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Eco-Safety Assessment of Glyoxal-Containing Cellulose Ether on Freeze-Dried Microbial Strain, Cyanobacteria, *Daphnia*, and Zebrafish

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***Running Head**;* Eco-safety assessment of glyoxal-containing cellulose ether on different aquatic organisms

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Abstract

The objective of this study is to investigate the aquatic-toxic effects of glyoxal-containing cellulose ether with four different glyoxal concentrations (0, 1.4, 2.3 and 6.3%) in response to global chemical regulations, e.g., European Union Classification, Labeling and Packaging (EU CLP). Tests of the aquatic-toxic effects of glyoxal-containing cellulose ether on 11 freeze-dried microbial strains, *Microcystis aeruginosa*, *Daphnia magna* and zebrafish embryos were designed as an initial stage for toxicity screening, and were performed with the standardized toxicity test guidelines. Glyoxal-containing cellulose ether showed no significant toxic effects in the toxicity tests for the 11 freeze-dried microbial strains, *Daphnia magna* and zebrafish embryos. Alternatively, 6.3% glyoxal-containing cellulose ether led to more than a 60% reduction of *Microcystis aeruginosa* growth after 7 days of exposure. Approximately 10% developmental abnormalities (e.g., bent spine) in zebrafish embryos were also observed in the group exposed to 6.3% glyoxal-containing cellulose ether after 6 days of exposure. These results imply that <6.3% glyoxal-containing cellulose ether results in non-toxic effects on the acute toxicity of aquatic organisms. However, ≥6.3% glyoxal-containing cellulose ether may affect the health of aquatic organisms with long-term exposure. In order to better evaluate the eco-safety of cellulosic products contained in glyoxal, further studies regarding the toxic effects of glyoxal-containing cellulose ether with long-term exposure are required. The results from this study allow us to evaluate the auatic-toxic effects of glyoxal-containing cellulosic products, under EU chemical regulations, on the health of aquatic organisms.

Keywords: *Aquatic-toxic effects; EU chemical regulation; Glyoxal-containing cellulose ether*

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1. Introduction

Cellulose ethers are water-soluble polymeric substances derived by the etherification of cellulose, which is one of the most widespread natural organic compounds. They are known as environmentally friendly polymers as a result of their properties (e.g., water solubility, pH stability and biodegradation).[1,2,3,4,5] Chemical modification of cellulose ethers has been performed to improve their physical and chemical properties (e.g., organic solubility, viscosity stability, water retention and non-ionic charges), and cellulose ethers in mixed with organic or inorganic chemicals have also been produced to enhance the cellulosic product quality by physical blending of functionalized additives.[2,5,6,7] These derivatives can be used in a wide range of industrial applications, including construction products, ceramics, paints, personal care products and pharmaceuticals.[2, 5, 8, 9] With the increasing use of chemicals in industrial applications, the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) proposed harmonized hazard communication elements, including safety data sheets (SDS) for chemicals. It was adopted in the chemical regulations of the European Union (EU) Registration, Evaluation, Authorization, and Restriction of Chemicals regulation (REACH) and Classification, Labeling and Packaging (CLP) regulation.[10, 11] Since most industrial applications of chemicals are based on mixtures containing more than a single chemical substance, the classification and labeling of chemical substances have been extended to a whole mixture of various substances from 2015.[11, 12, 13] Therefore, SDS for cellulosic products contained in functionalized additives under the EU CLP regulation are required.

