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

EnvironmentAsia 13(3) 2020 36-49
DOI 10.14456/ea.2020.40
ISSN 1906-1714; ONLINE ISSN: 2586-8861

Genetic Differentiation and Bioaccumulation Factor After Heavy Metal Exposure in Edible Aquatic Plants Near a Municipal Landfill

Patcharin Ruchuwararak[1,2], Somsak Intamat[1,3], and Lamyai Neeratanaphan[1,2]*

[1]*Research Group on Toxic Substances in Livestock and Aquatic Animals, Khon Kaen University, Thailand*
[2]*Department of Environmental Science, Faculty of Science, Khon Kaen University, Thailand*
[3]*Thatphanom Crown Prince Hospital, Nakornphanom, Thailand*

*Corresponding Author: hlamya@kku.ac.th
Received: March 13, 2020; Revised: April 24, 2020; Accepted: May 5, 2020

Abstract

This study investigated heavy metal contamination levels and the related genetic differentiation in *Limnocharis flava* and *Marsilea crenata* near a municipal landfill compared with those in a reference area using inductively coupled plasma optical emission spectrometry (ICP-OES) and inter-simple sequence repeats (ISSR) analysis. With the exception of Mn concentration in the water, the heavy metal concentrations (As, Cd, Pb, Cr, Mn and Zn) in the water and sediment of the landfill and reference areas were lower than the standards of Thailand. The heavy metal concentrations in the sediment from the landfill area were significantly higher than those from the reference area, except for Mn. In *L. flava* and *M. crenata* from both areas, As, Pb and Cr concentrations were higher than the food standards of FAO/WHO. In the landfill area, the highest bioaccumulation factor (BAF) values for As (3.92 ± 0.80), Cd (3.23 ± 0.77), Pb (1.51 ± 0.08) and Mn (1.47 ± 0.46) were found in *M. crenata*. The highest BAF values for Cr (0.80 ± 0.60) and Zn (6.97 ± 0.47) were found in *L. flava*. The genetic similarity values ranged from 0.56 - 0.99 in *L. flava* and from 0.71 - 0.98 in *M. crenata*. This result suggested that the heavy metal concentrations in the 2 aquatic plant species might be a factor in genetic differentiation. *M. crenata* might be better able than *L. flava* to endure the harsh conditions and adapt to survive in the municipal landfill area. *M. crenata* is suggested for use in phytoremediation of As, Cd, Pb and Mn.

Keywords: *Limnocharis flava*; *Marsilea crenata*; Heavy metal; ISSR marker; DNA change

1. Introduction

Human activities produced numerous amount of municipal solid waste (MSW). The variation, toxicity and quantity of MSW were enhanced by urbanization and economic expansion (Gidde *et al*., 2008). At least 33 percent from 2.10 billion tons of MSW in the world (as a tremendously conservative estimate) was not managed in the safe way for environment (Silpa *et al*., 2018). In Thailand, the quantity of MSW generated in 2018 was 27.8 million tons, which was a 16.4% increase from 2017, due to expanding urban communities and lifestyle changes, i.e., the shift from an agricultural to urban society (PCD, 2018). Khon Kaen province generated 710,298 tons of MSW in 2016 (TPCD, 2016).

Only 65.04% of the solid waste was properly disposed of (Khon Kaen municipality, 2016). An improper landfill was implemented for MSW disposal. This MSW generated landfill leachate, which is one of the main sources of heavy metals in the surrounding environments (Kanmani and Gandhimathi, 2013). The heavy metals found in the Khon Kaen municipal landfill include As, Pb, Cd, Cr, Mn and Zn (Promsid, 2014; Phoonaploy *et al*., 2016; Intamat *et al*., 2017; Sriuttha *et al*., 2017). The heavy metals contaminated in water and soil, and then accumulated in living organisms along the food chain, leading to biomagnification and health problems of human (Beaumont *et al*., 2008; Mohammad, 2012; Yang and Shu, 2015). Plants are used as bioindicators of heavy metal exposure (Stankovic *et al*., 2014). The advantages of using plants as bioindicators are as follows: they are eukaryotes, are endurable in environmental stresses, are easy to grow, are capable of processing complicated pollutant particles, and allow the assay of various environments (Kovalchuk and Kovalchuk, 2008). Aquatic plants are living organisms that absorb heavy metals through their roots, leading to a systemic accumulation of heavy metals. Four types of aquatic plants are classified as submerged, emergent, floating and marginal (Zuengsonthiporn and Jongrakthai, 2012). The differences in these plant growth forms and in the morphology of the plants, such as fibrous rooting systems and large surface areas that contact the heavy metal source, may determine the aquatic plants efficiency to absorb and accumulate heavy metals (Kulakow *et al*., 2000; Sarma, 2011). Many aquatic plants show their high potential to accumulate heavy metals and remove them from contaminated water (Basile *et al*., 2012). Because of the high tolerance and sensitivity of those aquatic plants to heavy metals, they have been used as bioindicators (Cardwell *et al*., 2002). The high accumulation of heavy metals might decrease plant growth, e.g. by inhibiting respiration and photosynthetic processes, and decrease chlorophyll synthesis (Dube *et al*., 2003; Peralta-Videa *et al*., 2009; Morsy *et al*., 2012). Furthermore, heavy metals might cause genetic differentiation both at the cellular level (cytotoxicity), such as chromosome abnormalities in the roots of *Elodea canadensis* (Zotina *et al*., 2015) and at the molecular level (genotoxicity), such as DNA damage in *Hydrilla verticillata*, *Ceratophyllum demersum*, *Colocasia esculenta* and *L. flava* (Gupta and Sarin, 2009; Boonmee *et al*., 2015; Neeratanaphan *et al*., 2016). Aquatic plants are suitable for use as genotoxicity indicators of environmental pollution. Heavy metals and chemical toxins induce genetic differentiation by increasing DNA polymorphism (Dhakshanamoorthy *et al*., 2011), resulting in DNA changes in aquatic plants. *L. flava* and *M. crenata* are local aquatic plants found in the Khon Kaen municipal landfill which local people consume these aquatic plants regularly. These aquatic plants are potentially suitable for studying genetic differentiation in the municipal landfill.

Simple sequence repeat (SSR), inter-smple sequence repeat (ISSR), randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers were developed and used to measure genetic differentiation (Hu *et al*., 2017). The use of ISSR is an acceptable and efficient approach for genetic variation assessment of plants because it is easy to apply, inexpensive, reliable, highly informative and repeatable (Reddy *et al*., 2002; Semagn *et al*., 2006; Wang *et al*., 2017). This approach has successfully detected DNA polymorphisms, which show alterations in DNA sequences and structures in plants contaminated with heavy metals (Mahfouz and Rayan, 2016). This study investigated heavy metal accumulation-related genetic differentiation of DNA using ISSR markers in *L. flava* and *M. crenata* from a municipal landfill compared with that from a reference area. The effects of heavy metals from a municipal landfill on two edible aquatic plants in this study can be used to help make guidelines and policies between local livelihoods and government agencies for sustainable management in aquatic ecosystems.

