EnvironmentAsia
The international journal published by the Thai Society of Higher Education Institutes on Environment

Available online at www.tshe.org/EA
EnvironmentAsia 7(2) (2014) 99-107

Assessment of Genotoxicity through ISSR Marker in *Pistia stratiotes* Induced by Lead

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Abstract

Assessment of DNA changes in *Pistia stratiotes* induced by lead (Pb), plants were grown in acclimatized conditions for 15 days. Each of 5 plants were then transferred to cultivate in three repeated 0.2, 0.3, 0.5 mg/l Pb experiments, four days and controls. Lead concentration in water was analyzed by inductively-coupled plasma - mass spectrometry (ICP-MS), physical and chemical factors including temperature, pH, dissolved oxygen (DO) and electro-conductivity (EC) before and after the experimentation. Inter-simple sequence repeat fingerprints (ISSR) were analyzed for genetic similarity values (S), and genomic template stability test (GTS) was evaluated for revealing correlation of genetic structure changes and induced Pb concentration levels. The results appeared that the mean Pb concentrations in water were decreased 0.0121, 0.1688 and 0.2338 mg/l in the 3 concentration experiments, respectively. Assumedly, these Pb values loosed were adsorbed and absorbed in the plants. The physical and chemical factors such as pH, DO and EC were decreased and temperature was increased. The 10 successfully ISSR primers produced a total of 577 bands used for dendrogram construction. The plants with Pb affected 0.2, 0.3 and 0.5 mg/l were correlated by each concentration grouping and the S reveals 0.85-0.90, 0.69-0.90 and 0.93-0.96, respectively. The S value and plant's individual grouping agreement reveal its true effect by each Pb concentration. GTS values were (-2.38) - (-23.81) signifying to have most changes in DNA structure, genetic erosion occurring thoroughly on its genome, and affecting its life. These effects agreed to its morphological characters including leaves lacking chlorophyll (yellow), beginning to decay and finally dying. The research shows that *P. stratiotes* can be used as a bio-indicator for Pb wastewater monitoring.

Keywords: lead; aquatic plant; DNA fingerprinting; genotoxicity

1. Introduction

One of the major concerns of this century is the preservation of environmental quality as environmental contamination occurs violently. Aquatic ecosystems are directly or indirectly ending as destinations of these substances, and they often present high pollutant concentrations that may be deleterious for organisms therein. Among these polluting substances, heavy metals are easily transported and accumulated in the environment. They arrive in aquatic ecosystems as dissolved and solid waste from domestic, industrial, and agricultural runoffs. Many industries, such as automotive, metal-producing, electroplating, battery and electric cable manufacturing, mining, tanning, steel and textile, release heavy metals such as cadmium, copper, chromium, nickel, and lead in waste waters (Demirezen *et al.,* 2007). Finally, wastewater effluents have contributed to the increment of metal loads deposited in aquatic sediments and affecting aquatic plants.

Lead in the aquatic environment has both natural and anthropogenic origins (Shotyk *et al.,* 2003; Inoue and Tanimizu, 2008). In 1990, 5.627.106 tons of lead were consumed worldwide (WHO, 1995). Mining, smelting and refining, as well as the manufacturing of lead-containing compounds and goods, can give rise to lead emissions (WHO, 1989). Many traditional methods are used to remove this toxic metallic ion from industrial wastewaters before discharge into natural water bodies including coagulation and precipitation, ion-exchange, membrane separation and electrolytic technologies. Coagulation and precipitation processes lead to a high consumption of reagents and leave behind "hazardous sludge" which needs to be safely disposed of (Volesky, 2003). Alternative technologies are required to reduce toxic metal concentrations into environmentally acceptable levels at affordable costs (Volesky, 2003; Aksu *et al.,* 2002). Biosorption could be considered for its economic edge as a possible alternative technique for metals removal. Biosorption is based on the passive sequestration firstly by non-living biomass, containing many types of different chemically active groups that show some tendencies to uptake other chemical substances or ions, attracting them from solution and binding them to the biomass surfaces (Volesky and Holand, 1995; Volesky, 2003). Biosorption of lead has been investigated using different kinds of aquatic plants. Such contaminations result in high concentrations

of heavy metals in plants, sediment, and water, which clearly demonstrate pollution by these metals (Aksoy *et al.,* 2005).

