GENETIC EROSION IN THE FRESHWATER SNAIL *FILOPALUDINA MARTENSI* IS AFFECTED BY LEAD AND CADMIUM

NEERATANAPHAN, L.[1*] – SUDMOON, R.[2] – CHAVEERACH, R.[2]

[1]*Department of Environmental Science, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*

[2]*Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*

**Corresponding author*
e-mail: hlamya@kku.ac.th

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Abstract. This study aims to determine the lead (Pb) and cadmium (Cd) concentrations and genetic variability in the tissues of the freshwater snail *Filopaludina martensi*. These measures can be biomarkers of environmental quality. The samples were collected over three seasons from five sites around the Ubonrat Dam. The results showed that the average concentrations of Cd and Pb found in the tissue of fresh water snails during the rainy season (0.1040 and 0.0106 µg/g, respectively) were higher than in the winter (0.0566 and 0.0030, respectively) and summer (0.0654 and 0.0059 µg/g, respectively). Random amplified polymorphic DNA (RAPD) was successfully applied to produce 440 total bands used for dendrogram construction and analysis of genetic similarity (S). The dendrogram separated the studied samples by seasons, showing the highest S values of 0.59-0.93 in the Rainy S1-S5 and S2-S3, 0.62-0.83 in the Winter S1-S4 and S1-S2, and 0.64-0.84 in the Summer S1-S5 and S2-S3. The highest S values in the rainy season that are lower values compare to samples from other seasons are those associated with the highest average concentrations of Cd and Pb in the tissues sampled in this season. The metal concentrations are below the standard control level, but they can still affect the genetic material of freshwater snails.
Keywords: *Lead*, *cadmium*, *freshwater snail*, *genotoxicity*, *genetic erosion*

Introduction

Heavy metals are often contaminants in marine and freshwater environments, where they are generally found in low concentrations. However, human activities have increased the levels of metal ions in many of these natural water systems. Industrial and domestic effluents in particular have contributed to the increased metal loads in coastal waters and are ultimately deposited into aquatic sediments (Ansari *et al.*, 2004; Pandey *et al.*, 2010). These metals accumulate in organisms, and some may become biomagnified in food chains (Singh *et al.*, 2010). Many aquatic organisms uptake metals directly from the water, so tissue concentrations reflect metal concentrations in the water. Carnivores at the top of the food chain, such as aquatic birds and mammals (including humans), however, obtain most of their pollutant burden from aquatic ecosystems by ingestion, especially of fish, which have a considerable potential for biomagnification (Richter and Nagel, 2006).

The determination of total heavy metal contents in sediments has not been performed up to now because the behavior of heavy metals is closely related to their chemical forms, and only those with high bioavailability can be absorbed by organisms and do great harm to other organisms and human beings. Thus, the investigation of heavy metal

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contamination in organisms more directly reflects the hazards to human health and the potential heavy metal pollution in water. Lying in the second trophic level in the water ecosystem, mollusks have long been known to accumulate both essential and non-essential trace elements from aquatic ecosystems (Liang *et al.*, 2004; Wagner and Boman, 2004; Nϕrum *et al.*, 2005). Mollusks, including freshwater snails and bivalves, have long been regarded as promising bio-indicators and bio-monitoring subjects. They are abundant in many terrestrial and aquatic systems, making them easily available for collection. They are highly tolerant to many pollutants and exhibit high accumulation properties, particularly for heavy metals. Mollusks living in the sea can accumulate heavy metals and may serve as excellent passive bio-monitors (Wang *et al.*, 2005).

The Pong River is the principle river in Northeastern Thailand. The Moon River is the continuation of this waterway into the lower northwest of this region, thus any water pollution would have a combined effect. People living in the Loei province, the Nongbualamphu province and the Khonkaen province utilize these rivers for consumption and agriculture. Despite reports of water pollution in the Pong River since 1986, there has still been a continuing crisis of water pollution as reported in 1992 (Faculty of Engineering, 2003). Sources of pollution came from several industries, and noted a high impact on the Pong River. Examples of these industries include a paper mill factory, a combined gas turbine power plant, a distillery plant, a sugar factory and wood working plants. In addition, there are several smaller industries located within the vicinity of the Pong River. These are all likely major causes of heavy metal contamination in the Pong River along with traces from pesticides, chemical fertilizers and other wastes from the community.