2. Materials and methods

2.1 Sampling sites

The study area was located in a reservoir that was 300 meters from the metropolitan municipal landfill and 17 km from the Khon Kaen municipality of Thailand at 16°35’41.30’ N latitude and 102°48’12.11’ E longitude (Figure 1). The geographic coordinate is UTM 48Q N 1,835,718; E 266,085, and the study area is located at a height of 200 meters up sea level. The MSW has been managed by sanitary landfills since 1988. The landfills have exceeded full capacity due to the objection of local people to using a new, proposed disposal area (Wirojanagud *et al*., 2004). Therefore, the very large amount of excess waste has caused many environmental problems, such as undesirable and unhygienic surroundings, including the occurrence of waste fires. The runoff from the heavy metals in the landfill leachate has contaminated 2 reservoirs to the north (Inmuang and Larbkam, 2011). The majority of the land near the municipal landfills was agricultural areas growing economic crops, such as sugarcane and rice. The reference area was a reservoir near an agricultural area in Khon Kaen province that was not affected by landfill leachate contamination.

2.2 Sample collection and preparation

Five replicates of water, sediment, *L. flava* and *M. crenata* samples from the municipal landfill and the reference area were collected in January 2017. The reference site was defined as the reservoir in the Khon Kaen province, where there was no leachate contamination. In the field, the water samples were immediately added to 3 drops of HNO_3 to fix the heavy metals (Kananke *et al*., 2015). The sediments were dehydrated in the open air,

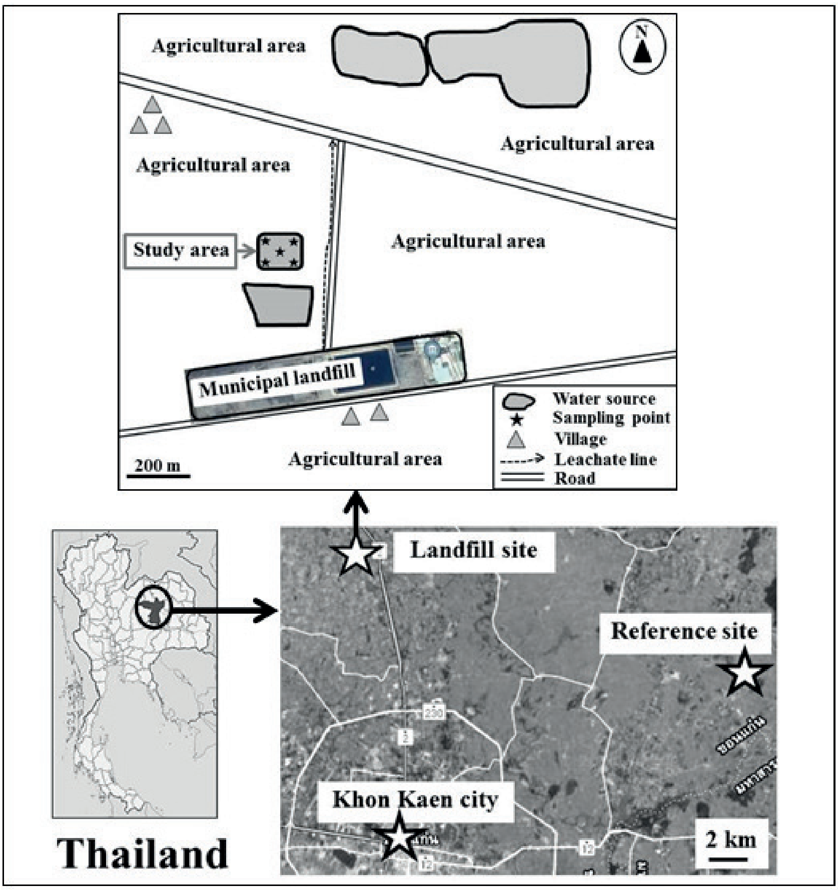

Figure 1. Location of the study area in a reservoir near the Khon Kaen municipal landfill.

ground with a mortar and sieved via a 2 mm screen (Odai *et al*., 2008). The aquatic plants were removed any materials by distilled water, dried in an oven for 24 hrs at 80°C, and ground with an agate mortar (Yang *et al*., 2013).

The aquatic plants in this study grow in wet conditions, such as paddy fields and shallow waters. *L. flava* is a perennial, erect, glabrous emergent aquatic weed, which grows 20-100 cm tall and roots in mud (Abhilash *et al*., 2009). *M. crenata* is a species of fern similar to a four-leaf clover, with stems up to 20 cm and leaves that float in deep water, grow erect in shallow water or spread on the ground (Zuengsonthiporn and Jongrakthai, 2011). The size of *M. crenata* is smaller than that of *L. flava.*

2.3 Heavy metal analysis

The water samples were digested with HNO_3 and boiled on a hot plate electronic for 30 min at 90°C. The dried sediments were digested with HNO_3, H_2O_2 and HCl, and boiled on a digestion block for 2 hrs at 180-220°C. The aquatic plants were digested with HNO_3 and H_2O_2 and boiled on a hot plate electronic for 2 hrs at 120°C. Each digested solution was diluted with deionized water and filtered with a filter paper (Odai *et al*., 2008; Yang *et al*., 2013). The 1 metalloid and 5 heavy metals (As, Cd, Pb, Cr, Mn and Zn) of the digested samples (water, sediments and aquatic plants) were detected by inductively coupled plasma optical emission spectrometry (ICP-OES). The ICP-OES detection limits and the wavelengths of As, Cd, Pb, Cr, Mn and Zn were followed on Thanomsangad *et al*. (2019).

The accuracy of heavy metals analysis was evaluated by the certified reference material (CRM) with the 311B methods (APHA, 2005). The recoveries of the heavy metals were 96-100%, which was acceptable (USEPA, 1994).

2.4 DNA extraction and ISSR analysis

DNA extraction: The shoots and buds from five replicates of fresh aquatic plants samples were preserved at -20°C. The aquatic plants were extracted by genomic DNA extraction kit (RBC Bioscience, Taiwan).

