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Toxicity of 17β-Estradiol to Green Algae and Cyanobacteria

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The toxicity of various concentrations of 17β-estradiol to *C. ellipsoidea* and *A. flos-aquae* was studied. The results showed that there was a highly significant relationship between the dry weight or chlorophyll-a (chl-a) content and OD 680 nm for the algae tested. When the 17β-estradiol concentration was ≥ 0.1 mg L^{-1} and ≤ 0.05 mg L^{-1} it inhibited and stimulated algal growth, respectively. EC_{50} decreased gradually from the 1st to the 7th days (18-104 mg L^{-1}) for *C. ellipsoidea* and from the 1st to the 5th days (16-448 mg L^{-1}) for *A. Spiroides*, then increased gradually from the 7th to the 15th days (31-104 mg L^{-1}) for *C. ellipsoidea* and from the 5th to 10th days (16-155 mg L^{-1}) for *A. Spiroides*. *C. ellipsoidea* was more sensitive than *A. flos-aquae* on the 1st to 3rd days and 7th to 10th days, but less sensitive on the 4th to 6th days. Both the toxicity and sensitivity were influenced by the testing time, implying that traditional algal tests (conducted at 72-96 h) do not provide sufficient information.

Key Words: 17β-Estradiol, Toxicity, Sensitive, Cyanobacteria, Green alga.

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are environmental contaminants that can interfere with the proper function of the endocrine system, which is linked with changes in the sex ratio, embryonic damage and reduced fecundity in a variety of vertebrate species. The endocrine and reproductive effects of these chemicals on organisms are believed to be attributable to several mechanisms *e.g.*, simulation of endogenous hormones, antagonism of the effects of endogenous hormones, alteration of the patterns of synthesis and metabolism of natural hormones and modification of hormone receptor levels. In addition, many environmental media, including river water environments are contaminated by various compounds that exert agonistic or antagonistic effects on the endocrine system[1]. Although the problem of environmental pollution associated with micropollutants, such as exogenous EDCs, has been reported by many researchers in recent years, many of its aspects have yet to be scientifically elucidated. This problem has an important impact on environmental

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protection as EDCs may cause major harm to the reproductive processes of human and animal species[2]. Steroidal estrogens have been shown to be the main contributors to the estrogenic activity observed in aquatic systems contaminated with sewage treatment effluents. Although the occurrence of steroid hormones in the environment has received a great deal of attention, little is known about their effect on algae in aquatic systems[3,4]. In order to determine the toxicity of 17β-estradiol to algae, a 15-day toxicity test on green algae and a 10 day toxicity test on cyanobacteria have been devised. In the present work, the concentration dependence of the toxicity of 17β-estradiol to *Chlorella ellipsoidea* and *Aphanizomenon flos-aquae* has been measured.

EXPERIMENTAL

Samples of 17β-estradiol used for testing (AR grade, Johnson Matthey Company) were dissolved in 99.5 % distilled acetone. *C. ellipsoidea* and *A. flos-aquae* were obtained from the Institute of Wuhan Hydrobiology, the Chinese Academy of Sciences. The medium for the algal growth inhibition test was prepared in accordance with the Chinese National Environmental Protection Agency Guidelines 201[5] using SE medium, which is composed of distilled water and the following chemical ingredients (mg L^{-1}): $NaNO_3$ (250), $K_2HPO_4 \cdot 3H_2O$ (75), $MgSO_4 \cdot 7H_2O$ (75), $CaCl_2 \cdot 2H_2O$ (25), KH_2PO_4 (175), NaCl (25), Na_2CO_3 (20), $Na_2SiO_3 \cdot 5H_2O$ (100), $FeCl_3 \cdot 6H_2O$ (5), A_5 solution (1 mL, H_3BO_3 2.86, $MnCl_2 \cdot 4H_2O$ 1.81, $ZnSO_4 \cdot 7H_2O$ 0.22, $CuSO_4 \cdot 5H_2O$ 0.079, $(NH_4)6Mo_7O_{24} \cdot 4H_2O$ 0.39 gL^{-1}) and Fe-EDTA (1 mL, Na_2EDTA 10, $FeCl_3 \cdot 6H_2O$ 0.81 gL^{-1}). The culture medium was sterilized at 121 ºC and 1.05 kg cm^{-2} for 0.5 h.

