Growth Inhibition and Recovery of *Lemna aequinoctialis* after Pulse Exposure to Cd^{2+}, Cr^{6+}, Pb^{2+} and Cu^{2+}

Jingbo XU[1, a], Fengyi ZHANG [2, b]

[1] School of Environment Northeast Normal University, Changchun, 130117, China

[2] School of Environment Northeast Normal University, Changchun, 130117, China

[a]email: xujb515@nenu.edu.cn, [b]email: zhangfy 282@nenu.edu.cn

Keywords: pulse exposure; heavy metal ion; growth inhibition; recovery; effect assessment of pollutants

Abstract. The exposures of aquatic organisms to pollutants are usually pulses yet the exposure concentrations in the standard toxicity tests are constant. It raises a question that whether these standard tests include the effects of pulse exposure? Therefore, 24-hour pulse exposure to 4 heavy metal ions (Cd^{2+}、Cr^{6+}、Pb^{2+}、Cu^{2+}) was given to *Lemna aequinoctialis*, and its effects on the growth of *L.aequinoctialis* was observed in the following 6 days. In the meantime, constant exposure through the methods of standard OECD tests was given and observation was made. Comparisons between the two tests were drawn. In the pulse exposure test, growth was inhibited at first with the increase of concentrations of Cd^{2+}、Cr^{6+}、Pb^{2+}、Cu^{2+}, but the growth rate reached the level of control group within the next 6 days. E_yC_{50} of the pulse group is 1 to 2.5 times higher than that of the OECD group. This research provides basis for the comparison between the effects of two exposure regimes at an experimental level. It is suggested that results can be applied in the effect assessment of intermittently released pollutants.

Introduction

The exposures of aquatic organisms to pollutants are usually pulses. For instance, raining following spraying herbicides results in runoff, and suspension of mist spray. The duration of a pulse can vary between a few hours and up to 1–2 days，and concentration is not constant, which depends on pollutants and recipient characters [4, 8, 10,18]. In comparison, standard toxicity tests for the basis of ecotoxicological risk assessment are operated in constant-concentration conditions and effect values are estimated. These types of tests are useful in hazard identification and classifying the toxic levels. However, because of difference of pulse modalities, questions raise that whether these methods can be extended to real situations, and whether can be used to set EQS (Environmental Quality Standards) [6]? To ensure the validity of results of standard tests and guarantee that aquatic organisms won't be influenced by pulse exposure, data of pulse exposure test is very crucial.

Till now, the mainly target of contaminants' pulse exposure is aquatic invertebrates [1, 2, 5, 7, 9, 11-13, 16, 17, 20]. In spite, their effects to primary producers are critical as well, because they offer food and habitats to animals and protect ecosystem. Nevertheless, there are only a few researches about effects of pollutants' pulse exposure to macrophytes[3, 4] and algae[21].

After application of contaminants, rapid transformations and phase distributions occur, which lead to quick reduction of exposure concentration and therefore cause pulse exposure [10, 17]. This is contrary to standard tests in the lab. Exposure concentration in the lab is constant, which is impossible for some instances [17]. Due to hydrolysis, photolysis and some other chemical transport process such as adsorption and evaporation, reduction of concentration is able to occur during the holding period, which is a pulse exposure test in essence. If test solution is renewed continuously, it can be treated as repeated pulse exposure test. To remedy this, time weighted mean (TWM) is calculated and acts as the assumed constant concentration.

However, there are only a few researches about pulsed effects of macrophytes and algae exposed

to heavy metals. *L. aequinoctialis* was exposed to Cd^{2+}、Cr^{6+}、Pb^{2+}、Cu^{2+} for 24 h followed by 6-d holding (the whole period lasts for 7 d in total) in the present study. Meanwhile, 7-d constant exposure tests of Cd, Cr, Pb, Cu were completed, and then comparison between results of pulse exposure and that of constant exposure were investigated. Furthermore, the meaning of consequences was discussed for environmental effects assessment of heavy metal ions.

Materials and method

Lemna aequinoctialis has fronds floating on the water surface freely, and 2~8 are together to form groups (3-6 together is more common). Fronds are light green with no green handle but a white trace connecting with frond, with 1.5~6.5 mm length and 0.5~1.5 mm width. *L.aequinoctialis* usually grows in warm temperate to tropical regions in the near shore of lakes, ponds, paddy fields and ditches.