The genotoxicity measurement process in living tissue at the DNA is advantageous because of its sensitivity and short response time (Gupta and Sarin, 2009). Heavy metals have an acute genotoxic potential in all living things. Immediately releasing these pollutants can cause morbidity and mortality in the exposed organism and can provoke order changes such as alterations to population dynamics and changes in biological diversity (An *et al.*, 2012). Random amplified polymorphic DNA (RAPD) is extremely efficient for DNA analysis in complex genomes as it is simple, inexpensive and minimally time-consuming. The resulting bands can be scored to evaluate genetic similarities and dissimilarities of samples through dendrogram construction. That is, RAPD bands are generally used to effectively indicate genetic relationships. The concept critical to cladistics is homology, which can be defined as a similarity resulting from common ancestry. Therefore, the cladogram designed depicts not only similarity but also evolutionary relatedness (Simpson, 2006).

The freshwater snail *Filopaludina martensi* Frauenfeld 1865 is a benthic fauna in the food chain ecosystem that lives and moves slowly to feed on the surface of sediments and thus accumulates heavy metals. This study aims to determine heavy metal accumulation in fresh water snail tissue and the genetic relationship shown by the similarity index, assessing other toxic contaminants through RAPD analysis.

Materials and methods

Sampling sites

The Pong River is the principle river in Northeastern Thailand, and the Moon River is the continuation of this waterway into the lower part of this region. These rivers support agricultural farms and many industries. The Bueng Jode reservoir receives a

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considerable amount of effluents from a paper mill factory before flowing into the Pong River. Five sampling sites were defined as the affected area of the Pong River (*Fig. 1*).

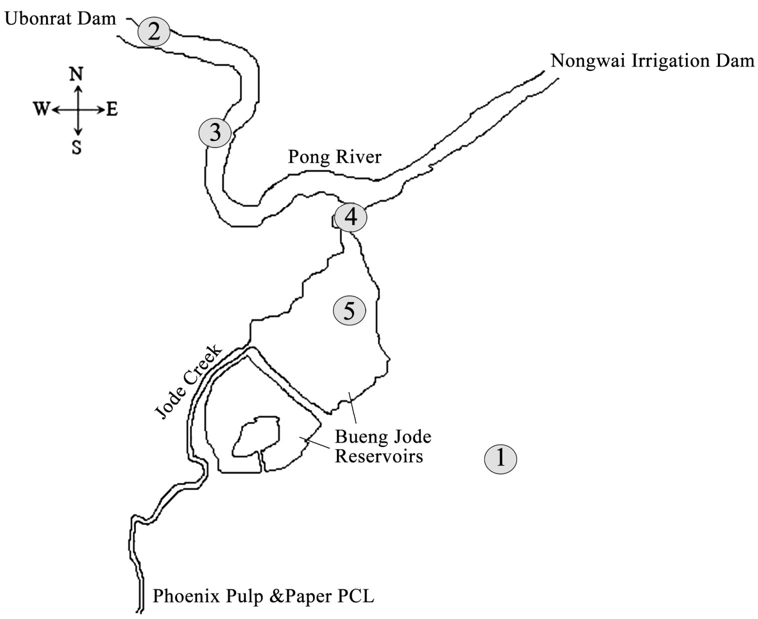

Figure 1. *Overview of the Pong River and locations of the five sampling sites as shown by numbers 1 to 5*

Sampling and sample analysis

Freshwater snails were collected from the five sites in three consecutive seasons in 2010. The concentrations of Cd and Pb were evaluated following these steps:

Three samples from each sampling site were homogenized, the soft tissues of freshwater snails were excised using a sterile stainless steel scalpel. These tissues were predigested with 3 ml of concentrated nitric acid overnight at 40 °C. After cooling, 2 ml of 30% hydrogen peroxide was added. There after the container was covered and placed in a high-pressure stainless steel bomb then put in an oven. The oven temperature was increased to160 °C and kept for 4 hrs. After cooling, the solution was diluted with Milli-Q water and transferred into a PET bottle to 50 g. Cd and Pb concentrations were determined with inductively coupled plasma-optical emission spectrometry (ICP-OES), PerkinElmer (Optima 4300 AV). For the wavelength analysis of ICP, wavelengths of Cd and Pb were 228.802 and 220.353 nm, respectively. The results are reported as dry weight. The accuracy of the results for heavy metal was evaluated with the certified reference material (CRM) using the 3111C method (APHA, 2005). Two aliquots of the CRM were spiked with a known amount of heavy metal spike standard, and one spike was analyzed according to 3111C method while the other was analyzed with the 3111B method (APHA, 2005). The metal recoveries were in the 96–100% range, which was acceptable (USEPA, 1994).