Glyoxal is one of the most extensively used cross linking agents in cellulosic products due to the advantage that its chemical reaction can enhance the solubility and dispersion of the polymer without chemical modification.[6, 7] However, the glyoxal toxicity for human health is an issue and may cause sensitization for eye and skin contact, and its properties have led to the regulation of glyoxal content in as functionalized products with glyoxal.[6, 14, 15, 16] For example, the use of glyoxal in paper and textile wall coverings is prohibited.[14] The European Commission

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(EC) defined that a content of up to 100 mg/L glyoxal in manufactured cosmetic products is safe.[15, 16] When more than 1% of glyoxal is used for the reaction process with cellulose ether, the treated cellulose ether products are obliged to describe under the EU CLP registration.[6, 15] These regulations imply that the use and production of glyoxal-containing products may result in glyoxal being released into the environment, and finally arouse the concern with respect to the potential impact on human and environmental health.[15, 16, 17]

The predominant target compartments for glyoxal-containing cellulosic products in the environment are hydrospheres rather than sediment, when considering production to final emission.[16, 17] Nevertheless, the aquatic hazards for glyoxal-containing cellulosic products are currently unclear, and the toxicity tests for these cellulosic products have been not performed for aquatic organisms. Cellulose is defined as a non-classification substance under the chemical regulation of the EU due to it being a natural organic compound.[1, 2, 3, 4, 5, 10, 11] Glyoxal is classified as a non-hazardous substance in the environment due to high half effective concentrations more than 100 mg/l for aquatic organisms, i.e., microorganisms, algae, *Daphnia* and fish.[16, 17, 18, 19, 20] However, the eco-safety assessment of glyoxal-containing cellulosic products is urgently needed in response to the EU CLP regulations.

Therefore, the aim of this study was to provide the aquatic toxic information available for glyoxal-containing cellulose ether based on a reliable evaluation of its hazardous properties. Surface-treated cellulose ethers with glyoxal were chosen in this study, since these glyoxal-containing cellulose ethers were specially developed to prevent lumping effects or to improve the rheological properties in wet blending applications, such as paints and emulsion. These cellulose ethers with four different concentrations of glyoxal were classified hazardous substances for human health by EU CLP regulations (Table 1). We evaluated the aquatic-toxic effects of glyoxal-containing cellulose ethers with four different concentrations by analysis of the toxicity tests, using four different aquatic organisms (i.e., 11 freeze-dried microbial strains,

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cyanobacteria, *Daphnia magna* and zebrafish embryos), which are widely applied aquatic toxicity tests due to the high sensitivity to various chemicals.[21, 22, 23, 24]

Table 1. Hazardous classification of glyoxal-containing cellulose ethers with four different glyoxal concentrations calculated by the EU CLP.

Classification	Glyoxal-containing cellulose ethers with four different concentrations			
	0 %	1.4 %	2.3 %	6.3 %
Physical hazards	-	-	-	-
Health hazards	-	Skin sens. 1, Muta. 2	Skin sens. 1, Muta. 2	Skin sens. 1, Muta. 2
Environmental hazards	-	-	-	-

'-' = Not calculable.

'Skin sens. 1'= May cause an allergic skin reaction.

'Muta. 2'= Suspected of causing genetic defects.

2. Materials and Methods

2.1. Test samples

Glyoxal-containing cellulose ethers with four different glyoxal concentrations (0, 1.4, 2.3 and 6.3%) were obtained from Lotte Fine Chemical Co., Ltd., Korea (cellulose, CAS 9004-65-3) (Table 2), and test solutions were prepared in accordance with the manufacturer manual (Lotte Fine Chemical Ltd., Korea).[25] Briefly, surface-treated test samples in powder form were dissolved using a magnetic stirrer in purified cold water for 1 h at room temperature, to ensure the complete removal of any undissolved powder samples before use in the toxicity tests with the different aquatic organisms.

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Table 2. Physicochemical characteristics of surface treated cellulose ethers with four different concentrations of glyoxal.

Test sample	Viscosity (mPas)	pH	Moisture (%)	NaCl (%)	Methoxyl (%)	Hydropropoxyl (%)	Total-glyoxal (%)
A	3.5	5.0 – 8.0	3	0.5	27 – 29	6 – 8	0
B							1.4
C							2.3
D							6.3

2.1.1. Tests for Aquatic-toxic effects

A schematic design for the aquatic-toxic effect tests is shown Figure 1. Tests for the aquatic-toxic effects of glyoxal-containing cellulose ethers on 11 freeze-dried microbial strains, *Microcystis aeruginosa*, *Daphnia magna* and zebrafish embryos have been covered by the standardized methods.[22, 23, 24, 26] The following points were investigated: the growth inhibition of microbial strains and cyanobacteria, *Daphnia* acute toxicity based on mortality and immobility, and zebrafish embryo toxicity based on mortality, hatchability and abnormalities.