L.aequinoctialis used in the present study was collected from Children Park and Little South Lake in Changchun, Jilin Province and identified by experts. Plants were culturing for 4 weeks and then used for tests. The test organisms collected from the field were transferred rapidly to containers with 20-L volume, and then added 10 L water and a little base mud in the original pool and 5 L tap water after aeration, saved and used the organisms in natural illumination and temperature and renewed once per year. To make up the loss of evaporation, 5 L tap water after aeration was added per 10 days. Lab temperature for culture was 25±5℃. Natural light with white fluorescent light was used, with intensity of 6500~10000 lux and photoperiod with 10-h light: 14-h dark.

In 7-d standard growth inhibition tests, dependent variable is frond number. It was operated as OECD Guideline 221[14]. There were 6 concentration gradients for each metal ion (see table 1).

TABLE I. CONCENTRATION GRADIENTS OF CD^{2+}, CR^{6+}, PB^{2+} AND CU^{2+}(WITH 3 REPEATS)

Ions	Concentration (mg/L)					
Cd^{2+}	0.05	0.1	0.2	0.4	0.8	1.6
Cr^{6+}	0.05	0.1	0.2	0.4	0.8	1.6
Pb^{2+}	0.2	0.4	0.8	1.6	3.2	6.4
Cu^{2+}	0.025	0.05	0.1	0.2	0.4	0.8

In pulse tests, exponentially growing subcultures of *L.aequinoctialis* were exposed in 6 concentrations with 3 replicates at each test concentration (there were 2 fronds for each measurement). 3 replicates were operated for control. Exposure was operated in 150 ml vessels with 100 ml test solution under conditions identical to culture conditions. Count frond number after 24-h exposure and then transform organisms into flasks with clean medium for rinsing. Next transferred fronds into a new 150 ml crystallizing dish without heavy metal ions, which was covered by a plastic petri dish. Medium was renewed after 3 days to ensure enough nutrients. Frond number was counted every day for continuous 6 days.

At the start of exposed period in pulse test, 4 ml test solution of the highest concentrations of each chemical was sampled for chemical analysis. To reduce the analytical work, these samples were combined before the F-AAS (flame a (time weighted mean) was calculated [15]. The TWM (time weighted mean) was calculated [14, 15].

To compare the treatments with controls at the end of the test, two tailed t-test was used. The coefficient of variation (CV) of the frond number for each treatment group was calculated at the end of the test. Average day-to-day growth rates $\bar{\mu}_d$ in the post exposure period were calculated. The biomass yield was used to draw concentration-response curves by software IBM SPSS Statistics 22.0. To calculate at which concentration the growth was reduced with 50 percent (EC_{50}) data was fitted to a three parameter log-logistic concentration-response model [14, 19].

Results and discussions

Frond number at each test concentration as a function of time in the post-exposure period was shown in fig. 1. Plants were exposed continuously in the first day and then transferred into clean

medium in the post-exposure period. CV in the four tests were at the range of 3~20 percent for controls (n=6) and 3~36 percent for treatments (n=3).The controls grow exponentially at an average growth rate of 0.45 day^{-1}, which is higher than the validity criteria of 0.275 day^{-1} [14]. And t-test for the biomass yield was operated (see fig. 1). The groups with significant difference ($p < 0.05$) were shown as *. For each heavy metal ion, there were no significant difference among controls and treatments at the three lowest concentrations ($p > 0.05$). For Cd^{2+} at 0.78mg/L, significant difference was observed in the 6th and 7th day with control, which was also in the 3rd to 6th day for Pb^{2+} at 0.63mg/L, in the 2nd to 5th day for Cu^{2+} at 0.16mg/L and in the 6th and 7th day for Cu^{2+} at 0.39mg/L. In total, for the four species, the higher exposed concentration was, the greater toxicity effect was.

Growth rates every day were shown in fig. 2, and recovery time of plants was able to be observed. Results of t-test were shown in fig. 2. The groups with significant difference ($p < 0.05$) were shown as *.