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Statistical analysis

The seasonal variations and sampling sites were analyzed using SPSS V.19. All the statistical tests were conducted at a 95% confidence level. The data normality was tested using the Kruskal-Wallis test.

DNA extraction

DNA was extracted from kidney tissue using the Genomic DNA Extraction Kit (RBC Bioscience, Taiwan) following the manufacturer's instructions. The extracted DNA was checked by 0.8% agarose gel electrophoresis and then diluted to a final concentration of 20 ng/µl.

DNA fingerprinting and analysis by RAPD marker

Amplifications were carried out on each sample in 25 µl reactions consisting of GoTaq Green Master Mix (Promega), 0.5 µM primer and 5 ng DNA template. Several RAPD primers were screened and the 12 primers that successfully amplified clear bands are as follows (5′ to 3′): CATCCCCCTG, GGACCCTTAC, GTCCCGACGA, AAGCCTCGTC, CAGGCCCTTC, TGCCGAGCTG, AATCGGGCTG, GGGTAACGCC, CAATCGCCGT, CAAACGTCGG, GACCTACCAC and TCAGTCCGGG. 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 min at 94 °C, 2 min annealing temperature at 40 °C, 2 min at 72 °C, and 7 min final extension at 72 °C using a thermal cycler (Swift^{TM} 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. The resulting RAPD bands were used for the dendrogram construction.

The RAPD bands from the primers that resulted in successful amplification were discerned on an agarose gel and were documented as diallele characters: present=1 and absent=0. These resulting bands were used to construct a dendrogram following NTSYSpc 2.10p (Rohlf, 1998). The dendrogram and genetic similarities were produced.

Results and Discussion

Cd and Pb concentrations

The Cd and Pb concentrations from the tissue of freshwater snail samples are shown in *Table 1*. The average concentration values of Cd and Pb in the winter, summer and rainy seasons are 0.0566, 0.0030; 0.0654, 0.0059; and 0.1040, 0.0106 µg/g of tissue, respectively. The contaminated values are higher in the rainy season than in the winter and summer. Statistical analysis shown in *Table 2* indicates that there are no significant differences between study sites or weather seasons with regard to Cd and Pb ($p>0.05$).

DNA fingerprinting of freshwater snail

The 12 successful RAPD primers generated clear and testable fingerprinting profiles. Examples of fingerprints are shown in *Figure 2-4*.

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Table 1. *Concentrations of Cd and Pb in freshwater snail tissue (µg/g, dry weight)*

Parameter	Cd content (µg/g)	Pb content (µg/g)
Winter season		
Site 1	0.0290	0.0011
Site 2	0.0310	0.0009
Site 3	0.0390	0.0027
Site 4	0.1100	0.0050
Site 5	0.0740	0.0053
Average	0.0566	0.0030
SD	0.0349	0.0021
Summer season		
Site 1	0.0930	0.0073
Site 2	0.1100	0.0051
Site 3	0.0360	0.0170
Site 4	0.0570	0.0000
Site 5	0.0310	0.0000
Average	0.0654	0.0059
SD	0.0349	0.0070
Rainy season		
Site 1	0.0630	0.0160
Site 2	0.0700	0.0210
Site 3	0.0340	0.0043
Site 4	0.3200	0.0030
Site 5	0.0330	0.0086
Average	0.1040	0.0106
SD	0.1219	0.0077

Table 2. *Values obtained from SPSS test*

Source of variation	p value (Cd)	p value (Pb)
Sample sites	0.393	0.792
Seasons	0.826	0.248

Significant value with a confidence level of 95%

Countable bands ranged in size from 100 bp to 2,500 bp. Only bands present in at least two of three samples from each studied site in each season were counted. These resulted in 440 total bands, which were used for dendrogram construction. The dendrogram distinguished studied samples into three groups based on the samples from five different sites (S1-S5) and seasonal grouping.