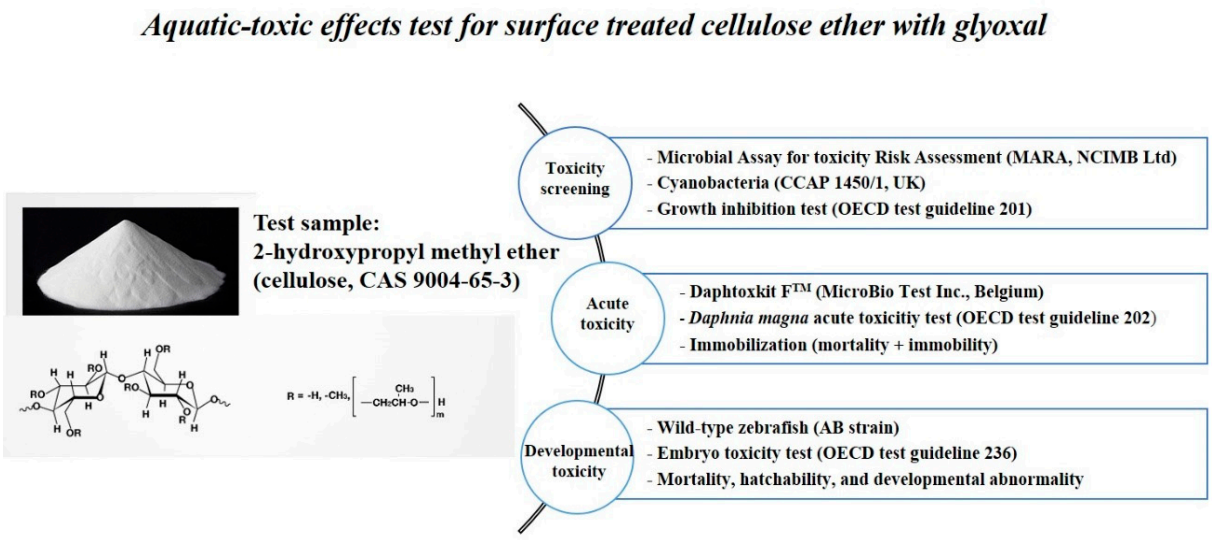

Figure 1. Schematic design for assessing the aquatic-toxic effects of glyoxal-containing cellulose ethers with four different glyoxal concentrations.

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2.1.2. Microbial array for toxicity risk assessment (MARA)

A MARA system involving 11 freeze-dried microbial strains was purchased from NCIMB Ltd, United Kingdom (Table 3). The MARA for test solutions (i.e., glyoxal-containing cellulose ether with four different glyoxal concentrations) was performed according to the manufacturer instructions.[26] Briefly, 11 freeze-dried microbial strains were pre-incubated in a 96-well MARA plate with 150 µl of aqueous nutrient peptone (2% phytone peptone) for 4 h at 30 °C. After pre-incubation, 200 µl of the test solution was transferred to the 96-well MARA plate with 100 µl of medium containing 0.01% redox indicator. A volume of 15 µl of each microbial strain was added to each well and incubated for 18 h at 30 °C. After 18 h of incubation, the MARA plates were scanned by a scanner (HP Scanjet G4050, Hewlett Packard, Palo Alto, CA, USA) using transmitted light with a resolution of 100. The average growth rate of the 11 freeze-dried microbial strains exposed to test solutions was determined with the reduction of tetrazolium red using MARA software in quadruplicate.

Table 3. Freeze-dried microbial strains for microbial assay for toxicity risk assessment (MARA, NCIMB Ltd., UK).