Aquatic plants have long been used for evaluation and monitoring of metals in water (Cardwell *et al.,* 2002) and also can be used in phytoremediation to reduce organic matter or remove metallic pollutants from water (Mishra and Tripathi, 2008). These heavy metals may be toxic to aquatic ecosystems and human health, and they also accumulate in organisms. The accumulations of these heavy metals in plants cause physiological, biochemical and genetic changes. The use of these modifications as an endpoint for the evaluation of toxicity of these pollutants was largely discussed in various organisms (Prasad *et al.,* 2001; Perry *et al.,* 2002; Dhir *et al.,* 2004; Pavlikova *et al.,* 2008).

Pistia stratiotes L. is a widely distributed free-floating perennial hydrophyte which can also become toxic when applied in high level amounts, and has no testing as a bio-indicator, and phytoremediation efficiency. It is interesting that these mentioned treatments grow well and rapidly reproduce in natural habitats. Genotoxicity measurements caused by heavy metals in living things, including aquatic plants, are mainly related to the sensitivity and short response time (Gupta and Sarin, 2009). Immediately releasing these pollutants has the capability of causing morbidity and mortality in the exposed organisms, and, possibly motivating order changes such as alterations to population dynamics and changes to biological diversity (An *et al.,* 2012). The genotoxic effects depend on the oxidative state of the metal, its concentration and duration of its exposure. In general, effects are more pronounced at higher concentrations and at longer duration of exposures (Patra, 1999; Bandyopadhyay, 2000; Bhowmik, 2000).

Various molecular approaches, DNA fingerprints based on Inter Simple sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD) methods are generally used to effectively indicate genetic relationships. Banding patterns can be scored for genomic template stability (GTS) evaluation in order to detect various types of DNA damage and mutations shown by researches including the cells of bacteria, plants and animals (Savva, 1998; Atienzar *et al.,* 1999). Recently, Gupta and Sarin (2009) used RAPD bands for GTS evaluation in *Hydrilla verticillata* and *Ceratophyllum demersum* and treated it with Cd, Hg and Cu to show DNA damage. Zhou et al. (2011) also used RAPD bands for GTS evaluation indicating DNA damage in *Euplotes svannus* (Protozoa, Ciliophora) induced by nitrofurazone in marine ciliates. For the ISSR method, Ungherese *et al.* (2010) used it for studying relationship between heavy metals pollution and genetic diversity in the sandhopper, *Talitrus saltator* in Mediterranean populations.

In this work, the principal aims were to assess the impact of Pb contamination in *P. stratiotes* in terms of DNA damage by GTS evaluation values using the ISSR marker.

2. Materials and Methods

2.1. Plant acclimatization

Aquatic plant, *P. stratiotes* were collected from a natural pond, in northeastern, Thailand and transferred to the field laboratory for 15 days of acclimatization in filtered deionized water. There are no fertilizer supplemented as the experiment has to use plants without polluted condition. Also, they can survive from accumulated food and other organic matter decomposed from their plant parts.

2.2. Experimental set-up

Experimental conditions, add Pb levels 0.2, 0.3 and 0.5 mg/l in the filtered deionized water with 5 mature plants of expected equal reproductive size following Gupta and Chandra method (Gupta and Chandra, 1996). Each concentration was done with three replicates, growing in the field laboratory for 4 days. For the Pb preparation, the reagent was dissolved in 5 liters of deionized water to get the desired contamination level.