A tool for amplifying the DNA amount was polymerase chain reaction (PCR) via 40 ISSR primers. The base sequences of 40 ISSR primers are shown in Table 1. The total volume of the PCR mixture (10 μl) consisted of 3.8 μl of deionized water, 5 μl of GoTaq Green Master Mix, 0.2 μl of primers and 1 μl of extracted DNA. The ISSR reaction was performed using a thermal cycler (FlexCycler2). First, the PCR mixture was incubated at 94°C for 3 min, followed by 35 cycles of 30 s at 94°C for denaturation, 30 s at 40°C for annealing, and 2 min at 72°C for extension, and, finally, 1 cycle of 7 min at 72°C for final extension. The final DNA products were stored at -20°C (Tanee *et al*., 2016).

Detection of the DNA products: The electrophoresis of the DNA products was performed on 0.8% agarose gel for the extracted DNA and 1.2% agarose gel for the PCR product, and then visualized with RedSafe™ nucleic acid staining solution. The DNA bands were imaged under UV light (DUT-48 Blue light Transilluminator) (Mahfouz and Rayan, 2016).

2.5 Statistical analysis and data calculation

The heavy metal concentrations in the water, sediment and aquatic plant samples were calculated the mean values and standard deviations (S.D.) by Microsoft Office Excel 2010 program. The bioaccumulation factor (BAF), calculated as the heavy metal concentrations in the plant tissue divided by the heavy metal concentrations in the sediment, presented the efficiency of the aquatic plants for accumulating heavy metals from the sediment (Barron, 2003; Abdul and Thomas, 2009) The heavy metal concentrations in the water, sediment and 2 aquatic plant species of the landfill and reference areas were analyzed by the Mann-Whitney U-test at a 95% confidence level.

The genetic differentiation of the aquatic plants was evaluated by the genetic similarity (S) values. The S values were calculated as the new band appearances and normal band disappearances in the plants from the study area compared to those in reference plants that were not exposed to landfill leachate contamination. NTSYSpc 2.10 program was used to the S values and dendrogram construction from the data bands (Rohlf, 2000).

Table 1. The base sequences of 40 ISSR primers

Primers	Base sequence of primer	Primers	Base sequence of primer
1	CTCTCTCTCTCTCTCTTG	P5	CACACACACACAGT
A4	AGAGAGAGAGAGAGAA	P6	CACACACACACAAG
A5	AGAGAGAGAGAGAGAGC	P7	CACACACACACAGG
A6	AGAGAGAGAGAGAGAGT	P8	GAGAGAGAGAGAGG
A7	CACACACACACACACACC	P9	GTGTGTGTGTGTGG
A8	CACACACACACACACACAA	P10	GAGAGAGAGAGACC
A9	CACACACACACACACAT	P11	GTGTGTGTGTGTCC
A10	CACACACACACACACAG	P12	CACCACCACGC
A11	AGAGAGAGAGAGAGAAA	P13	GAGGAGGAGGC
A12	AGAGAGAGAGAGAGAAC	P 14	CTCCTCCTCGC
A13	AGAGAGAGAGAGAGAAG	P15	GTGGTGGTGGC
A14	AGAGAGAGAGAGAGAAT	P16	ACTGACTGACTGATCG
I1	CCTACCACACACACACACA	P17	GACAGACAGACAGACA
I2	AGAGAGAGAGAGAGCTGCT	P18	GTGTGTGTGTGTGTGTC
I3	CACACACACACACACACA	P19	ACACACACACACACACG
I4	AGAGAGAGAGAGAGAGAG	P20	ACACACACACACACACCG
P1	AGAGAGAGAGAGAGAGG	P21	CCCTCCCTCCCTCCCT
P2	CTCTCTCTCTCTCTCTAC	P22	CCCCGTGTGTGTGTGT
P3	CTCTCTCTCTCTCTCTGC	P23	AGAGAGAGAGAGAGAG
P4	CACACACACACAAC	P24	GAGAGAGAGA

Table 2. Heavy metal concentrations in the water and sediment samples from the landfill and reference areas.

Study area	Heavy metal concentrations					
	As	Cd	Pb	Cr	Mn	Zn
Water	(mg/L)					
Landfill	0.01 ± 0.00	0.01 ± 0.00[a]	ND	ND	3.92 ± 2.38	0.05 ± 0.03
Reference	0.01 ± 0.00	ND[b]	ND	ND	1.72 ± 0.36	0.05 ± 0.04
Standard*	0.01	0.05	0.05	0.05	1.00	1.00
Sediment	(mg/kg)					
Landfill	1.18 ± 0.52[a]	0.47 ± 0.19[a]	3.20 ± 0.65[a]	6.97 ± 1.28[a]	77.06 ± 29.92[a]	9.86 ± 2.53[a]
Reference	0.57 ± 0.09[b]	0.20 ± 0.02[b]	0.50 ± 0.10[b]	3.19 ± 0.40[b]	713.79 ± 17.11[b]	2.87 ± 0.14[b]
Standard**	3.90	37.00	400.00	300.00	1,800.00	-

Remarks: * Water quality standards of water sources, Pollution Control Department, Ministry of Natural Resources and Environment, Thailand (TPCD), 1994.
** Soil quality standard, TPCD, 2004.
ND = not detected
Different letters in the same column indicate significant differences.

3. Results and discussion

3.1 Heavy metal concentrations in the water and sediment

The heavy metal (As, Cd, Pb, Cr, Mn and Zn) concentrations in the water and sediment samples from the landfill and reference areas are shown in Table 2. With the exception of Mn, the heavy metal concentrations from both areas were not higher than the water standards of Thailand (TPCD, 1994). The heavy metals translocated to the floating and submerged plants since the study area is covered by plants. Rhizofiltration is the process to remove heavy metals from the water by plants absorb, precipitate and adsorb heavy metals with roots or submerged parts of aquatic plants (Rawat *et al*., 2012; Masarovičová and Kráľová, 2018). The study area was not directly exposed to leachate. The leachate flows into a small drainage stream that is far from the study area, at a distance of approximately 100 meters. The important cause of heavy metal contamination in the water around this study area is groundwater. In addition, heavy metal contamination in the sediment led to decreased heavy metal accumulation in the water.

The heavy metal concentrations in the sediment from the landfill and reference areas were lower than the soil standards of Thailand (TPCD, 2004). The heavy metals translocated to plants and rice crops, as the study area was located in a rice field with other plants. Phytoextraction is the process to reduce heavy metals from the soil by plants accumulate a high volume of heavy metals in their shoots (Masarovičová and Kráľová, 2018). With the exception of Mn, the heavy metal concentrations from the landfill area were higher significantly than those from the reference area. The main source of heavy metal concentrations in the landfill area is solid waste, which contained household hazardous waste such as lubricators, chemical containers, and light bulbs (Keeratitorn, 2004). Manure, which is used as fertilizer in the agricultural areas, was the major source of Mn in the reference area (Amanullah *et al*., 2010). Mn has the highest concentration in the water and sediment samples existing in organic and inorganic forms. Mn is used in the chemical manufacturing, in the textile and leather production, in the processing of glass and batteries production, and as a fertilizer (Millaleo *et al*., 2010). The high concentration of Mn could be explained by the waste segregation and collection systems. The solid waste was not separated, resulting in the disposal of hazardous waste and municipal waste. In addition, fertilizer is commonly used in this area.