MARA Number	Microbial strain	
#1	*Microbacterium sp*	(NCIMB 30255)
#2	*Brevundimonas diminuta*	(NCIMB 30256)
#3	*Citrobacter freundii*	(NCIMB 30257)
#4	*Comamonas testosteroni*	(NCIMB 30258)
#5	*Enterococcus casseliflavus*	(NCIMB 30259)
#6	*Delftia acidovorans*	(NCIMB 30260)
#7	*Kurthia gibsonii*	(NCIMB 30261)
#8	*Staphylococcus warneri*	(NCIMB 30262)
#9	*Pseudsomonas chlororaphis*	(NCIMB 30263)
#10	*Serratia rubra*	(NCIMB 30264)
#11	*Pichia anomola*	(NCIMB 30265)

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2.1.2. Algal growth inhibition test

Cyanobacteria (i.e., *Microcystis aeruginosa*) was purchased from the Culture Collection of Algae and Protozoa, Cambria UK strain (CCAP 1450/1), and cultivated at 25 ± 1 °C with 16 h of light and 8 h of darkness (16L:8D) in Blue-Green medium (BG11, Sigma-Aldrich, Germany) until use for the algal growth inhibition test (total volume of $\geq 1\times10^5$ cell/mL). Based on the findings in the MARA system, algal growth inhibition tests for 6.3% glyoxal-containing cellulose ether were conducted based on the protocol described in the OECD Guideline 201.[23] At 0, 3 and 7 days during an exposure period of 7 days, the numbers of algal cells were counted using a Neubauer chamber (Celeromics, UK), and the concentrations of chlorophyll-a, a parameter for determining the algal growth,[27] were measured using an UV-Vis spectrometer (Lambda 35 UV/Vis system, Perkin-Elmer, Waltham, MA, USA). The growth inhibition of *M. aeruginosa* (i.e., the growth rate at the end of the experiment period minus the growth rate at the start of the experiment period) for 6.3% glyoxal-containing cellulose ether was determined in triplicate.

2.1.3. Daphnia magna acute toxicity test

Daphnia magna ephippia was obtained from Daphtoxkit F™ (MicroBioTests Inc., Gent, Belgium) and was cultured for 72 h in International Organization for Standardization standard freshwater Daphtoxkit F™. The toxicity test with *D. magna* neonates within 8 h after hatching were conducted in six-well cell culture plates (Cellstar®, greiner bio-one, Germany) filled with 10 mL of each test solution.[28] During the experimental period, *D. magna* neonates were maintained at 23 ± 0.5 °C under a light cycle of 16 h light and 8 h darkness (16L: 8D). The toxicity to *D. magna* was investigated after an exposure period of 48 h to glyoxal-containing cellulose ether. [22] During the experimental period, the dead and immobilized individuals for

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each cellulose ether solution were recorded for calculating the immobilization [immobilization= (numbers of dead and immobilized individuals /number of initial individuals)×100] after exposure to each cellulose ether solution. Tap water filtered through a Millipore® 0.22 µm GSWP filter (Merck KGaA, Germany) after sterilization at 130 °C for 1 h was used as a control medium. Toxicity for each cellulose ether was determined in quadruplicate (10 daphnids per replicate).

2.1.4. Zebrafish embryo toxicity test

Wild-type zebrafish (AB strain) breeding and maintenance was performed under standard conditions.[25, 28] Tests for embryo toxicity were conducted in six-well cell culture plates (Cellstar®, greiner bio-one, Germany) filled with 10 mL of each test solution. Embryos at 8 h post fertilization were maintained under a long photoperiod (16L: 8D light/dark cycle) at a 26.5 ± 0.5 °C water temperature for 6 days after exposure to glyoxal-containing cellulose ether. Tap water filtered through a Millipore® 0.22 µm GSWP filter (Merck KGaA, Germany) after sterilization at 130 °C for 1 h was used as the control group. The mortality, hatchability and developmental abnormalities in each test solution were recorded to assess the embryo toxicity of each glyoxal-containing cellulose ether.[25, 28] The embryo toxicity for each cellulose ether was determined in quadruplicate (10 embryos per replicate).