2.3. Physical and chemical factors and Pb analysis in water

Physical and chemical factors including temperature, pH, dissolved oxygen (DO) and electro-conductivity (EC) were measured before (the first day) and after (the fourth day) of experimentations. The water samples were done by pH meter (EcoScan pH5, Eutech), DO meter (YSI 550A) and EC meter (CH-8603, Mettler Toledo), respectively.

Pb analysis after experimentations, water samples of 40 ml were taken from all the experimental sets at 24 hrs interval for Pb analysis (Jarvis *et al.,* 1992). The concentration of Pb was determined in water by induction coupled plasma-mass spectrometry (ICP-MS; model 7500C). The wavelength analysis of ICP for Pb was 220.353 nm. The accuracy of the results for metal was evaluated with the certified reference material (CRM) with the 3111C method (APHA, 2005). Two aliquots of the CRM were spiked with a known amount of metal spike standard and one spike was analyzed according to 3111C method and other with the 3111B (APHA, 2005). The metal recoveries were in the 96–100 % which was acceptable (USEPA, 1994).

Table 1. Physical and chemical factors measured in water before and after added Pb (mean+SD)

Experimental conditions (Pb: mg/l)	Temperature (°C)		pH		DO (mg/l)		EC (µS/cm)	
	before	after	before	after	before	after	before	after
Control 1, without Pb, with plants	33.50+0.10	34.80+0.10	7.63+0.15	7.02+0.02	7.40+0.20	7.20+0.10	23.80+0.10	10.35+0.03
Control 2, with Pb 0.2, without plants	34.10+0.02	38.00+0.10	8.54+0.04	7.51+0.21	8.00+0.20	7.00+0.26	166.00+8.72	195.10+4.42
Control 3, with Pb 0.3, without plants	33.80+0.64	36.90+0.20	8.54+0.17	7.88+0.38	8.00+0.15	7.10+0.63	195.50+1.55	184.60+3.57
Control 4, with Pb 0.5, without plants	34.40+1.25	36.60+0.82	8.70+0.17	8.52+0.03	8.50+0.10	9.00+0.21	156.60+2.05	177.90+5.69
Experiment with Pb 0.2, with plants	33.10+1.50	37.00+0.74	7.53+0.53	7.50+0.39	7.60+0.44	7.50+0.46	32.30+4.93	18.60+2.25
Experiment with Pb 0.3, with plants	32.70+0.17	36.40+0.49	7.25+0.09	6.94+0.36	8.10+0.44	7.70+0.31	24.60+9.47	14.60+4.78
Experiment with Pb 0.5, with plants	32.70+0.06	37.20+0.65	6.93+0.06	6.77+0.04	7.30+0.06	7.00+0.17	36.10+7.58	27.80+5.80

2.4. DNA analysis

Three repeated experimentations on *P. stratiotes* 0, 0.2, 0.3 and 0.5 mg/l Pb, and controls were undergone through DNA extraction, ISSR banding patterns and analysis of DNA fingerprint profiles by GTS evaluation. DNA was extracted from the plant tissues using Genomic DNA Extraction Kit (RBC Bioscience, Taiwan) following manufacturer's instruction details. The extracted DNA was checked by 0.8 % agarose gel electrophoresis then diluted to a final concentration of 20 ng/µl.

DNA fingerprinting by ISSR marker was carried out on the plant samples each in 25 µl reactions consisting of GoTaq Green Master Mix (Promega), 0.5 µM primer and 5 ng DNA template. Thirty-eight ISSR primers were screened and the ten primers that successfully amplified clear bands are as follows (5′ to 3′): $(AG)_8G$, $(AG)_8C$, $(AG)_8T$, $(AG)_7AAC$, $(CA)_9A$, $(AG)_7AAT$, $(GTG)_3GC$, $(AC)_8CG$, $(CA)_9G$ and $(GT)_8C$. The reaction mixture was incubated at 94 °C for 3 min and the amplification was performed with the following thermal cycles: 35 cycles of denaturation for 1 minute at 94 °C, annealing for 2 minutes at 50 °C, extension for 2 minutes at 72 °C, followed by a final extension for 7 minutes at 72 °C using a thermal cycler (Swift Maxi Thermal Cycler, ESCO MICRO Pte. LtD). Amplification products were detected by 1.2 % agarose gel electrophoresis in TAE buffer and visualized using Ethidium bromide staining. The resulting ISSR bands from the successfully amplified primers and discerned from the agarose gel were documented as diallelic characters: present=1, absent=0; the ISSR are considered the dominant markers. These resulted bands were used to construct a dendrogram by the NTSYS - pc 2.1 program (Rohlf, 1998).