3.2 Heavy metal concentrations in L. flava and M. crenata

Heavy metal (As, Cd, Pb, Cr, Mn and Zn) concentrations in the 2 aquatic plant species are shown in Table 3-4. Plants uptake high heavy metal concentrations from the water and sediment, and accordingly accumulate the heavy metals into their tissues (Jiwan and Ajay, 2011), leading to high heavy metal concentrations in the aquatic plants from the landfill area. *M. crenata* showed the highest concentrations of As (4.64 ± 0.95 mg/kg), Cd (1.51 ± 0.36 mg/kg), Pb (4.83 ± 0.25 mg/kg), and Mn (113.49 ± 35.33 mg/kg). The concentrations of Cr (5.57 ± 4.19 mg/kg) and Zn (68.81 ± 4.68 mg/kg) were highest in *L. flava.* The morphology of *M. crenata* includes many fibrous roots, branching roots from nodes and internodes, a large surface area that contact the source of the heavy metals, and foliar uptake ability (Kulakow *et al*., 2000; Zuengsonthiporn and Jongrakthai, 2011; Tripathi *et al*., 2012). These four characteristics may lead to *M. crenata* having the highest concentration of heavy metals. Only the rhizomes of *L. flava* are fibrous. In *M. crenata*, the accumulation of heavy metals occurs through the whole plant surface, which is in contact with the water; therefore, there is a higher concentration of heavy metals in *M. crenata* than in *L. flava,* which accumulates heavy metals only through its roots (Mishra *et al*., 2008). These results suggest that the plant growth forms (e.g. floating and emergent plants) are associated with the level of heavy metal concentrations. Moreover, low concentrations of heavy metals in water samples are due to high concentrations of heavy metals in aquatic plants. Plants internally distribute heavy metals in different ways; localize selected heavy metals mainly in stems and roots, store and accumulate heavy metals for later distribution in a nontoxic form (Memon *et al*., 2001). Plants were classified into three types according to metal accumulated organ and growing on soil contaminated with metals; metal exclusion, metal indication and metal accumulation (Baker and Walker, 1990).

The Cd and Pb concentrations in *L. flava* from the landfill area were significantly higher than the reference area. The As, Cd and Pb concentrations in *M. crenata* from the landfill area were significantly higher than the reference area. Most of the nonessential heavy metals (As, Cd and Pb) had higher concentrations in the 2 aquatic plant species from the landfill area than the reference area. Mn, which is an essential heavy metal, had a significantly lower concentration in the 2 aquatic plant species from the landfill area than the reference area. The concentrations of As, Pb and Cr were higher than the food standards of FAO/WHO and Thailand in the 2 aquatic plant species from the landfill and reference areas. The local people who consume aquatic plants are considered to get health risk. Heavy metals are toxic to health and have impacts such as cancer of the skin, lung and bladder (As); neurotoxicity (Pb); and cancer of several tissues and organs (Cr) (Peralta-Videa *et al*., 2009).

3.3 Bioaccumulation factor (BAF) of heavy metals in L. flava and M. crenata

The BAF is used for evaluating the potential of aquatic plants to accumulate heavy metals or be hyperaccumulator plants, i.e., to have BAF values > 1 (Sun *et al*., 2009). The BAF of heavy metals in *L. flava* and *M. crenata* from the landfill area are shown in Figure 2. The BAF value of Zn was higher than 1 in both species. The highest BAF values of As (3.92 ± 0.80), Cd (3.23 ± 0.77), Pb (1.5 ± 0.08), and Mn (1.47 ± 0.46) were found in *M. crenata*.

With the exception of Cr, all the BAF values of heavy metals in *M. crenata* were higher than 1. The previous study, the hyperaccumulation of heavy metals in *M. crenata* was observed (Das *et al*., 2013). The highest BAF values of Cr (0.80 ± 0.60) and Zn (6.97 ± 0.47) were found in *L. flava*. Previous studies of *L. flava* at this study area found BAF values greater than 1 for As, Cd, Pb and Cr, specifically, 131.30 ± 15.35, 7.19 ± 1.69, 26.52 ± 1.26 and 128.47 ± 18.47, respectively (Intamat *et al*., 2017; Sriuttha *et al*., 2017). High BAF values, or the designation as hyperaccumulator plants, demonstrate the capability of a plant to remove heavy metals from the sediment and to accumulate them in its tissues. The high accumulation of heavy metals in aquatic plants decrease their growth and cause DNA damage or genetic differentiation (Dhakshanamoorthy *et al*., 2011; Morsy *et al*., 2012).

Table 3. Heavy metal concentrations in *L. flava* from the landfill and reference areas.

Study area	Heavy metal concentrations (mg/kg)					
	As	Cd	Pb	Cr	Mn	Zn
Landfill						
Individual 1	1.03	0.03	1.65	3.05	69.75	67.52
Individual 2	0.85	0.10	1.48	3.19	69.10	65.54
Individual 3	2.16	0.07	3.26	12.98	53.75	77.05
Individual 4	0.23	0.02	1.59	3.99	76.20	67.48
Individual 5	0.98	0.08	1.96	4.66	77.65	66.47
Means	1.05	0.06^{a}	1.99^{a}	5.57	69.29^{a}	68.81
S.D.	0.70	0.03	0.73	4.19	9.48	4.68
Reference						
Individual 1	0.63	0.02	1.09	4.49	586.88	77.89
Individual 2	0.55	ND	0.96	2.98	593.07	48.01
Individual 3	0.95	0.03	1.00	5.16	652.19	53.54
Individual 4	0.38	0.01	1.01	3.99	583.16	51.02
Individual 5	ND	0.01	1.04	4.87	581.93	51.98
Means	0.50	0.02^{b}	1.02^{b}	4.30	599.44^{b}	56.49
S.D.	0.35	0.01	0.05	0.86	29.80	12.13
Standard	0.1*	0.2*	1**	1***	-	100*

Remarks: * Joint FAO/WHO food standards program, Codex Alimentarius Commission, 2011.
** Standard of foods containing contaminants, Notification of the Ministry of Public Health, Thailand, No. 98, 1986.
*** International standards in vegetables by the FAO of the United Nations, 2001.
ND=not detected
Different letters in the same column indicate significant differences.