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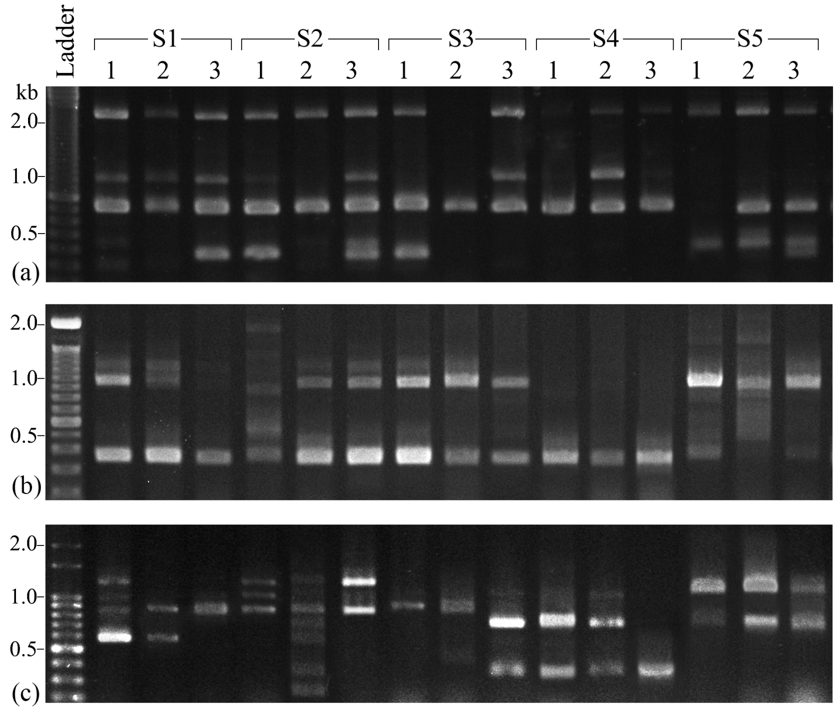

Figure 2. *Examples of RAPD fingerprints from all freshwater snail samples studied in the winter from primers CATCCCCCTG (a), CAATCGCCGT (b) and CAAACGTCGG (c)*

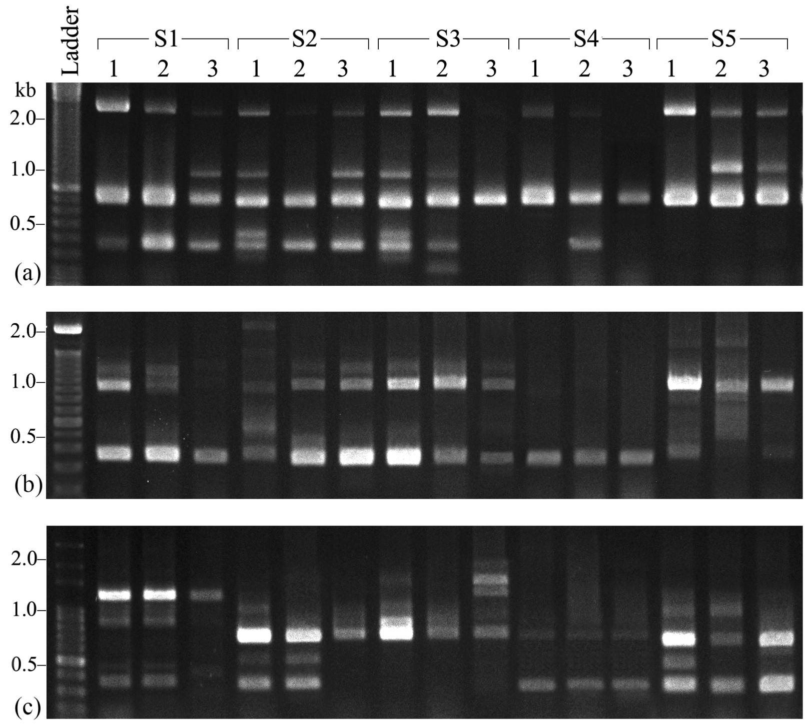

Figure 3. *Examples of RAPD fingerprints from all freshwater snail samples studied in the summer from primers CATCCCCCTG (a), CAATCGCCGT (b) and CAAACGTCGG (c)*