Each change observed in ISSR profiles such as disappearance or appearance of bands in comparison to controls were given the arbitrary score of +1 and the average was calculated for each metal using number of primers used. Primers that did not produce changes in ISSR profiles or which were too difficult to score were not used in calculation. Genomic template stability (GTS, %) was calculated using the equation: GTS = 100 - (100 x *a/n*) x 100 % where, *a* is ISSR changes detected in each sample treated and *n* is the number of total bands in the control (Atienzar *et al.* (1999).

3. Results and Discussion

3.1. Physical and chemical factors and Pb concentrations in water

Physical and chemical factors measured before and after 4 days of experimentations are shown in the Table 1. The overall values showed both decrease and increase. The pH, DO and EC values after adding Pb were decreased, but temperature was increased in all controls and in diverse Pb concentration experiments.

Pb analysis after experimentations added Pb concentrations following setting 0.2, 0.3 and 0.5 mg/l with plants treatment are shown in the Table 2, the mean Pb concentrations in water were decreased and remained in water as averages 0.0039+0.0005, 0.0060+0.0005 and 0.0059+0.0008 mg/l, respectively. The percentage values expected to adsorb/absorb by plants average 6.05, 56.26 and 46.76 %, respectively.

As we know that, aquatic plants can be used as efficient bio-indicator and removal of heavy metals quoted in the introduction. In the research, lead adsorption/absorption properties of *P. stratiotes* were investigated. The physical and chemical factors including pH, DO and EC were overall seemly decreased except for temperature which was increased and measured on the fourth day of experiments.

The pH values decreased because the various parts of the plant *P. stratiotes*, including stolons, roots and leaves have slowly rotted becoming organic matter with a majority of nitrogen, phosphorous and potassium and other minor compounds dissolved in water.

Temperature measured was likely higher with as much sunlight as there was at that time. However, it was still in the range of 32.50-38.00 °C which plants in the tropical area have photosynthesis ability. Growth and photosynthesis of submerged aquatic plants is affected by a number of environmental variables, such as water temperature, pH, CO2 supply, nutrient availability, and irradiance (Sand-Jensen, 1989; Bornette and Puijalon, 2010). Plants show an optimum of temperature for photosynthesis, and from them, the carbon assimilation decreases (Allen, 2001). Therefore, the temperature factor is unaffected on the *P. stratiotes* death.

DO values also decreased, lower than the first day of experiments because of *P. stratiotes*, its stolons, roots and leaves have slowly rotted, becoming organic matter with a majority of nitrogen, phosphorous and potassium and other minor compounds dissolved in water, as same pH measured. Microorganisms used these compounds for substrate and oxygen for catabolism. In case oxygen is enough for microorganism activity, the water is good, and the water becomes polluted when oxygen is not enough.

EC values were lower than the first day experiment because of some nutrient in the water that appeared in the same way as explained in the pH and DO values. Actually, in experiments with plants and Pb, EC values decrease as plants use organic matters in nutrient forms as mentioned in pH and DO values. In contrast, control 2 and 4, with Pb and without plants reducing EC values