Table 4. Heavy metal concentrations in *M. crenata* from the landfill and reference areas.

Study area	Heavy metal concentrations (mg/kg)					
	As	Cd	Pb	Cr	Mn	Zn
Landfill						
Individual 1	5.02	2.10	5.09	1.77	77.39	66.12
Individual 2	3.61	1.60	4.79	1.82	73.80	72.10
Individual 3	3.88	1.31	4.98	3.38	142.15	49.00
Individual 4	6.00	1.32	4.84	3.34	146.57	48.32
Individual 5	4.67	1.21	4.43	2.72	127.54	48.11
Means	4.64[a]	1.51[a]	4.83[a]	2.61[a]	113.49[a]	56.73
S.D.	0.95	0.36	0.25	0.79	35.33	11.50
Reference						
Individual 1	1.56	0.59	4.30	7.74	720.63	78.23
Individual 2	1.69	0.57	4.04	7.43	734.03	70.55
Individual 3	1.24	0.57	4.50	8.04	675.01	71.51
Individual 4	1.75	0.63	4.37	7.74	752.84	66.31
Individual 5	1.40	0.59	3.43	8.84	727.02	38.37
Means	1.53[b]	0.59[b]	4.13[b]	7.96[b]	721.90[b]	64.99
S.D.	0.21	0.02	0.43	0.54	28.86	15.49
Standard	0.1*	0.2*	1**	1***	-	100**

Remarks: * Joint FAO/WHO food standards program, Codex Alimentarius Commission, 2011.
** Standard of foods containing contaminants, Notification of the Ministry of Public Health, Thailand, No. 98, 1986.
*** International standards in vegetables by the FAO of the United Nations, 2001.
Different letters in the same column indicate significant differences.

3.4 DNA analysis of the ISSR profiles of L. flava and M. crenata

DNA analysis in the 2 aquatic plant species with 40 primers from the inter-simple sequence repeats (ISSR) found 22 primers of *L. flava* that could detect the 1,175 total DNA bands. The DNA bands, containing 172 characteristics, were divided into 89 monomorphic bands and 83 polymorphic bands. The 19 primers of *M. crenata* detected 1,213 total DNA bands. The DNA bands, containing 146 characteristics, were divided into 94 monomorphic bands and 52 polymorphic bands. The ISSR profiles from primers P4, P10 and P12 from the *L. flava* and *M. crenata* samples in the reference and landfill areas are shown in Figure 3.

The dendrogram construction from the total DNA bands of *L. flava* and *M. crenata* clearly separated the landfill samples from the reference samples, as shown in Figure 4. The dendrogram of *M. crenata* separated the sample results into 2 main groups. The first group comprised individuals 1.1 - 1.5 from the reference area; the second group comprised individuals 2.1 - 2.5 from the landfill area. The dendrogram of *L. flava* separated the sample results into 3 main groups. The first group comprised individuals 1.1 - 1.5 from the reference area, the second group comprised individuals 2.1, 2.2, 2.4 and 2.5 from the landfill area. Whereas the individual 2.3 was separated into the third group. The separation of individual 2.3 was related to high heavy metal concentrations, especially of Cr (12.98 mg/kg), Pb (3.26 mg/kg) and As (2.16 mg/kg). This is in accordance with previous studies, which have found that high Cr concentrations increase DNA changes in many plants (Rodriguez *et al*., 2011). Furthermore, the mixture of heavy metals has been found to be more toxic to living organisms than individual heavy metals (Salem *et al*., 2014).

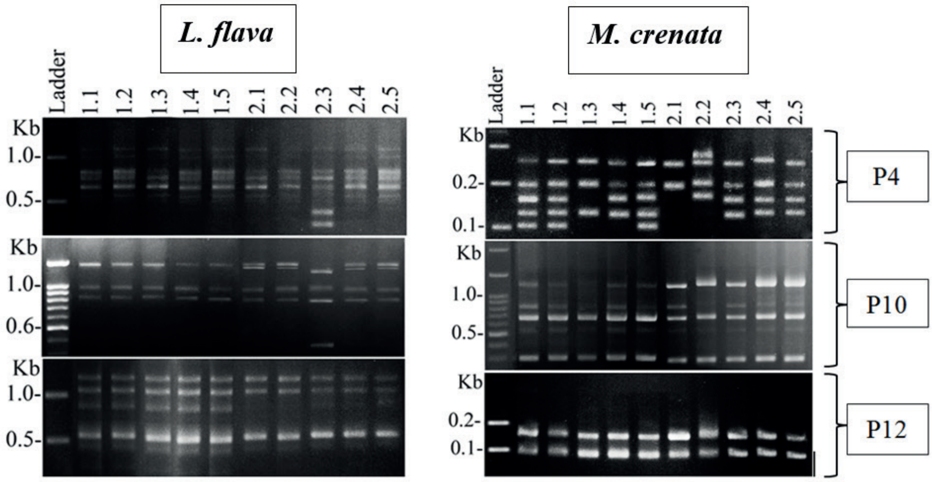

Figure 3. ISSR profiles from primers P4, P10 and P12 of the *L. flava* and *M. crenata* samples from the reference area (1.1 - 1.5) and landfill area (2.1 - 2.5).