2.1.5. Statiscal analysis

All errors are expressed as mean ± standard error of mean (SEM). Comparison between the toxicities for each glyoxal-containing cellulose ether was carried out using a *post hoc* Student-Newman-Keuls test in the one-way ANOVA (SigmaPlot version 12.5, Systat Software, Inc., San Jose, CA, USA). Statistical significance was set at $P < 0.05$.

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3. Results and discussion

3.1. MARA and cyanobacteria algal growth inhibition

The toxicity of each glyoxal-containing cellulose ether for 11 freeze-dried microbial strains and cyanobacteria are shown in Table 4 and Figure 2. There was no remarkable difference between the growth rates of the 11 freeze-dried microbial strains exposed to glyoxal-containing cellulosic products of ≤ 2.4%, due to the growth rates being more than 75% in all strains (Table 4). However, when exposed to glyoxal-containing cellulosic products of 6.3%, the growth rate of microbial strain #2 (*Brevundimonas diminuta*, NCIMB 30256) was less than 75% and tended to decrease in a does dependent manner (Table 4, Figure 2A). In addition, the inhibition rate of *M. aeruginosa* growth in the 6.3% glyoxal-containing cellulose ether-exposed group for 7 days was significantly higher (i.e., 60.6%) when compared to that of the control group, and the decrease of growth showed an exposure-time dependent manner (Figure 2B). The difference between microbial strains and *M. aeruginosa* on the growth inhibition after exposure to glyoxal-containing cellulose ethers may be a result of exposure time rather than the glyoxal concentration. These results imply that glyoxal-containing cellulose ether with 6.3% glyoxal may have bactericidal properties or may not be totally biodegradable during the short-term, unlike in previous reports. [1, 2, 3, 4, 5, 15, 16, 17]

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Table 4. Average growth rate of freeze-dried microbial strains exposed to surface treated cellulose ethers with four different concentrations of glyoxal.

MARA Number	Average values of growth rate ± SEM (N = 4)			
	Tested cellulose ether with four different concentrations of glyoxal (%)			
	A (0)	B (1.4)	C (2.3)	D (6.3)
#1	100.0 ± 0.0	100.0 ± 0.0	99.3 ± 0.8	100.0 ± 0.0
#2	86.3 ± 1.4	83.0 ± 0.8	77.5 ± 1.2	72.0 ± 3.1†
#3	98.5 ± 1.2	96.5 ± 2.0	98.3 ± 0.9	92.3 ± 3.7
#4	100.0 ± 0.0	99.5 ± 0.5	99.8 ± 0.3	98.0 ± 1.2
#5	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	79.3 ± 1.2
#6	98.8 ± 0.6	97.0 ± 0.8	87.8 ± 1.9	86.3 ± 1.9
#7	100.0 ± 0.0	100.0 ± 0.0	99.5 ± 0.5	100.0 ± 0.0
#8	98.5 ± 1.0	99.0 ± 0.6	91.0 ± 1.8	91.3 ± 2.0
#9	77.3 ± 1.9	77.5 ± 1.6	75.0 ± 2.0	86.7 ± 1.8
#10	95.8 ± 2.5	94.0 ± 2.2	93.3 ± 1.9	87.3 ± 1.8
#11	100.0 ± 0.0	100.0 ± 0.0	98.0 ± 2.0	98.7 ± 0.9

†Growth rate is lower than 75%, which was the lowest growth rate in this study.