Although some pulsed exposure concentrations were higher than E_rC_{50} in OECD Growth Inhibition Test (see table 2). Cd^{2+} and Cr^{6+} pulsed exposures at these concentrations didn't cause effects on growth of plants in post-exposure period yet. For Cu^{2+}, there was no inhibition at the lowest four exposed concentrations compared to controls in post-exposure period. For Pb^{2+}, growth rates were inhibited at all treatments except the lowest two concentrations, but recovery was rapid to reach to the level of control. Based on above observation, inhibited plants generally recovered relatively rapidly, which may due to relatively strong metabolic ability for the four metal ions in *L.aequinoctialis.* There was no inhibition induced at low concentration for these ions, which suggested that effect was caused only if the concentration reached to the threshold value level. For the four ions, growth rate decreased sensitively while concentration was a little higher than E_rC_{50} value in standard test.

TABLE II. E_YC_{50} WITH 95% CONFIDENCE INTERVAL (MG/L) (24-H PULSED EXPOSURE TEST AND OECD TEST) AND THE RATIO(E_YC_{50} IN PULSED EXPOSURE/ E_YC_{50} IN OECD TEST).

	E_yC_{50} in pulsed exposure	E_yC_{50}in OECD test	ratio	E_rC_{50} in OECD
Cd^{2+}	0.76 (0.55~0.87)	0.38 (0.32~0.43)	2. 00	0.45 (0.38~0.53)
Cr^{6+}	0.84 (0.60~1.08)	0.40 (0.35~0.47)	1. 68	0.50 (0.37~0.67)
Pb^{2+}	0.96 (0.96~0.11)	0.86 (0.81~0.93)	1.12	0.102(0.93~0.11)
Cu^{2+}	0.40 (0.20~0.60)	0.16(0.15~0.17)	2. 50	0.18(0.17~0.19)

Values of EC_{50} were based on increasing biomass yield (the increasing frond numbers during the whole test). The two tests both lasted for 7 d. E_rC_{50} with 95% confidence interval (mg/L) values in OECD test were shown in the last column

To compare the data of pulsed exposure tests and OECD tests, EC_{50} based on increasing biomass yield that was the increasing frond numbers during the whole test (E_yC_{50}) was calculated in each test (see table 2). Generally, EC_{50} based on growth rate of frond number (E_rC_{50}), namely E_rC_{50}, was used more. In this situation, the growth rate was constant during the test, and there were no connections with control in respects of absolute level of concentration, slope of concentration-effect curve and duration of the test [14]. However, the growth rate in the whole process in pulsed exposure was not constant, so method of increasing biomass yield was more suitable without above presumes. Just as prediction and description in OECD (2006), E_yC_{50} values in OECD tests were all smaller than E_rC_{50}. By comparing E_yC_{50} values of pulsed exposure and OECD test (see table 2), it was found that E_yC_{50} values in pulsed exposure was 1~2.5 folds larger than those in OECD tests, probably because exposed duration in pulsed exposure was shorter than that in OECD test. However, because of exposed duration and the difference of increasing growth rate in post exposure period, maybe the gap between E_yC_{50} in two tests was not only 1~2.5 folds.

On the basis of experiments to discover the difference between pulsed and continuous exposures, pulse tests provide a new thought when effects of chemicals to the environment were assessed. In WFD (Water Framework Directive) [6], which is a guideline document about obtaining EQS, the concept of intermittent release is that releasing duration is not more than 24 h and once per month at most annually on average. Otherwise, the upper limit is five times per month, and interval between two releases is 6 d at least [6]. In order to obtain the Water Quality Criterion of pulsed release, which is also known as MAC-EQS (Maximun Allowable Concentration EQS), at least three short duration tests to three trophic levels were operated, and AF (assessment factor) 100 was applied in

the lowest $L(E)C_{50}$ in these tests. AF value used to obtained AA-EQS (annual average EQS) and MAC-EQS in common methods is 10 folds higher than that of pulsed release, so "safe" concentration of short-period exposure is 10 folds higher than that of continuous exposure[6].

