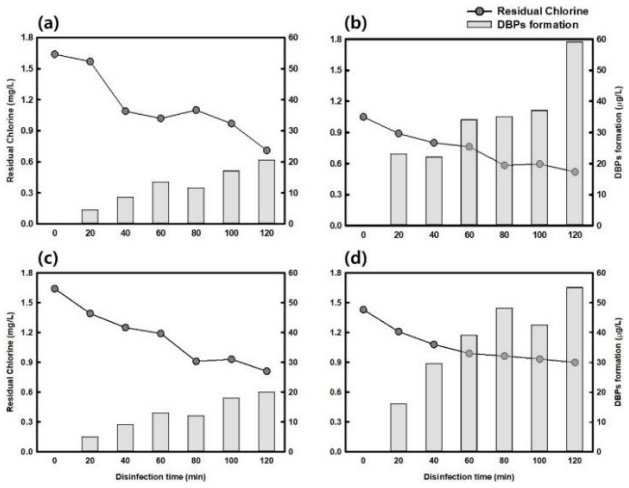

Fig. 3 Residual chlorine concentration and DBPs formation over time in the solutions of (a) MCLR (b) SRN (c) Humic acid and (d) Chuso

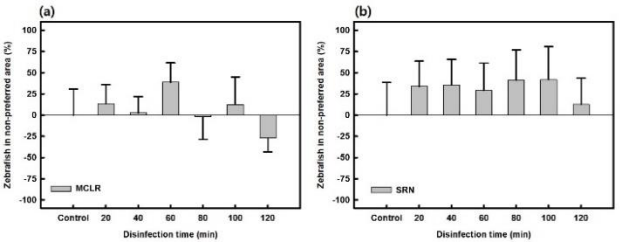

Fig. 4 Percentage of fish observed in non-preferred wavelength according to disinfection time (a) MCLR and (b) SRN

Previous study has also shown that exposure to external substances affects the cognitive ability of zebrafish (Ryan *et al.* 2017). Zebrafish are readily available and easy to manage because their breeding environment is relatively

Table 3 Correlation analysis of zebrafish movement

		Disinfection Time	Mean Velocity	Mean Acceleration	Mean Deacceleration	Active Time
Disinfection Time	PCC*	1	-0.257	-0.273	0.262	-0.109
	p-value		0.011	0.006	0.009	0.288

* PCC: Pearson Correlation Coefficient

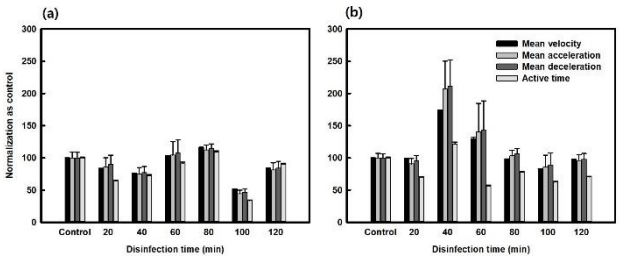

Fig. 5 Analysis of zebrafish behavior based on changes in (a) MCLR and (b) SRN over time

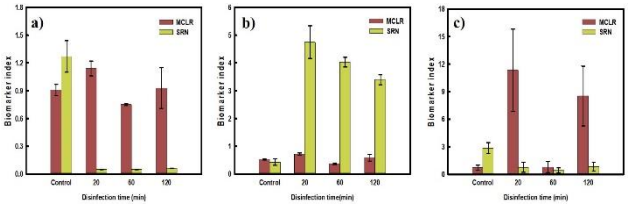

Fig. 6 Changes in selected metabolite biomarkers after exposure to disinfected natural organic matter a) Membrane mobility, b) Inflammation and c) Cardiovascular system

insensitive, unlike that of algae or *Daphnia magna* (Adema 1978). Moreover, zebrafish share 22% of their genes and more than 90% of their homology with humans (Seung 2015, Soyeon *et al.* 2009). Therefore, zebrafish possess most of the organs and genes that humans do, so the toxicity of aquatic life for humans can be determined and quantified from zebrafish toxic exposure. Additionally, zebrafish could distinguish between certain wavelengths when their visual system is developed at 5 days post-fertilization (dpf).