Algal cells were propagated photoautotropically in a 250 mL Erlenmeyer flask containing 100 mL of liquid SE medium and incubated in a rotary shaker (100 rpm) at 22 ºC with illumination by cool-white fluorescent lights with light intensities of 450 μmol m^{-2} s^{-1} in continuous mode[6]. For the toxicity tests, 30 mL aliquots of the medium containing the algal cells (initial cell concentration OD_{680} = 0.008) were inoculated into sterile 50 mL Erlenmeyer flasks. A wide range of concentrations of the estradiol was examined in previous testing in order to find an adequate toxicity range for the tested substance. Then, similar concentrations were tested according to the results of the previous test[7]. The cultures were then treated with various 17β-estradiol concentrations and incubated for 15 days on an orbital shaker running at 100 rpm at 22 ºC and under a continuous light intensity of 450 μmol m^{-2} s^{-1}. Cell counts were correlated with absorbance over time for 24 h on a Shimadzu UV-1600 spectrophotometer. The most suitable wavelength to use for monitoring culture growth was 680 nm, which is supported by the finding of Kasai *et al*.[8] that cell number and A680 nm are highly correlated and the growth of the algal cells was monitored spectrophotometrically. Measurement at each 17β-estradiol concentration was replicated three times. Appropriate control systems containing no 17β-estradiol were included in each experiment. Controls and treated cultures grew under the

same temperature, photoperiod and shaking conditions as the stock culture. In each experiment, per cent inhibition values, relative to the growth in control systems, were calculated using optical density. Chlorophyll-a (chl-a) content analysis came after filtration of 20 mL of the medium through 0.45 μm pore size Whatman GF/C membranes and extraction with 90 % acetone. Chl-a content in the samples was estimated using a spectrophotometer in a trichromatic method[9]. The dry weight of algae was determined with a digital balance after the cells were filtered on a 0.45 μm membrane and dried at 105 ºC for 8 h. Statistical analysis was conducted using Microsoft Excel 2003.

RESULTS AND DISCUSSION

Correlation between chlorophyll-a content or dry weight and the absorbance: There was a highly significant relationship between the dry weight or chl-a content and OD 680 nm for the two algae. The linear regression equations are shown in Fig. 1 and coefficients of correlation (R^2) were > 0.98 and $p < 0.001$. The growth of the algal biomass was calculated indirectly from OD 680 nm data.