Table 2. Pb concentrations (mean+SD) in water in each experimental condition*

Experimental conditions (Pb: mg/l)	Pb concentration (mg/l)	Pb remaining in water (mg/l)	Pb missing (mg/l)	Pb Plant adsorb/absorb (mg/l)	% Pb Plant adsorb/absorb
Control 1, without Pb, with plants	0	0	0		
Control 2, with Pb 0.2, without plants	0.2	0.0160±0.002	0.1840±0.002		
Control 3, with Pb 0.3, without plants	0.3	0.1748±0.0017	0.1252±0.0017		
Control 4, with Pb 0.5, without plants	0.5	0.2397±0.0111	0.2603±0.0017		
Experiment with Pb 0.2, with plants	0.2	0.0039±0.0005		0.0121±0.0005	6.05±0.11
Experiment with Pb 0.3, with plants	0.3	0.0060±0.0009		0.1688±0.0009	56.26±0.30
Experiment with Pb 0.5, with plants	0.5	0.0059±0.0008		0.2338±0.0008	46.76±0.16

*Remark: Plant adsorption/absorption Pb = Pb concentration - (Pb remaining in water + Pb missing from each concentration in control) since all experiments were done in the open field like controls assuming that there are microorganisms in them as controls.

following reasons mentioned, the EC values were assumed to be varied following organism activities. Microorganisms can use organic matter in the form of nutrients, otherwise they cannot use that organic matter, thus contaminating the water, as tested in the field. Therefore, the EC values of the control 2 and 4 increase from 166.00 to 195.10 and 156.60 to 177.90. However, those values can fluctuate. They can actually increase or decrease in either direction.

A disappearing Pb pathway in the research is summarized in that it binds well to a large number of molecules like amino acids, several enzymes, DNA and RNA of the plants; thus it disrupts many metabolic pathways (Patra *et al.,* 2004).

Pb is unlikely to affect aquatic plants at levels that might be found in the general environment. This experiment, chose the concentrations of lead 0.2, 0.3 and 0.5 mg/l because it is in the form of simple salts. Lead is acutely toxic to aquatic invertebrates at concentrations above 0.1 and more than 40 mg/l for freshwater organisms and above 2.5 and more than 500 mg/l for marine organisms (WHO, 1989).

Some Pb reduction was likely adsorbed and absorbed in *P. stratiotes*, at that time. Some Pb in controls 2, 3 and 4 were lost causing a decrease in values of pH, DO and EC factors. Assumedly, some microorganisms came to develop after a period of growing plants in the field experiments. These organisms should be identified in order to use them for Pb treatments in the future.

The experiments also assumed that there is some Pb lost in the same way as control, and then, Pb concentration that plants adsorb/absorb was evaluated by the starting concentration removed with Pb remaining in water, plus Pb missing by microorganisms from controls. In higher concentrations of Pb, the higher amount of Pb was lost by the *P. stratiotes* plants, however, the plant treatment efficiency by adsorption/absorption should be limited by a value.

3.2. Assessment of DNA changes in Pistia stratiotes

The 10 successfully ISSR primers mentioned generated clear and invaluable fingerprinting profiles as indicated by sample shown in Fig. 1. The total produced 577 bands were used for dendrogram construction shown by the Fig. 2. The dendrogram distinguished control out to be out group. Additionally, it gathered the same experiment and differentiated plant groups as experiments 1, 2, and 3 in a 0.2 mg/l concentration; experiments 4, 5 and 6 in a 0.3 mg/l concentration; and experiments 7, 8 and 9 in a 0.5 mg/l concentration. The pair wise genetic relationships in the term similarity (S) of all experiments are shown clearly in Table 3.
The studied aquatic plant and Pb affected 0.2, 0.3 and 0.5 mg/l shows correlation by the grouping S revealing 0.85-0.90, 0.69-0.90 and 0.93-0.96 in each group, respectively.