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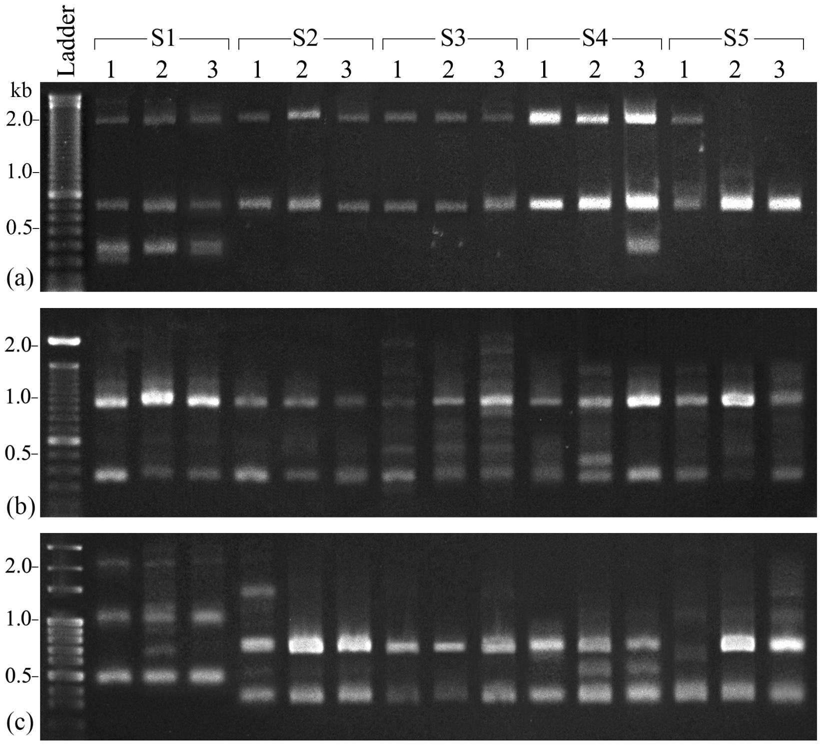

***Figure 4.** Examples of RAPD fingerprints from all freshwater snail samples studied in the rainy season from primers CATCCCCCTG (a), CAATCGCCGT (b) and CAAACGTCGG (c)*

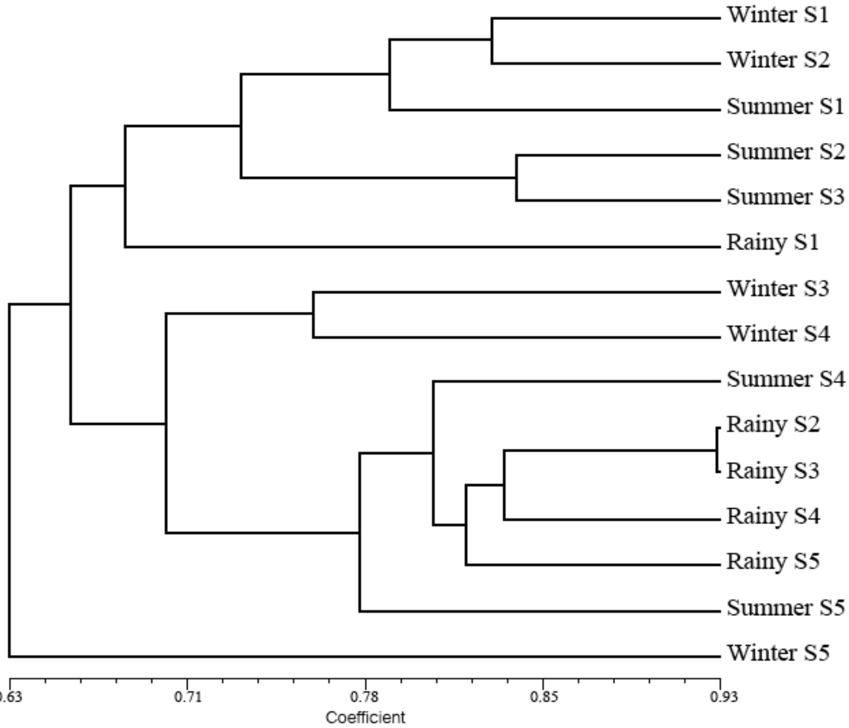

***Figure 5.** The dendrogram constructed from twelve RAPD primers by the NTSYSpc 2.10p program showing the genetic relationships between the five studied sample sites at different seasons*

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Table 3. *Relationships of all pairwise studied samples at different seasons indicating genetic similarity values*