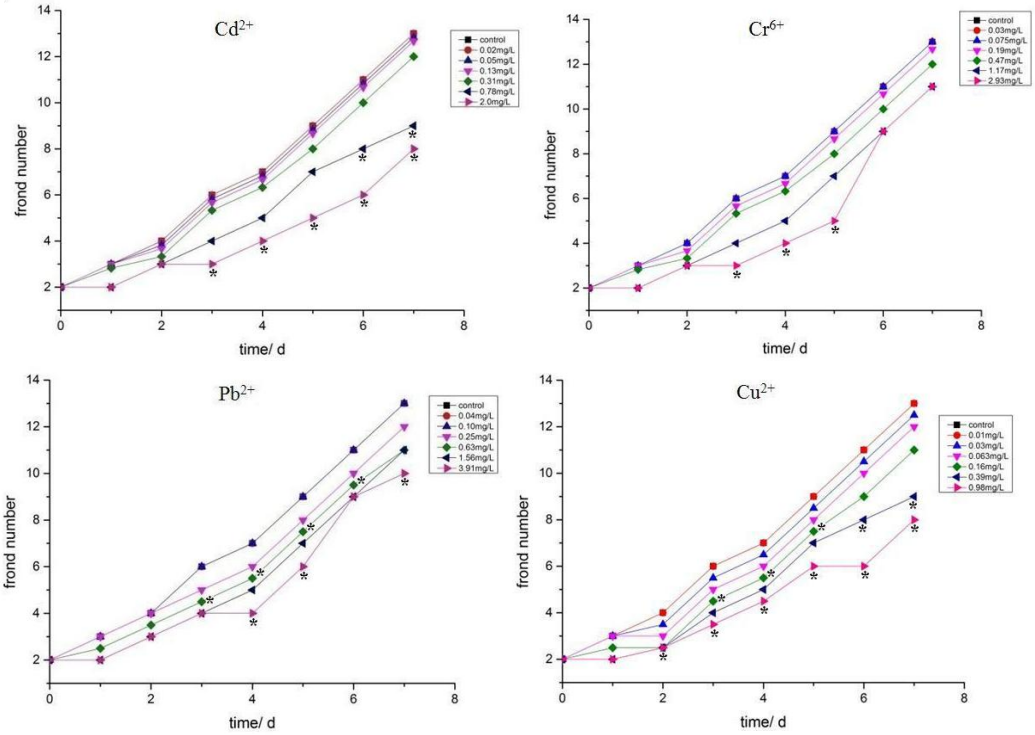

Fig.1.The growing curves of fronds number at each test concentration against time for the four metals. The parallel part of the curve meant the same growth rate.