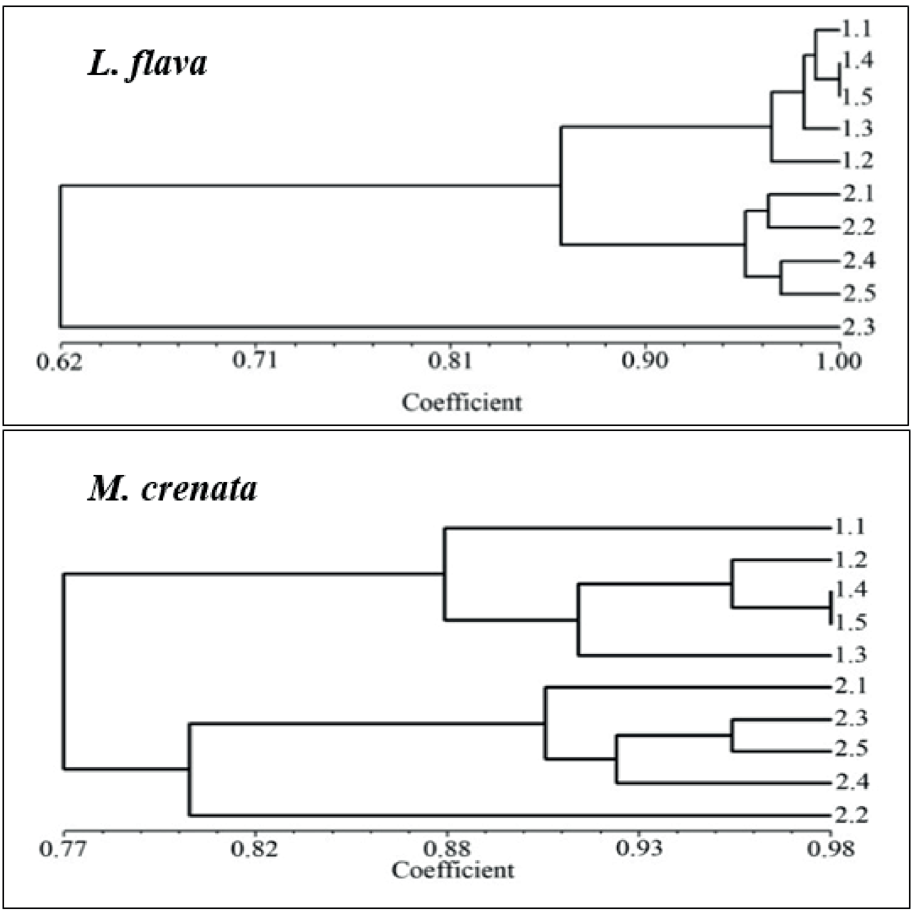

Figure 4. The dendrograms of *L. flava* and *M. crenata* samples from the reference area (1.1 - 1.5) and the landfill area (2.1 - 2.5).

The genetic relationships between the reference area and the landfill area are described in terms of genetic similarity. The values of genetic similarity within species should be from 0.85 - 1.00 (Neeratanaphan *et al*., 2016). In this study, the range of genetic similarity values in *L. flava* and *M. crenata* were 0.56 - 0.99 and 0.71 - 0.98, respectively, demonstrating their genetic differentiation. The genetic similarity between the groups of the 2 aquatic plant species showed that the genetic similarity in the reference area was higher than that in the landfill area. In aquatic plants, heavy metals are a cause of genotoxic effects, such as DNA damage, DNA strand breakage and cause a reduction in the DNA repair mechanism (Whiteside *et al*., 2010; Ventura *et al*., 2013; Tanee *et al*., 2016). The level of DNA damage has been shown to depend on heavy metal concentrations (Rodriguez *et al*., 2011). The high concentrations of heavy metals led to high genetic differentiation or low genetic similarity. The genetic similarity between groups of *L. flava* was low. Individual 2.3 was separated from the other groups. The heavy metal concentrations varied in individual 2.3. The highest Cr, Pb and As concentrations were found in individual 2.3. This result suggested that high heavy metal concentrations affected the DNA material in *L. flava*. Cr, Pb and As induce many stress responses and damage to components in cellular level of different plant, such as DNA, proteins and membranes (Finnagan and Chen, 2012). In *M. crenata,* the genetic differentiation was lower than that in *L. flava*. However, the heavy metal concentrations (As, Cd and Pb) were higher than in *L. flava*. These results show the high ability of *M. crenata* to endure and adapt to the conditions of the municipal landfill, which was contaminated with high heavy metal concentrations, by repairing DNA damage (Manova and Gruszka, 2015). The complicated results for the 2 aquatic plant species suggests that the genetic similarity values and the level of DNA damage might relate not only to the heavy metal concentrations but also to many factors, such as the type of heavy metal, reactions among different heavy metals and toxic substances (chemicals or pesticides), and plant species (Beyersmann and Hartwig, 2008; Oves *et al*., 2016). This study was conducted in the field, and some factors could not be controlled. With the results of this study, it might be summarized that genetic differentiation had occurred in the 2 aquatic plant species living in the municipal landfill. The heavy metal contamination in this area might be the main cause of genetic differentiation. The two species of aquatic plants assessed in this study, but especially *M. crenata*, can endure and adapt to the environmental conditions of the municipal landfill. *M. crenata* showed a higher ability than *L. flava* to endure and adapt to the environmental conditions and survive in the toxic area.

4. Conclusion

Contamination of heavy metals in the environment such as water and sediment including aquatic plants around the municipal landfill area were found in this study. The heavy metal concentrations in the water and sediment were not higher than the standards of Thailand, except for the Mn concentration in the water. The As, Pb and Cr concentrations in the aquatic plants were higher than the food standards of FAO/WHO and Thailand. The aquatic plants may be a source of human health hazards for the people who live around the municipal landfill. The highest concentration and BAF values of the nonessential heavy metals (As, Cd and Pb) were found in *M. crenata*. The highest concentrations and BAF values of the essential heavy metals (Cr and Zn) were found in *L. flava*. The heavy metal contamination from the municipal landfill induced genotoxicity in the aquatic plants. The genetic similarity in the aquatic plant samples was low. The genetic similarity in the reference area was higher than that in the landfill area in the samples from the 2 aquatic plant species. *M. crenata* showed a higher ability to endure and adapt to the environmental conditions and survive in the toxic area. *M. crenata* is suggested for use in phytoremediation.

References

Abdul HBO, Thomas BV. Translocation and bioaccumulation of trace metals in desert plants of Kuwait Governorates. Research Journal of Environmental Sciences 2009; 3: 581-587.

Abhilash PC, Pandey VC, Srivastava P, Rakesh PS, Chandran S, Singh N, Thomas AP. Phytofiltration of cadmium from water by *Limnocharis flava (L.)* Buchenau grown in free-floating culture system. Journal of Hazardous Materials 2009; 170: 791-797.

Amanullah MM, Sekar S, Muthukrishnan P. Prospects and potential of poultry manure. Asian Journal of Plant Sciences 2010; 9(4): 172-182.

American Public Health Association (APHA). Standard methods for the examination of water and wastewater. American Public Health Association, Washington DC, USA. 2005.

Baker AJM, Walker PL. Ecophysiology of metal uptake by tolerant plants. In: Shaw AJ, editor. Heavy metal tolerance in plants: Evolutionary aspects. CRC Press, Boca Raton. 1990.

Barron MG. Bioaccumulation and bioconcentration in aquatic organisms. In: David J, Hoffman BA, Rattner G, Allen B and John C, editors. Handbook of ecotoxicology. Lewis Publishers, London. 2003.

Basile A, Sorbo S, Conte B, Cobianchi RC, Trinchella F, Capasso C, Carginale V. Toxicity, accumulation, and removal of heavy metals by three aquatic macrophytes. International Journal of Phytoremediation 2012; 14: 374-387.

Beaumont JJ, Sedman RM, Reynolds SD, Sherman CD, Li LH, Howd RA, Sandy MS, Zeise L, Alexeeff GV. Cancer mortality in a Chinese population exposed to hexavalent chromium in drinking water. Epidemiology 2008; 19(1): 12-23.