A different pattern of behavioral response of zebrafish after exposure to the different DOM samples was found. When the chlorine concentration is greater than 1 ppm, the MCLR was removed and degraded by chlorine more than 90% when contact time is over 15 minutes (Lionel *et al.* 2006, Michael *et al.* 2019). Thus, it is assumed that there was no impact by toxicity of MCLR compared to DBPs when the disinfection time increased. Fig. 4(a) shows that the longer the zebrafish were exposed to DBPs from MCLR, the higher the percentage found in non-preferred wavelength locations, suggesting that DBPs exposure caused cognitive problems. The cognitive function of zebrafish was found to decrease the most in comparison to the control group after 60 min of contact with MCLR. In contrast, zebrafish exposed to DBPs from the SRN samples were consistently located in blue area (short wavelength; Fig. 4b).

3.2.2 Movement

The movements of zebrafish can be used as a new aquatic ecotoxicology assessment method using small aquatic organisms (Yushi *et al.* 2015). Fig. 5 shows the results of the analysis of zebrafish movement as a function of disinfection time with chlorination. In the case of DBPs from MCLR with chlorine, the trend for all observations (mean acceleration, mean deceleration, mean velocity, and active time) appeared to be relatively constant over time. At 80 min, all observed factors increased by about 10% in comparison with that observed at 60 min. However, at 100 min, the zebrafish movements decreased to less than 50% compared to the control group. In the case of the DBPs from SRN with chlorine, all factors increased at 40 minutes. Mean acceleration and deceleration have increased by twice as much as the control group. After 40 mins, all factors showed decreasing trend. Among the variables, active time was the most sensitive measuring factor for the zebrafish movement. The reaction of *zebrafish* movement by DBPs from two DOM was different. The movement of organisms, including fish, is controlled by complex factors, including both endogenous and exogenous (Mingzhe *et al.* 2018).

However, it is difficult to determine whether the reaction is due to temper, syndrome or coping styles based on this movement test alone (Koolhaas *et al.* 1999, Careau *et al.* 2008, Reale *et al.* 2007). Thus, the analysis of the changes in motion requires corroboration with an accurate analysis of their effects by checking for changes in the physiology. Comparing the p-value of each movement analysis, average acceleration and deacceleration are the most significant factors correlated with the disinfection time (as shown in Table 3). Sudden changes in the speed of fish movements are associated with nerves in the body, and thus may be related with in vivo inflammation (Fig. 6B). The longer the disinfection time, the more likely the effects of DBPs produced by MCLR will appear in organisms. However, in the case of NOM, the DBPs produced by composite materials appear to have a stronger impact after a relatively shorter disinfection time.

3.3 Lipid profile analysis

A lipid profile analysis was conducted to observe the physiological changes in zebrafish caused by DBPs, and to determine if there is a link between changes in zebrafish cognition and zebrafish behavior. The weight of the lipid metabolite relative to the overall lipid mass of standardized zebrafish samples was analyzed. In this study, changes in the analyzed lipid profile was investigated if these can be as correlated with the behavior changes of zebrafish after DBP exposure. For the analysis of potential lipid biomarkers, lipids were divided into saturated fatty acids and unsaturated fatty acids (single or complex). In this study,

the saturated fatty acids of lipids analyzed in the zebrafish were C16:0 and C18:0. C16:1 and C18:1 were the single unsaturated fatty acids analyzed. C18:1 is a fatty acid called oleic acid, one of the fatty acids of Omega-9. The main role of this in the body is to reduce blood pressure and lower the level of cholesterol. The biomarkers were identified by saturated and unsaturated fatty acid; C16:0 (Palmitic acid) and C18:0 (Stearic acid) as saturated fatty acid and C16:1 (Palmitoleic acid), C18:1 (Oric acid) for single unsaturated fatty acid (Loris *et al.* 2017). Among the complex unsaturated fatty acids, the omega-3 fatty acids were C20:5 (Eicosapentaenoic acid; EPA) and C22:6 (Docosahexaenoic acid; DHA), and the omega-6 fatty acids were C18:2, C20:3, and C20:4. The external changes were suspected to be caused by the influence of the fish's brain, and C20:5 and C22:6 identified in the lipid profile were found to vary with the contact time of the disinfectant. C20:5 (EPA) and C22:6 (DHA) both play an important role in the function of the brain, metabolic activity and survival. Low concentration of EPA and DHA results in low growth and survival rates for organisms (Takeshi, 1993, Ganesaratnam *et al.* 2000). Cell membrane fluidity was evaluated as the ratio between saturated fatty acids and single unsaturated fatty acids. The higher the content of saturated fatty acids relative to unsaturated fatty acids, the lower the fluidity of the cell membrane. Inflammatory activations can be identified by the proportion of omega-3 relative to omega-6. A rise in the ratio of omega-6 to omega-3 indicates an induction in the production of inflammatory medium in the lipid metabolite pathway (Simopoulos 2002). The higher the concentration of omega-3 fatty acids, the lower the risk of developing cardiovascular disease. According to membrane mobility and inflammation biomarkers in Table 4, as the disinfection time increased, the changes in the biomarker's numbers were more likely to decrease or increase in the DBPs from complex DOM (SRN) compared to single DOM (MCLR). For the cardiovascular biomarker, biomarker's number was reduced in the DBPs from complex DOM but showed a tendency to change the lipid profile by disinfection time when single DOM was present. There is no effect of cardiovascular system on MCLR's toxicity (Best *et al.* 2001). Thus, it showed that change of cardiovascular system in zebrafish by DBPs from MCLR and research on this is needed in future studies. The results confirmed that the compounds of DBPs from single and complex DOM could cause damage to zebrafish physiology as contact with chlorine increases with time (Fig. 7).