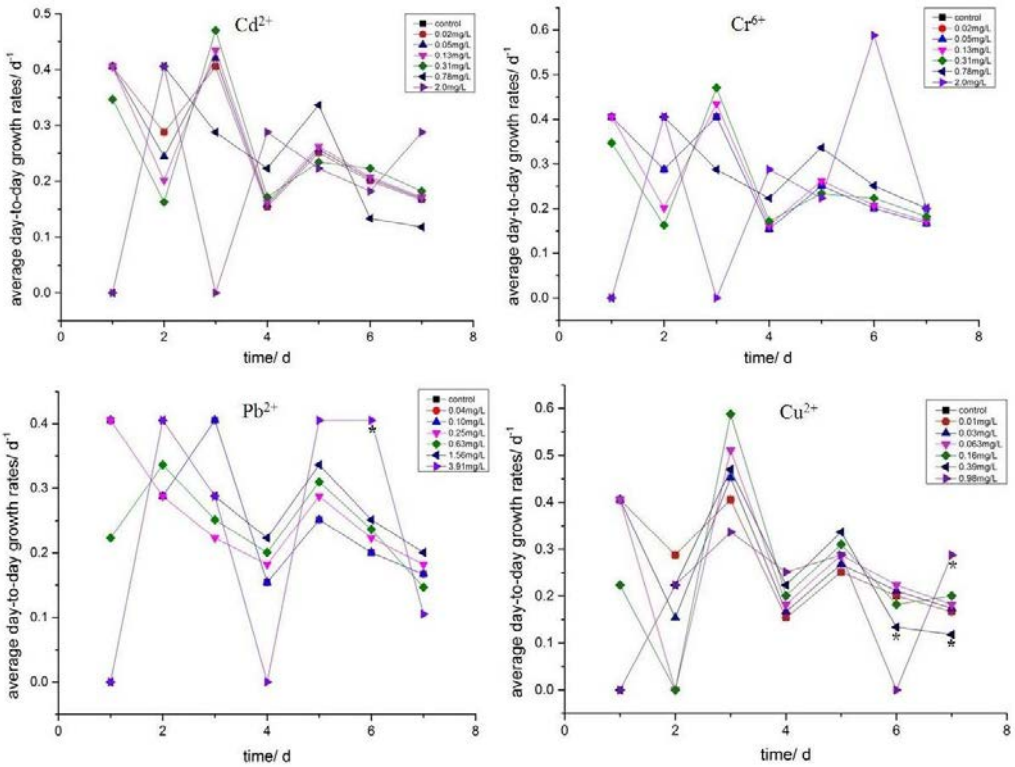

Fig.2.Average day-to-day growth rates $\bar{\mu}_d$ in the post exposure period for ions at each concentration.

In spite that *Lemna* test is not common algae test, similar test is still used as a part of algae test. Generally, AA-EQS is based on no observed effect concentration (NOEC) of long-time test, but under the condition without support of NOEC of other trophic levels, NOEC of algae test shouldn't be used [6], so E_yC_{50} value is the basis on AA-EQS. E_yC_{50} of pulsed test was only 1~2.5 folds to

that of 7-d standard test, but not 10 folds or more as previous thoughts. In this situation, therefore, decreasing AF value from 1000 to 100 decreases the protection to environment. Based on this, there is a doubt that if it is reasonable to decrease AF from 1000 to 100 for all compounds. In other words, if it will be better to decide experimentally AF value as in the present study? WFD didn't consider recovery of plants while setting EQS [10], but the present research suggested that recovery of *L.aequinoctialis* should be paid attention to. Fig. 1 and fig. 2 clearly show that plants still recovered in spite of decrease of biomass yield due to pulsed exposure. For all heavy metal ions and all concentration, growth rate of treatments nearly reached to the level of controls on 3rd day. E_yC_{50} mainly reflected the loss of biomass yield hence. On the other hand, we can't ignore the effect of pulsed exposure only because of rapid recovery of organisms after exposing. The gap of E_yC_{50} between continuous exposure and pulsed exposure was relatively small (not more than 10 times), and *L.aequinoctialis* as a kind of sensitive aquatic macrophyte can recover growth after exposure to heavy metal ions for once. By analyzing the two facts, it can be concluded that the method to obtain MAC-EQS of heavy metal ions is enough to protect ecosystem at present.

Conclusion

In this research, *L.aequinoctialis* was exposed to Cd^{2+}, Cr^{6+}, Pb^{2+} and Cu^{2+}, and standard test and 24-h pulsed test were operated respectively. Then their effects to increasing of biomass yield were compared. E_yC_{50} of 24-h pulsed exposure was 1~2.5 folds to that of standard 7-d continuous exposure test. In pulsed exposure test, concentration a little higher than E_rC_{50} of standard test caused decrease of growth rate, but in general, growth rates of these plants were able to recover to the level of control on 6th day. The present study offered experimental basis to compare the influences caused by the two exposed mechanisms. However, our research didn't consider the sensitivity of the organisms. Nevertheless, even if the result obtained from this method was not able to apply to assess the effects of intermittent release, present method to obtain MAC-EQS of these heavy metals was still enough to protect ecosystem.

Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (No. 31570507). The authors wish to thank Dr Zhang Leilei (Laboratory of School of Environment Northeast Normal University) for her test technical assistance.

References

[1] Andersen T H, Tjørnhøj R, Wollenberger L, et al. Acute and chronic effects of pulse exposure of *Daphnia magna* to dimethoate and pirimicarb[J]. Environmental toxicology and chemistry, 2006, 25(5): 1187-1195.

[2] Ashauer R, Boxall A B A, Brown C D. Modeling combined effects of pulsed exposure to carbaryl and chlorpyrifos on Gammaruspulex[J]. Environmental science & technology, 2007, 41(15): 5535-5541.