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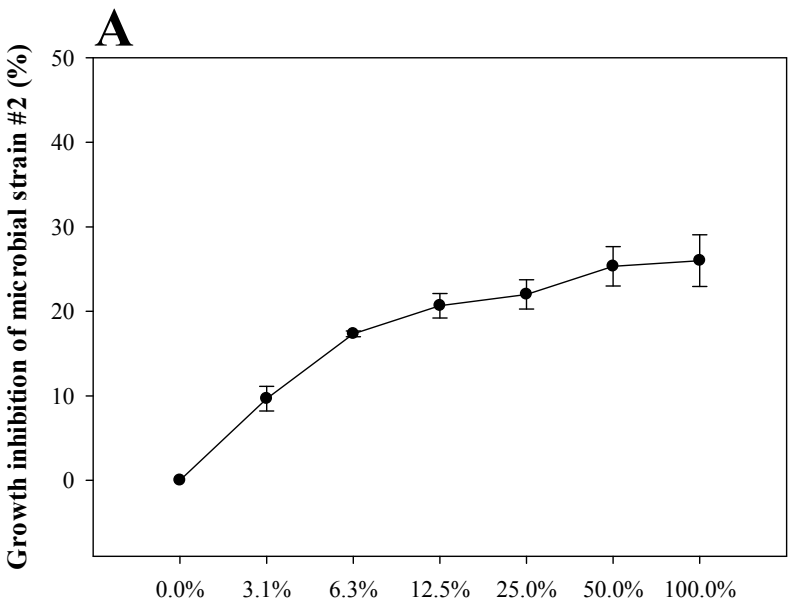

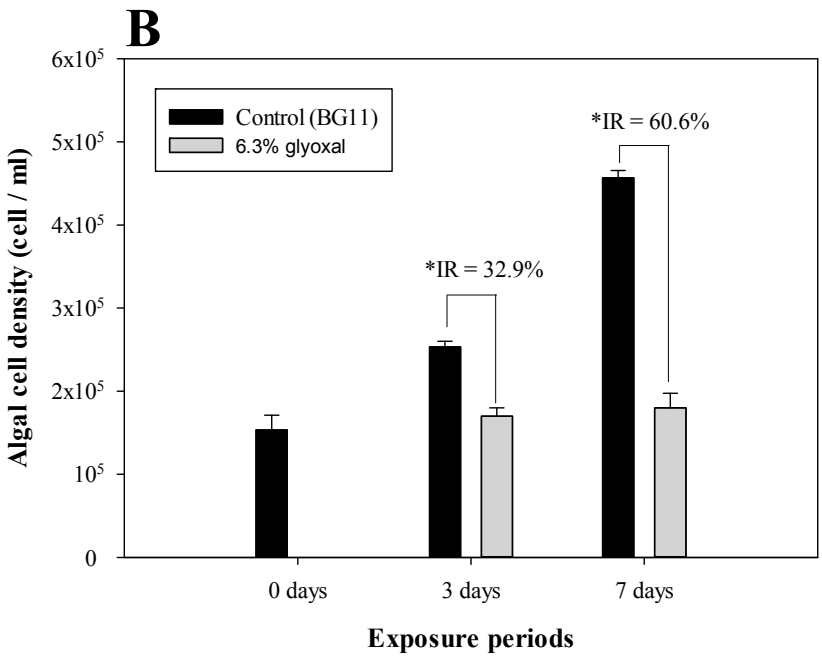

Figure 2. Growth inhibition rate of microbial strain #2 (A) and *Microcystis aeruginosa* (B) after exposure to 6.3% glyoxal-containing cellulose ether. The inhibition rates (IR) of *Microcystis aeruginosa* growth at 3 and 7 days after exposure to 6.3% glyoxal-containing cellulose ether were 32.9% and 60.6%, respectively. *denotes significant differences between algal cell density ($P < 0.05$).

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3.2. Daphnia magna acute toxicity and zebrafish embryo toxicity

After 48 h exposure to glyoxal-containing cellulose ethers with four different concentrations, the mortality and immobility of *D. magna* were rarely observed in the glyoxal-containing cellulose ethers-exposed group, due to the results that normal behavior was observed for more than 85% in all groups (Figure 3A). The zebrafish embryo toxicity was also not observed in the glyoxal-containing cellulose ethers-exposed group throughout the experimental period. The survival rate and hatching rate of zebrafish embryos exposed to glyoxal-containing cellulose ethers with four different concentrations were higher than 90% and 85%, respectively (Figure 3B). It appears that glyoxal-containing cellulose ethers had no affected on *D. magna* acute toxicity and zebrafish embryo toxicity,[16, 17] even if 6.3% glyoxal-containing cellulose ether inhibited the *M. aeruginosa* growth. The results indicate that the toxicity to glyoxal-containing cellulose ethers are sensitively reflected to *M. aeruginosa*, rather than waterflea (*Daphnia magna*) or zebrafish embryos.[17, 29]