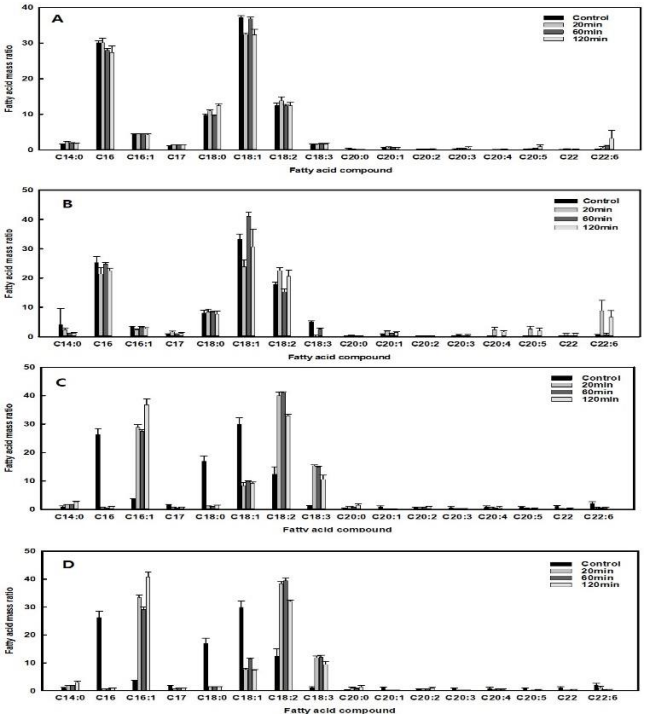

Fig. 7 Changes in selected metabolite biomarkers after exposure to disinfected natural organic matter a) Membrane mobility, b) Inflammation and c) Cardiovascular system

4. Conclusions

Several studies showed that DBPs from chlorination can reach up to 160 ppb in drinking water (Xing and William, 2018) and its negative potential effect to health is well known (Chad *et al.* 2007). In this study, researchers identified the toxic effects of DBPs on zebrafish, health indication organism from several different organic matter origins, specifically microcystin-LR (a simple organic matter) and Suwannee river NOM (a complex organic matter). One of the key findings of this research was that the external changes (i.e. cognitive function and movement) of exposed zebrafish was dependent on the origin of DBPs from different organic matter. To correlate the behavior, change of zebrafish with the internal metabolite profile of fish, lipid profile changes as function of disinfection contact time was monitored. Several biomarkers (i.e. for membrane mobility, inflammation, cardiovascular system) were identified in this study, and as disinfection time increased, it was seen that changes with respect to the control were observed, and the changes were also different depending on the composition of the substance. The physiological responses in the zebrafish as seen through the lipid profile analysis were also different relative to control depending on the source of DBP, and disinfection time. For instance, changes in biomarkers associated with membrane mobility, inflammation and cardiovascular system were significantly greater upon exposure to SRN DBPs. Furthermore, a decreasing trend in biomarker index for all three parameters were also observed with increasing disinfection time. Therefore, lipid profile analysis confirmed in vivo toxicity in zebrafish during the exposure. Still, a lot of research on the application of this assessment method is being developed, such that observation of these changes in biomarkers can demonstrate its possibility of being applied in risk assessment monitoring.

Acknowledgments

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