The GTS analysis from *P. stratiotes* in each experiment starting from (-2.38) - (-23.81) shown in

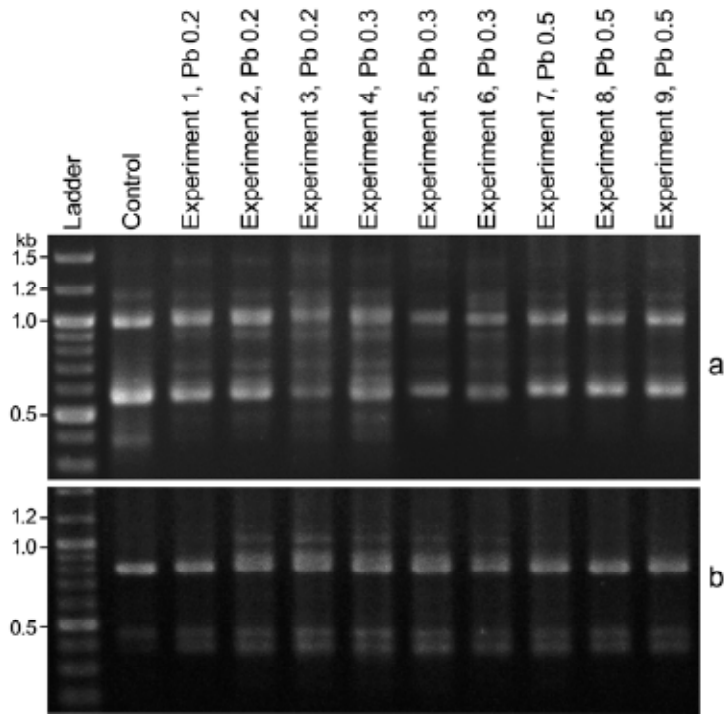

Figure 1. Examples of ISSR fingerprints of P. stratiotes in control and experimental conditions from primers (AG)8T (a) and (AG)7AAT (b)

Table 4 signifying to have most changes in DNA structure.

The ISSR marker is the suitable tool for study the dendrogram constructed from a total of 577 ISSR bands, separated the control to be out of the group which is agreed upon in very different conditions from the experiments, gathered a group of a concentration plants, and separated a group of a different concentration of plants as the 0.2, 0.3 and 0.5 mg/l affected Pb plants. Moreover, the S of 0.85-0.90, 0.69-0.90 and the 0.93-0.96 in each plant concentration group were correlated to the plant grouping revealing their true effect by each Pb concentration.

GTS values are also evaluated by efficient ISSR tool indication (-2.38) - (-23.81) signifying to have most changes in DNA structure. Genetic erosion occurred thoroughly, and its genome was affected its life. The GTS values were varied in each individual with the same condition due to their durability characteristics. So, the genome break was assumed unequal.