[3] Belgers J D M, Aalderink G H, Arts G H P, et al. Can time-weighted average concentrations be used to assess the risks of metsulfuron-methyl to Myriophyllumspicatum under different time–variable exposure regimes?[J]. Chemosphere, 2011, 85(6): 1017-1025.

[4] Cedergreen N, Streibig J C. The toxicity of herbicides to non-target aquatic plants and algae: assessment of predictive factors and hazard[J]. Pest management science, 2005, 61(12): 1152-1160.

[5] Cold A, Forbes V E. Consequences of a short pulse of pesticide exposure for survival and reproduction of Gammaruspulex[J]. Aquatic Toxicology, 2004, 67(3): 287-299.

[6] European Commission, 2011. Technical Guidance for Deriving Environmental Quality

Standards, Guidance Document No.27 (TGD-EQS-2011).European Commission, Directorate-General Health & Consumer Protection, Brussels, Belgium, p.1.

[7] Forbes V E, Cold A. Effects of the pyrethroidesfenvalerate on life‐cycle traits and population dynamics of Chironomusriparius—importance of exposure scenario[J]. Environmental toxicology and chemistry, 2005, 24(1): 78-86.

[8] Heckmann L H, Friberg N. Macroinvertebrate community response to pulse exposure with the insecticide lambda-cyhalothrin using in-stream mesocosms[J]. Environmental toxicology and chemistry, 2005, 24(3): 582-590.

[9] Handy R D. Intermittent exposure to aquatic pollutants: assessment, toxicity and sublethal responses in fish and invertebrates[J]. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 1994, 107(2): 171-184.

[10] Hommen U, Baveco J M, Galic N, et al. Potential application of ecological models in the European environmental risk assessment of chemicals I: review of protection goals in EU directives and regulations[J]. Integrated environmental assessment and management, 2010, 6(3): 325-337.

[11] Mugni H, Ronco A, Bonetto C. Insecticide toxicity to Hyalellacurvispina in runoff and stream water within a soybean farm (Buenos Aires, Argentina)[J]. Ecotoxicology and environmental safety, 2011, 74(3): 350-354.

[12] Naddy R B, Johnson K A, Klaine S J. Response of Daphnia magna to pulsed exposures of chlorpyrifos[J]. Environmental toxicology and chemistry, 2000, 19(2): 423-431.

[13] Naddy R B, Klaine S J. Effect of pulse frequency and interval on the toxicity of chlorpyrifos to Daphnia magna[J]. Chemosphere, 2001, 45(4): 497-506.

[14] OECD, 2006. OECD Guidelines for Testing of Chemicals.Lemna sp. Growth Inhibition Test. OECD Guideline 221.Organisation for Economic Cooperationand Development, Paris, France.

[15] OECD, 1998. OECD Guidelines for Testing of Chemicals.Daphnia magna Reproduction Test. OECD Guideline 211.Organisation for Economic Cooperation and Development, Paris, France.

[16] Parsons J T, Surgeoner G A. Acute toxicities of permethrin, fenitrothion, carbaryl and carbofuran to mosquito larvae during single‐or multiple‐pulse exposures[J]. Environmental toxicology and chemistry, 1991, 10(9): 1229-1233.

[17] Reinert K H, Giddings J M, Judd L. Effects analysis of time‐varying or repeated exposures in aquatic ecological risk assessment of agrochemicals[J]. Environmental Toxicology and Chemistry, 2002, 21(9): 1977-1992.

[18] Rosenkrantz R T, Cedergreen N, Baun A, et al. Influence of pH, light cycle, and temperature on ecotoxicity of four sulfonylurea herbicides towards Lemnagibba[J]. Ecotoxicology, 2013, 22(1): 33-41.

[19]Seefeldt S S, Jensen J E, Fuerst E P. Log-logistic analysis of herbicide dose-response relationships[J]. Weed technology, 1995: 218-227.

[20]Shuang L, Jingbo X, Lianxi S, The trans-generation effect during pulsed cadmium exposure:Tolerance and induction of hsp70, Ecotoxicology and environmental safety.107(2014)300-305

[21]Vallotton N, Eggen R I L, Chèvre N. Effect of sequential isoproturon pulse exposure on Scenedesmusvacuolatus[J]. Archives of environmental contamination and toxicology, 2009, 56(3): 442-449.