Beyersmann D, Hartwig A. Carcinogenic metal compounds: Recent insight into molecular and cellular mechanisms. Archives of Toxicology 2008; 82: 493-512.

Boonmee S, Neeratanaphan L, Tanee T, Khamon P. The genetic differentiation of *Colocasia esculenta* growing in gold mining areas with arsenic contamination. Environmental Monitoring and Assessment 2015; 187(227): 1-8.

Cardwell A, Hawker D, Greenway M. Metal accumulation in aquatic macrophytes from Southeast Queensland, Australia. Chemosphere 2002; 48: 653-663.

Das K, Mandal C, Ghosh N, Dey N, Adak MK. Cadmium accumulation in *Marsilea minuta* Linn. and its antioxidative responses. American Journal of Plant Sciences 2013; 4: 365-371.

Dhakshanamoorthy D, Selvaraj R, Chidambaram ALA. Induced mutagenesis in *Jatropha curcas* L. using gamma rays and detection of DNA polymorphism through RAPD marker. Comptes Rendus Biologies 2011; 334: 24-30.

Dube BK, Tewari K, Chatterjee J, Chatterjee C. Excess chromium alters uptake and translocation of certain nutrients in Citrullus. Chemosphere 2003; 53: 1147-1153.

Finnagan PM, Chen W. Arsenic toxicity: The effect on plants metabolism. Frontiers in Plant Science 2012; 3(182): 1-18.

Gidde MR, Todkar VV, Kokate KK. Municipal solid waste management in emerging mega cities: A case study of Pune city. Indo Italian Conference on Green and Clean Environment, MAEER's MIT College of Engineering, Pune, India. 2008.

Gupta M, Sarin BN. Heavy metal induced DNA changes in aquatic macrophytes: Random amplified polymorphic DNA analysis and identification of sequence characterized amplified region marker. Journal of Environmental Sciences 2009; 21: 686-690.

Hu S, Li G, Yang J, Hou H. Aquatic plant genomics: Advances, applications, and prospects. International Journal of Genomics 2017; 2017: 1-9.

Inmuang U, Larbkam J. Water quality and perception of the population about water pollution problems at communities near the landfill area of the Khon Kaen municipality, Khon Kaen province. KKU Journal for Public Health Research 2011; 4: 51-62.

Intamat S, Buasriyot P, Sriuttha M, Tengjaroenkul B, Neeratanaphan L. Bioaccumulation of arsenic in aquatic plants and animals near a municipal landfill. International Journal of Environmental Studies 2017; 74: 303-314.

Jiwan S, Ajay SK. Effects of heavy metals on soil, plants, human health and aquatic life. International Journal of Research in Chemistry and Environment 2011; 1: 15-21.

Kanmani S, Gandhimathi R. Assessment of heavy metal contamination in soil due to leachate migration from an open dumping site. Applied Water Science 2013; 3: 193-205.

Kananke TC, Wansapala J, Gunaratne A. Pb and Cr contaminations of irrigation water, soil, and green leafy vegetables collected from difference areas of Colombo district, Srilanka. Pakistan Journal of Nutrition 2015; 14(9): 593-602.

Keeratitorn S. Analysis of hazardous waste stream in Khon Kaen metropolitan municipality. Master Thesis in Environmental Engineering, Graduate School, Khon Kaen University. 2004.

Khon Kaen Municipality. Strategic development plan 2017-2020. Khon Kaen Municipality, Khon Kaen, Thailand. 2016.

Kovalchuk I, Kovalchuk O. Transgenic plants as sensors of environmental pollution genotoxicity. Sensors 2008; 8: 1539-1558.

Kulakow PA, Schwab AP, Banks MK. Screening plant species for growth on weathered, petroleum hydrocarbon-contaminated sediments. International Journal of Phytoremediation 2000; 2: 297-317.

Mahfouz H, Rayan WA. Assessment of lead stress using genome template stability in *Hordeum vulgare*. Egyptian Journal of Genetics and Cytology 2016; 45: 297-321.

Manova V, Gruszka D. DNA damage and repair in plants from models to crops. Frontiers in Plant Science 2015; 6: 1-26.

Masarovičová E, Kráľová K. Woody species in phytoremediation applications for contaminated soils. In: Ansari AA, Gill SS, Gill R, Lanza GR and Newman L, editors. Phytoremediation: Management of environmental contaminants, volume 6. Springer Nature Switzerland AG, Gewerbestrasse, Cham. 2018.

Memon AR, Aktoprakligül D, Zdemür A, Vertii A. Heavy metal accumulation and detoxification mechanisms in plants. Turkish Journal of Botany 2001; 25: 111-121.

Millaleo R, Reyes-Díaz M, Ivanov AG, Mora ML, Alberdi M. Manganese as essential and toxic element for plants: Transport, accumulation and resistance mechanisms. Journal of Soil Science and Plant Nutrition 2010; 10(4): 476-494.

Mishra S, Srivastava S, Tripathi RD, Dwivedi S, Trivedi PK. Thiol metabolism and antioxidant systems complement each other during arsenate detoxification in *Ceratophyllum demersum* L. Aquatic Toxicology 2008; 86: 205-215.

Mohammad PI. Lead pollution: A risk factor for cardiovascular disease in Asian developing countries. Pakistan Journal of Pharmaceutical Sciences 2012; 25(1): 289-294.

Morsy AA, Salama KHA, Kamel HA, Mansour MMF. Effect of heavy metals on plasma membrane lipids and antioxidant enzymes of *Zygophyllum* species. EurAsian Journal of BioSciences 2012; 6: 1-10.

Neeratanaphan L, Boonmee S, Srisamoot N, Tanomtong A, Tengjaroenkul B. Analysis of genetic similarity of *Limnocharis flava* individuals growing around a gold mining area with arsenic contamination. Applied Ecology and Environmental Research 2016; 14: 105-114.

Odai SN, Mensah E, Sipitey D, Ryo S, Awuah E. Heavy metals by vegetables cultivated on urban waste dumpsites: Case study of Kumasi, Ghama. Research Journal of Environmental Toxicology 2008; 2(2): 92-99.

Oves M, Saghir KM, Huda QA, Nadeen FM, Almeelbi T. Heavy metals: Biological importance and detoxification strategies. Journal of Bioremediation and Biodegradation 2016; 7(334): 1-15.

Peralta-Videa JR, Lopez ML, Narayan M, Saupe G, Gardea-Torresdey J. The biochemistry of environmental heavy metal uptake by plants: Implications for the food chain. International Journal of Biochemistry and Cell Biology 2009; 41: 1665-1677.