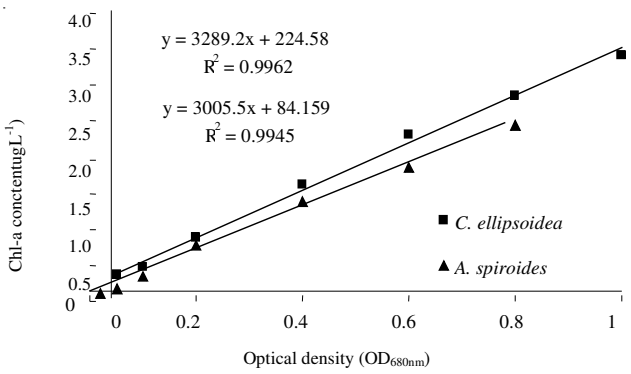

Fig. 1a. Relationship between OD 680 and Chl-a content of two algae

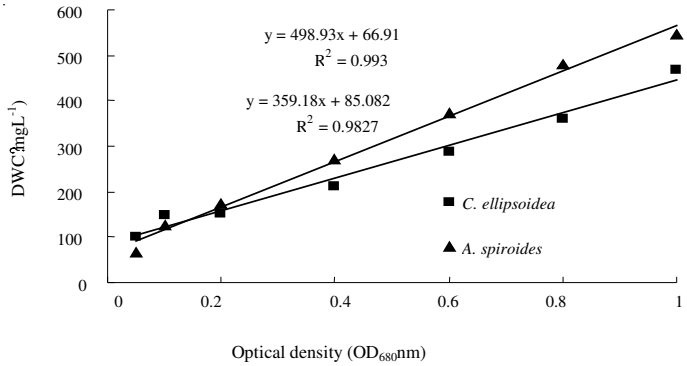

Fig. 1b. Relationship between OD 680 and DWC of two algae

Percentage inhibition of the two algae by 17β-estradiol at various concentrations and times: The toxicities of 17β-estradiol to *C. ellipsoidea* and *A. flos-aquae* are shown in Figs. 2 and 3. When the concentration of 17β-estradiol was 50 mg L^{-1} (Figs. 2a and 3a), the maximal growth inhibition ratios were 75 % for *C. ellipsoidea* (on the 3rd day) and 65 % for *A. flos-aquae* (on the 4th day). However, the growth inhibition ratios of the two tested algae decreased gradually from the 5th to 15th days and a ratio of 45 % occurred on the 15th day for *C. ellipsoidea*. When the concentration was 20 mg L^{-1} (Figs. 2a and 3a), the maximal growth inhibition ratios were 55 % (on 6th day) for *C. ellipsoidea* and 54 % (on 5th day) for *A. flos-aquae*. The growth inhibition ratios of the two tested algae decreased gradually from the 7th to 15th days and a ratio of 37 % occurred on the 15th day for *C. ellipsoidea*.

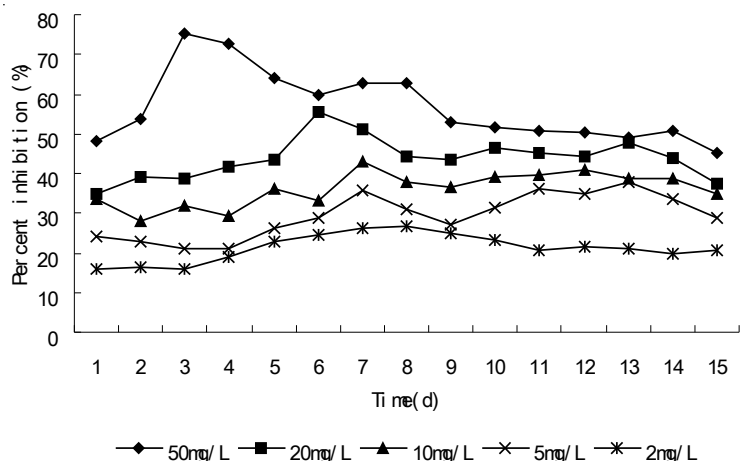

Fig. 2a. Toxicity to *C. ellipsoidea* at various concentrations (2-50 mg L^{-1}) and time

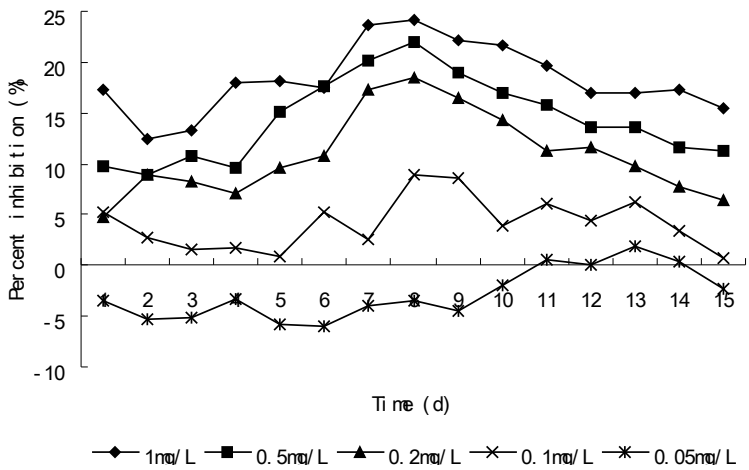

Fig. 2b. Toxicity to *C. ellipsoidea* at various concentrations (0.05-1 mg L^{-1}) and time

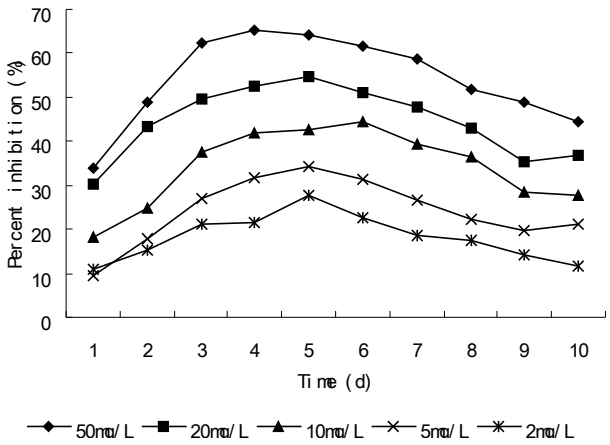

Fig. 3a. Toxicity to *A. flos-aquae* at various concentrations (2-50 mg L^{-1}) and time