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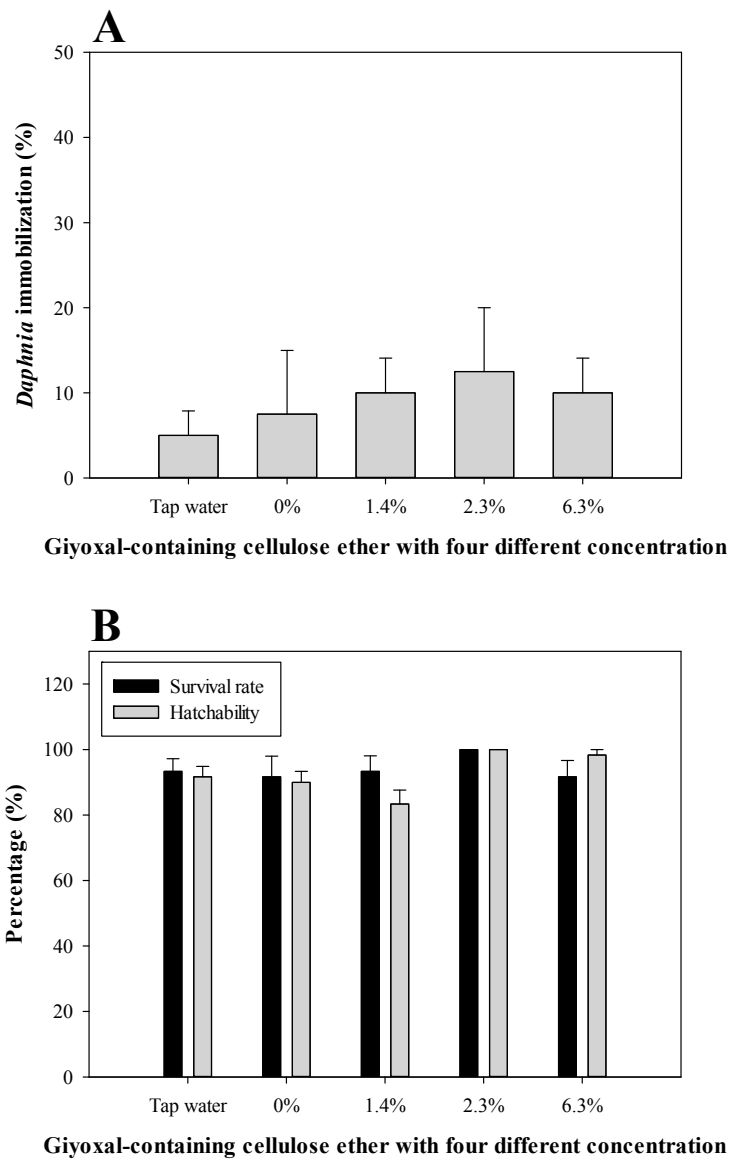

Figure 3. Acute toxicity of *Daphnia magna* after 48 h (A) and zebrafish embryo toxicity after 6 days (B) to glyoxal-containing cellulose ether with four different glyoxal concentrations after exposure.

Alternatively, while there was no acute toxicity of glyoxal-containing cellulose ether for the zebrafish embryos, developmental abnormalities, such as bent spines, were observed in the glyoxal-containing cellulose ethers-exposed group, in particular 2.3% and 6.3% glyoxal-containing cellulose ethers (Figure 4). However, there is no significant difference between the embryo developmental abnormalities in all groups. It is possible that glyoxal-containing cellulose ethers do not result in acute toxicity in mortality and hatchability on zebrafish embryos,

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but may cause developmental abnormalities after 6 days of exposure. Unfortunately, studies examining the development toxicity of glyoxal-containing cellulose ethers on zebrafish embryos are rare and their toxicity mechanisms are also unknown. Further studies on the effects induced by glyoxal-containing concentration during zebrafish embryo development are therefore required.