Phoonaploy U, Intamat S, Tengjaroenkul B, Sriuttha M, Tanomtong A, Neeratanaphan L. Evaluation of abnormal chromosomes in rice field frogs (*Fejervarya limnocharis*) from reservoirs affected by leachate with cadmium, chromium and lead contamination. EnvironmentAsia 2016; 9(2): 26-38.

Pollution Control Department (PCD). Thailand state of pollution report 2018. Ministry of Natural Resources, Bangkok. 2018.

Promsid P. Chromosomal aberration assessment of fish in reservoir affected by leachate in municipal landfill. Master of Science Thesis, Department of Environmental Science, Faculty of Science, Khon Kaen University. 2014.

Rawat K, Fulekar MH, Pathak B. Rhizofiltration a green technology for remediation of heavy metals. International Journal of Innovation in Bio-Sciences 2012; 2(4): 193-199.

Reddy MP, Sarla N, Siddiq EA. Inter-simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 2002; 128: 9-17.

Rodriguez E, Azevedo R, Fernandes P, Santos C. Cr (VI) induces DNA damage, cell cycle arrest and polyploidization: A flow cytometric and comet assay study in *Pisum sativum*. Chemical Research in Toxicology 2011; 24(7): 1040-1047.

Rohlf FJ. NTSYS-pc: Numerical taxonomy and multivariate analysis system, version 2.1. Exeter Software Setauket, New York. 2000.

Salem ZB, Capelli N, Grisey E, Baurand PE, Ayadi H, Aleya L. First evidence of fish genotoxicity induced by heavy metals from landfill leachates: The advantage of using the RAPD-PCR technique. Ecotoxicology and Environmental Safety 2014; 101: 90-96.

Sarma H. Metal hyperaccumulation in plants: A review focusing on phytoremediation technology. Journal of Environmental Science and Technology 2011; 4(2): 118-138.

Semagn K, Bjørnstad A, Ndjiondjop MN. An overview of molecular marker methods for plants. African Journal of Biotechnology 2006; 5: 2540-2568.

Silpa K, Lisa YC, Perinaz B, Frank VW. What a waste 2.0: A global snapshot of solid waste management to 2050, urban development. World Bank, Washington DC. 2018.

Sriuttha M, Tengjaroenkul B, Intamat S, Phoonaploy U, Thanomsangad P, Neeratanaphan L. Cadmium, chromium, and lead accumulation in aquatic plants and animals near a municipal landfill. Human and Ecological Risk Assessment: An International Journal 2017; 23: 350-363.

Stankovic S, Kalaba P, Stankovic AR. Biota as toxic metal indicators. Environmental Chemistry Letters 2014; 12: 63-84.

Sun Y, Zhou Q, Wang L, Liu W. Cadmium tolerance and accumulation characteristics of *Biden spilosa* L. as a potential Cd-hyperaccumulator. Journal of Hazardous Materials 2009; 161: 808-814.

Tanee T, Sudmoon R, Thamsenanupap P, Chaveerach A. Effect of cadmium on DNA changes in Ipomoea *aquatica* Forssk. Polish Journal of Environmental Studies 2016; 25: 311-315.

Thailand Pollution Control Department (TPCD). Surface water quality standards. Notification of the National Environmental Board, No. 8, Ministry of Natural Resources and Environment, Bangkok. 1994.

Thailand Pollution Control Department (TPCD). Soil quality standards for habitat and agriculture. Notification of the National Environmental Board, No. 25, Ministry of Natural Resources and Environment, Bangkok. 2004.

Thailand Pollution Control Department (TPCD). Thailand state of pollution report 2016. Ministry of Natural Resources and Environment, Bangkok. 2016.

Thanomsangad P, Tengjaroenkul B, Sriuttha M, Neeratanaphan L. Heavy metal accumulation in frogs surrounding an e-waste dump site and human health risk assessment. Human and Ecological Risk Assessment: An International Journal 2019; 2019: 1-16.

Tripathi P, Dwivedi S, Mishra A, Kumar A, Dave R, Srivastava S, Shukla MK, Srivastava PK, Chakrabarty D, Trivedi PK, Tripathi RD. Arsenic accumulation in native plants of West Bengal, India: Prospects for phytoremediation but concerns with the use of medicinal plants. Environmental Monitoring and Assessment 2012; 184: 2617-2631.

US Environmental Protection Agency (USEPA). Sample preparation procedure for spectrochemical determination of total recoverable element. Method 2002. Environmental Monitoring System Lab, Cincinnati, OH. 1994.

Ventura L, Giovannini A, Savio M, Donà M, Macovei A, Buttafava A, Carbonera D, Balestrazzi A. Single cell gel electrophoresis (comet) assay with plants: Research on DNA repair and ecogenotoxicity testing. Chemosphere 2013; 92(2013): 1-9.

Wang P, Zhang Y, Zhao L, Mo B, Luo T. Effect of gamma rays on *Sophora davidii* and detection of DNA polymorphism through ISSR marker. BioMed Research International 2017; 2017: 1-6.

Whiteside JR, Box CL, McMillan TJ, Allison SL. Cadmium and copper inhibit both DNA repair activities of polynucleotide kinase. DNA Repair (Amst) 2010; 2: 83-89.

Wirojanagud W, Srisatit T, Bowyer J, Yuberk N, Howen L, Kemmadamrong P, Pitaksanurat S, Ratanachai C. Solid waste benchmarking study of 13 Thai municipalities. United States Agency for International Development, Washington DC. 2004.

Yang L, Li Y, Xj G, Ma X, Yan Q. Comparison of dry ashing, wet ashing and microwave digestion for determination of trace elements in Periostracum serpentis and Periostracum cicadae by ICP-AES. Journal of the Chilean Chemical Society 2013; 58: 1876-1879.

Yang H, Shu Y. Cadmium transporters in the kidney and cadmium induced nephrotoxicity. International Journal of Molecular Sciences 2015; 16: 1484-1494.

Zotina T, Medvedeva M, Trofimova E, Alexandrova Y, Dementyev D, Bolsunovsky A. Chromosomal abnormalities in roots of aquatic plant *Elodea canadensis* as a tool for testing genotoxicity of bottom sediments. Ecotoxicology and Environmental Safety 2015; 122: 384-391.

Zuengsonthiporn S, Jongrakthai T. Biology and distribution of water clover *(Marsilea)* and weedy potential of alien water clover. Annual Report 2011 of Plant Protection Research and Development Office, Department of Agriculture, Ministry of Bangkok. 2011.

Zuengsonthiporn S, Jongrakthai T. Aquatic plant for protection of native plant. Annual Report 2012 of Plant Protection Research and Development Office, Department of Agriculture, Ministry of Bangkok. 2012.