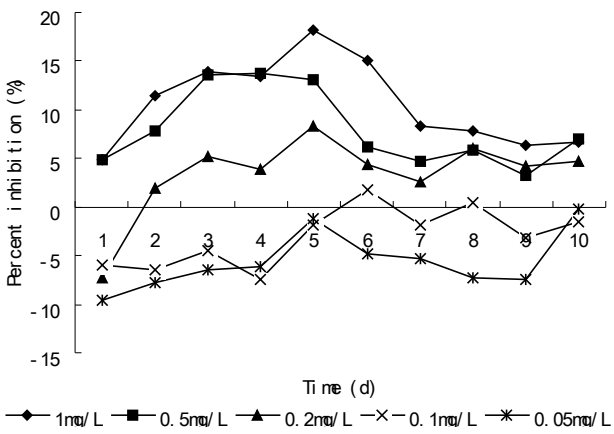

Fig. 3b. Toxicity to *A. flos-aquae* at various concentrations (0.05-1.00 mg L^{-1}) and time

The maximal growth inhibition ratios of 43 % (on 7th day) for *C. ellipsoidea* and 44 % (on 6th day) for *A. flos-aquae* occurred at 10 mg L^{-1} 17β-estradiol (Figs. 2a and 3a). The inhibition ratios for the two algae decreased gradually from the 8th to 15th days and were 34 and 28 %, respectively, on the 10th and 15th days. The maximal growth inhibition ratios of 37 % (on 13th day) for *C. ellipsoidea* and 34 % (on 5th day) for *A. flos-aquae* were measured at 5 mg L^{-1} (Figs. 2a and 3a). On the 10th and 15th days, inhibition ratios of 28 and 21 % occurred, respectively. The maximal growth inhibition ratios of 26 % (on 8th day) for *C. ellipsoidea* and 27 % (on 5th day) for *A. flos-aquae* occurred when 17β-estradiol was 2 mg L^{-1} (Figs. 2a and 3a). On the 10th and 15th days, inhibition ratios of 20 and 11 % occurred for the green algae and cyanobacteria, respectively.

At 1 mg L^{-1} (Figs. 3a and 3b), the maximal growth inhibition ratios of 24 % (on 8th day) for *C. ellipsoidea* and 18 % (on 5th day) for *A. flos-aquae* occurred. On the

10th and 15th days, inhibition ratios were 15 and 7 % for the green algae and cyanobacteria, respectively. At 0.5 mg L^{-1} (Figs. 3a and 3b), the maximal inhibition ratios of 21 % for *C. ellipsoidea* (on 8th day) and 13 % for *A. flos-aquae* (on 5th day) were measured. Inhibition ratios of 11 and 7 % for the green algae and cyanobacteria, respectively, occurred on the 10th and 15th days. When the concentration was 0.2 mg L^{-1} (Figs. 3a and 3b), the maximal growth inhibition ratio of 8 % occurred on the 8th day and the 5th day for *A. flos-aquae* and *C. ellipsoidea*, respectively. On the 10th and 15th days, inhibition ratios of 6 and 4 % occurred for the green algae and cyanobacteria, respectively.

At 0.1 mg L^{-1} (Figs. 3a and 3b), the maximal growth inhibition ratios of 8 % for *C. ellipsoidea* occurred on the 8th day and 1 % for *A. flos-aquae* occurred on 6th day. However, there was a slight negative inhibiting effect (stimulating algal growth) on the 1st-4th day and 6th-10th day and a stimulating effect occurred on the 4th day (7 %) for the cyanobacteria. When the 17β-estradiol concentration was 0.05 mg L^{-1} (Figs. 3a and 3b), there was a slight stimulating effect on the 1st-10th day and a moderate stimulating effect up to 6 % on the 6th day for the green algae and 7 % on 9th day for the cyanobacteria.