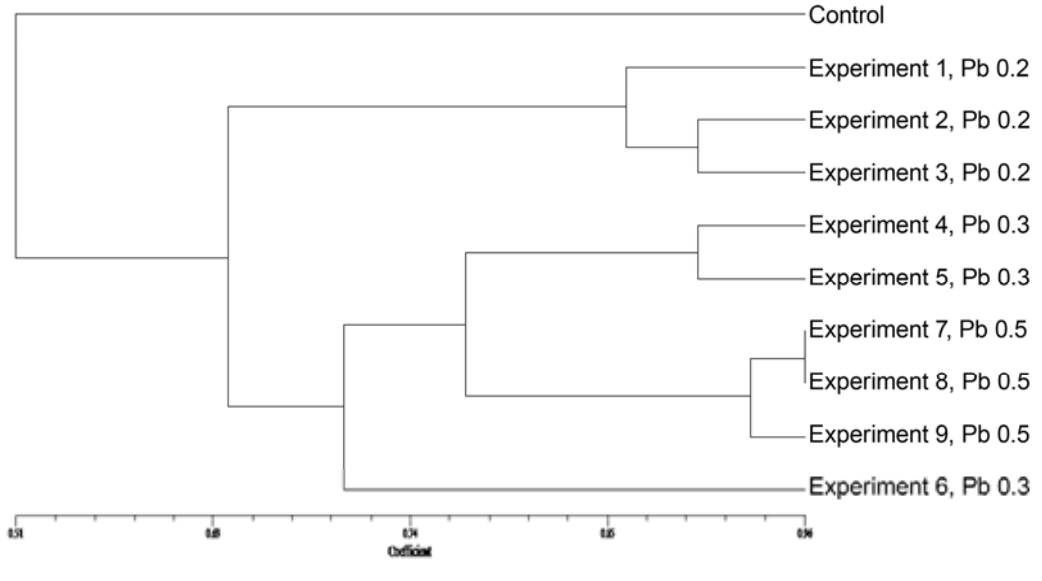

Figure 2. The dendrogram constructed from eight ISSR primers by the NTSYSpc 2.10p program showing genetic relationships between control and experimental conditions

Table 3. The Relations of all pair wise in experimental conditions indicated by similar genetic values

	Control	Experiment 1, Pb 0.2 mg/l	Experiment 2, Pb 0.2 mg/l	Experiment 3, Pb 0.2 mg/l	Experiment 4, Pb 0.3 mg/l	Experiment 5, Pb 0.3 mg/l	Experiment 6, Pb 0.3 mg/l	Experiment 7, Pb 0.5 mg/l	Experiment 8, Pb 0.5 mg/l	Experiment 9, Pb 0.5 mg/l
Control	1.00									
Experiment 1, Pb 0.2 mg/l	0.56	1.00								
Experiment 2, Pb 0.2 mg/l	0.51	0.87	1.00							
Experiment 3, Pb 0.2 mg/l	0.53	0.85	0.90	1.00						
Experiment 4, Pb 0.3 mg/l	0.49	0.62	0.65	0.75	1.00					
Experiment 5, Pb 0.3 mg/l	0.47	0.64	0.67	0.73	0.90	1.00				
Experiment 6, Pb 0.3 mg/l	0.57	0.61	0.60	0.62	0.69	0.75	1.00			
Experiment 7, Pb 0.5 mg/l	0.48	0.61	0.60	0.64	0.77	0.79	0.68	1.00		
Experiment 8, Pb 0.5 mg/l	0.51	0.61	0.58	0.62	0.79	0.79	0.66	0.96	1.00	
Experiment 9, Pb 0.5 mg/l	0.52	0.66	0.61	0.65	0.72	0.76	0.73	0.93	0.93	1.00

Table 4. Percentage of Genomic Template Stability (GTS) of P. stratiotes in each experimental condition

Experimental conditions (Pb: mg/l)	GTS
Experiment 1, Pb 0.2 (1)	-4.76
Experiment 2, Pb 0.2 (2)	-16.67
Experiment 3, Pb 0.2 (3)	-11.90
Experiment 4, Pb 0.3	-19.05
Experiment 5, Pb 0.3	-23.81
Experiment 6, Pb 0.3	-2.38
Experiment 7, Pb 0.5	-21.43
Experiment 8, Pb 0.5	-16.67
Experiment 9, Pb 0.5	-14.29

These effects agreed to its morphological characters including leaves lacking chlorophyll (yellow), beginning to decay and finally dying. The research shows that *P. stratiotes* can be used as a bio-indicator for Pb wastewater monitoring.

4. Conclusions

Aquatic plants have long been used as bio-indicators for assessment of genotoxicity and environmental pollution as shown by one of *P. stratiotes* induced by Pb. The physical and chemical factors including pH, DO and EC were decreased except for temperature which was increased after experimentation since of causes and factors occurred from the lives of the plants. With these 0.2, 0.3 and 0.5 mg/l experiments, the plants cannot endured to grow and finally dying. The dead of plants are accorded with most changes in DNA structure, genetic erosion occurring thoroughly on its genome, and affecting its life. Therefore, *P. stratiotes* is considered a good bio-indicator sensitive to Pb contamination and not appropriate for wastewater treatment.

Acknowledgements

This research was granted by Khon Kaen University under Incubation Researcher Project and Genetics and Environmental Toxicology Research Group.

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Received 27 March 2014
Accepted 6 May 2014

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