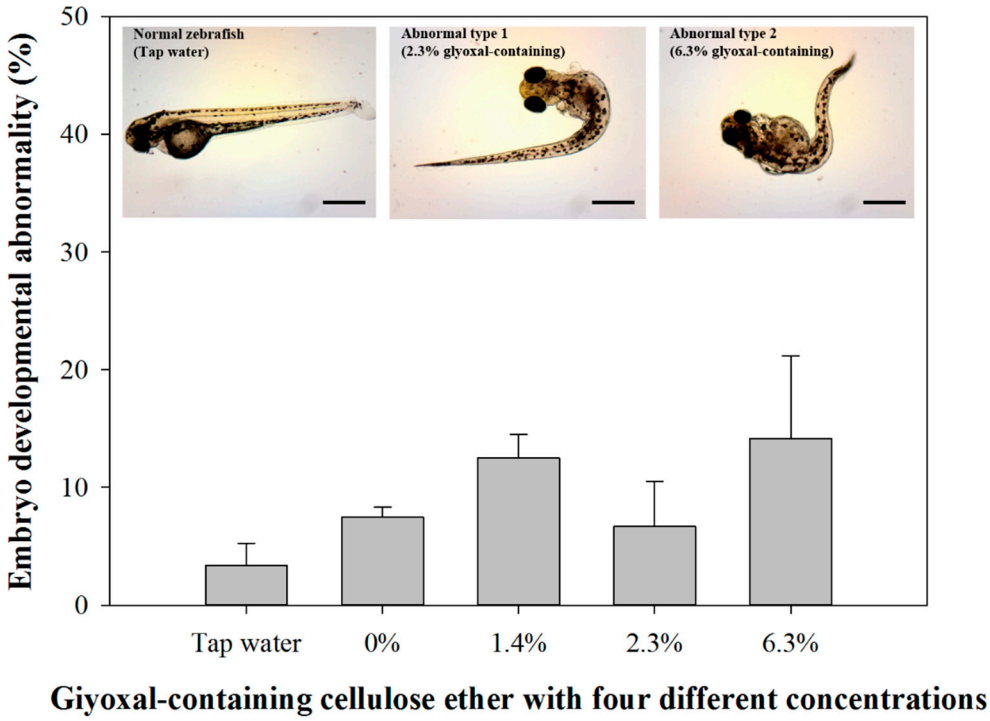

Figure 4. Zebrafish embryo toxicity to glyoxal-containing cellulose ether with four different glyoxal concentrations after 6 days of exposure. All scale bars are 500 μm.

4. Conclusions

A major finding from this study is the non-toxic effect of ≤6.3% glyoxal-containing cellulose ethers by toxicity tests using different aquatic organisms (i.e., 11 freeze-dried microbial strains, *D. magna* and zebrafish embryo), although ≥1.4% glyoxal-containing cellulose ethers were classified as a hazardous substances on human health by EU CLP. However, 6.3% glyoxal-containing cellulose ether may influence *M. aeruginosa* growth due to

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the >60% growth inhibition after 6 d of exposure. These effects may depend on exposure period due to the result that the growth inhibition of *M. aeruginosa* was higher in 6.3% glyoxal-containing cellulose ether for 6 days of exposure (growth inhibition of 60.6%) compared to 3 days of exposure (growth inhibition of 32.9%). In order to clearly evaluate the ecological effects of glyoxal-containing cellulose ethers, further studies of the growth inhibition of *M. aeruginosa* by long-term exposure is required. Consequently, the findings from this study are able to available the effect data of glyoxal-containing cellulosic products under EU chemical regulations, on the health of aquatic organisms.

Author Contributions

All authors have contributed to the conception and design of the experiments and have given their approval to the final version of the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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