Toxicity of 17β-estradiol to the two algae at various concentrations and times: The toxicities of 17β-estradiol to the two algae are shown in Table-1. For *C. ellipsoidea* (Table-1), the EC_{50} values decreased gradually from the 1st to 7th days (18-104 mg L^{-1}) and then increased gradually from the 7th to 15th day (31-104 mg L^{-1}). With respect to A. spiroides (Table-1), the EC_{50} values decreased gradually from the 1st to 5th days (16-448 mg L^{-1}) and increased from the 5th to 10th days (16-155 mg L^{-1}). The increases could be attributed to the use of 17β-estradiol as a carbon source by the algae. Therefore, the highest toxicities of 17β-estradiol were 18.34 mg L^{-1} to *C. ellipsoidea* at 7 days and 16.26 mg L^{-1} at 5 days to *A. spiroides*.

TABLE-1
TOXICITY OF 17β-ESTRADIOL TO CYANOBACTERIA *A. spiroides*

Days	Regression equation*	Coefficient correlation	Significance level	EC_{50} (mgL^{-1})	EC_{50}CE/ EC_{50}AS
1	Y = 0.0768X + 1.0923	0.904	0.004	447.32	0.232
2	Y = 0.0842X + 1.2805	0.910	0.000	94.25	0.653
3	Y = 0.1009X + 1.5657	0.950	0.000	25.88	0.965
4	Y = 0.1108X + 1.7086	0.968	0.000	18.31	1.433
5	Y = 0.1045X + 1.6523	0.980	0.000	16.26	2.008
6	Y = 0.1109X + 1.6996	0.980	0.000	20.06	1.417
7	Y = 0.1088X + 1.6362	0.965	0.000	29.15	0.629
8	Y = 0.0917X + 1.4001	0.937	0.000	54.59	0.573
9	Y = 0.0839X + 1.2631	0.932	0.000	112.19	0.583
10	Y = 0.0772X + 1.1776	0.934	0.000	154.21	0.287

*: Y and X stand for per cent inhibition and natural logarithm of concentration, respectively.

Sensitivity of the two algae to 17β-estradiol at various concentrations and times: Interesting variations among individual species of *C. ellipsoidea* and *A. flos-aquae* were found in response to 17β-estradiol. The ratio of EC_{50} values for the green algae and cyanobacteria (EC_{50}CE/EC50AS, Table-1) varied with time. *C. ellipsoidea* was more sensitive than *A. flos-aquae* on the 1st-3rd day and 7th-10th day, but less sensitive on the 4th-6th day. The latter may lead to algal bloom owing to algal overabundance and present a higher aquatic ecological risk. However, a stimulating effect occurred only on the 1st-5th day and decreased gradually until completely gone on the 13th-15th day. Moreover, the effective concentrations were higher than those presently detected in the actual aquatic environment by orders of magnitude.

The results showed that the growth inhibition rate of the algae changed with the testing time upon exposure to 17β-estradiol at various concentrations. For the green algae, the maximal inhibition rate was delayed by the decrease in 17β-estradiol concentration and basically occurred on the 8th day. For the cyanobacteria, the same occurred, but basically on the 5th day. The full life-cycles of *C. ellipsoidea* and *A. flos-aquae* were found to be 15 and 10 days from our previous tests. In this test, a different time was chosen to test the two algae because the most suitable culture time is 15 days for *C. ellipsoidea* and 10 days for *A. flos-aquae* under the specific culture conditions (composition of the medium, temperature, illumination and vibration speed). That is to say that a full life-cycle was completed exactly during the culture time. The magnitude of the toxicity value was influenced by the testing time itself. In environmental risk assessment, the ultimate aim is to provide sufficient information for decision-making with the purpose of protecting the environment from the unwanted effects of chemicals[10]. Traditional acute algal tests are normally based on short-term tests, initially concentrating on gathering base-set data from 3-4 days tests with a few species as lethal endpoints. As a consequence, crucial information is not considered in the early stages of a risk assessment process. This could lead to an erroneous risk assessment in worst-case situations[10]. We recommend that algal tests should be based on eco-toxicological tests that could provide information from the full life-cycle of the tested individual species, which should be confirmed in previous tests. At the minimum, the testing time should not be shorter than a life-cycle.

It is believed that, in the algae toxicity test, the algal growth biomass is very small on the 1st-2nd days, so that when calculating the EC_{50} value the error is magnified by the algal growth inhibition ratio owing to the small biomass of the control (to which was not added any toxic algal media). This caused a greater inaccuracy in the value. Traditional acute algal tests that are normally performed from 3-4 days are feasible based on short-term tests with a few species because of the decrease in errors in the test. However, the results of our tests showed that the high point in toxicity was not achieved at 3-4 days. As a consequence, 3-4 days could not be considered as the crucial time period in the early stages of a risk

assessment process. In addition, the toxicity profile was not consistent with the risk assessment of aquatic ecology: though some toxic compounds are not at highly toxic levels, they could present toxicity persistently over a long test process. This could lead to an erroneous risk assessment in traditional acute algal tests, owing to difficulties in decomposition and absorption by the algae. They added a higher ecological risk to the aquatic systems. The present work showed that various algae had different toxicities to the same agent and these varied with time. Because the toxicity could reverse as time went by, this phenomenon caused a great deal of fluctuations in the aquatic ecological risk.

In 1998, the US poultry industry generated almost 12 million Mg of broiler litter containing appreciable concentrations of estradiol, testosterone and sex hormones of environmental concern, most of which was applied to grasslands as fertilizer. Finlay-Moore *et al.*[1] measured estradiol concentrations in soil and runoff water from large grazed and ungrazed grasslands amended with broiler litter and the results showed background concentrations of estradiol were 50-150 ng L^{-1} in runoff water and 55 ng kg^{-1} in soil. Depending on the litter application rate and time from application, the estradiol concentration in the soil increased up to 675 ng kg^{-1}. During a winter recharge event, five springs in northwest Arkansas were sampled to analyze 17β-estradiol content in their waters by Peterson *et al.*[11] and they found that 17β-estradiol was present in five springs representing three different water-bearing formations and concentrations ranged from 6-66 ng L^{-1}. It is well known that the toxic concentration of 17β-estradiol to humans is only 1 ng L^{-1}. Our tests showed that concentrations of 17β-estradiol over 0.1 mg L^{-1} could inhibit algal growth and a concentration of 0.1 mg L^{-1} in a water environment could stimulate the growth of cyanobacteria slightly, while a concentration of 0.05 mg L^{-1} could stimulate the growth of both the green algae and cyanobacteria. It is inferred that 17β-estradiol at 0.05 mg L^{-1} or lower could stimulate algal growth because it is used as a carbon source or to enhance the self-recovery capability of algae.

As a consequence, it was found that, whether 17β-estradiol was inhibiting or stimulating algal growth at various concentrations, the two effects tended to weaken and then disappear as time went by (15 days for *C. ellipsoidea* and 10 days for *A. flos-aquae*). It can be seen that under the specific culture conditions (composition of the medium, temperature, illumination and vibration speed), the growth end-points were the same for the algae; that is to say that algae grew and reached the same biomass whether in the control or experimental groups and were not influenced by the exogenous chemicals. From present results, it is concluded that the growth rule of algae obeys a certain law of nature and it is proposed that it as the principle of preconcerted equal growth biomass.

Conclusion

The toxicity of 17β-estradiol at various concentrations (0.05-50 mg L^{-1}) to the green algae *C. ellipsoidea* during 15 days and to cyanobacteria *A. flos-aquae* during 10 days was continuously tested. The conclusions from the present work in a

condensed form are as follows: (**i**) There was a highly significant relationship between both dry weight and chl-a content with OD 680 nm for the two algae. The growth of algal biomass could be calculated indirectly by using OD 680 nm. (**ii**) With 17β-estradiol concentrations at or above 0.1 mg L^{-1}, algal growth of *C. ellipsoidea* and *A. flos-aquae* was inhibited, while at or less than 0.05 mg L^{-1} algal growth was stimulated. (**iii**) EC_{50} decreased gradually from the 1st to the 7th days (18-104 mg L^{-1}) for *C. ellipsoidea* and from the 1st to the 5th days (16-448 mg L^{-1}) for *A. spiroides*, then increased gradually from the 7th to the 15th days (31-104 mg L^{-1}) for *C. ellipsoidea* and from the 5th to the 10th days (16-155 mg L^{-1}) for *A. spiroides*. (**iv**) *C. ellipsoidea* was more sensitive than *A. flos-aquae* on the 1st to the 3rd days and the 7th to the 10th days, but was less sensitive on the 4th to the 6th days. (**v**) The EC_{50} value and the sensitivity were influenced by the testing time. Official acute algal tests are normally based on short-term test periods focused on gathering base-set data initially from 72-96 h tests with few species as lethal endpoints. As a consequence, crucial information is not considered in the early stages of the risk assessment process. This could lead to an erroneous risk assessment in the worst-case situations.

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