	Winter S1	Winter S2	Winter S3	Winter S4	Winter S5	Summer S1	Summer S2	Summer S3	Summer S4	Summer S5	Rainy S1	Rainy S2	Rainy S3	Rainy S4	Rainy S5
Winter S1	1.00														
Winter S2	0.83	1.00													
Winter S3	0.72	0.74	1.00												
Winter S4	0.62	0.64	0.76	1.00											
Winter S5	0.66	0.68	0.65	0.66	1.00										
Summer S1	0.76	0.82	0.68	0.65	0.63	1.00									
Summer S2	0.66	0.68	0.61	0.68	0.62	0.76	1.00								
Summer S3	0.69	0.76	0.64	0.67	0.67	0.81	0.84	1.00							
Summer S4	0.64	0.68	0.69	0.77	0.68	0.69	0.77	0.76	1.00						
Summer S5	0.61	0.61	0.66	0.76	0.69	0.64	0.74	0.66	0.80	1.00					
Rainy S1	0.62	0.68	0.61	0.58	0.52	0.65	0.73	0.72	0.64	0.61	1.00				
Rainy S2	0.60	0.64	0.63	0.77	0.60	0.65	0.75	0.72	0.83	0.80	0.62	1.00			
Rainy S3	0.57	0.59	0.64	0.76	0.61	0.62	0.76	0.71	0.82	0.81	0.63	0.93	1.00		
Rainy S4	0.67	0.65	0.60	0.72	0.61	0.66	0.78	0.77	0.78	0.75	0.67	0.84	0.83	1.00	
Rainy S5	0.53	0.57	0.62	0.74	0.55	0.58	0.67	0.64	0.80	0.73	0.59	0.86	0.81	0.79	1.00

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The first group is the Winter S5 only; the second group comprised the Rainy S2, Rainy S3, Rainy S4, Rainy S5, Summer S4, Summer S5, Winter S3 and Winter S4 samples; the third group comprised the Rainy S1, Summer S1, Summer S2, Summer S3, Winter S1 and Winter S2 samples. The dendrogram (*Fig. 5*) shows genetic similarity between all studied samples according to the pairwise genetic relationships shown in *Table 3*. The dendrogram separated the studied samples by seasons and showed the highest S (0.59-0.93) of the studied samples in the rainy season, Rainy S1-S5 and S2-S3. The other two sites are 0.62-0.83 in the Winter S1-S4 and S1-S2, and 0.64-0.84 in the Summer S1-S5 and S2-S3, respectively.

Toxin-free food is essential for people's wellbeing and animal health. Freshwater snails have long been used as an environmental indicator. However, there are no current studies addressing ecotoxicology of the Pong River or of the potentially harmful impacts of pollutants on food. This research is much needed for supporting agricultural farms and people's life styles in Northeastern Thailand, along with the many industries surrounding them. Consequently, these concerns were addressed in this study by measuring metal concentrations in tissues. The freshwater snail, *F. martensi* is a much-relished ingredient in Thailand's cuisine. To ensure quality food and a safe environment around the Pong River, this experiment examined the concentration of heavy metals Cd and Pb in the freshwater snail's tissues and determined the cellular biomarker as effective for these objective examinations. The study showed that the average concentrations of Cd and Pb found in freshwater snail tissue in the rainy season were 0.1040 and 0.0106 μg/g, respectively, and these concentrations are lower in the winter, at 0.0566 and 0.0030 μg/g, respectively, and in the summer, at 0.0654 and 0.0059 μg/g, respectively. However, there are no significant differences in heavy metal concentrations according to SPSS V.19 analysis (Kruskal-Wallis) for each site (p=0.393 for Cd, p=0.792 for Pb) or for each season (p=0.826 for Cd, p=0.248 for Pb). Accordingly, the dendrogram distinguished the studied samples into the three groups (the Winter S5; the Rainy S2, Rainy S3, Rainy S4, Rainy S5, Summer S4, Summer S5, Winter S3 and Winter S4; the Rainy S1, Summer S1, Summer S2, Summer S3, Winter S1 and Winter S2) from the different samples from different sites and different seasons, grouping them independently from the heavy metal concentrations. Fortunately, these metal concentration values are below both Thailand's standard control level (2.0 μg/g (Thailand Pollution Control Department, 1986)) and the Food and Agricultural Organization (FAO) (2.0 μg/g in Cd and 1.0 – 6.0 μg/g in Pb.) The rainy season has running water leaching heavy metals such as Cd and Pb from communities and industries into the Pong River, which are then deposited in sediments and accumulate in the tissue of freshwater snails. This likely accounts for the increased concentration of Cd and Pb during the rainy season. The freshwater snails in this area are affected by many industries, agricultural fertilizers and insecticides, but can still be of good quality. This animal can endure these two heavy metals, and survive in the environment around the Pong River, which is a moderately contaminated area. This study, in sum, is a field investigation, and there are other possible measures of toxicity from the environment beside these two heavy metals.

Understanding the effect of pollutants on genetic variability is fundamental for preservation. The evolutionary potential of natural populations, the effects of toxic contaminants on the environment, and the effect of Cd and Pb on the freshwater snail habitats were investigated at the molecular level using RAPD marker analysis. Genomic DNA modifications such as damage and structure variations were detected, and these

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modifications can be biomarkers. The highest S values (0.59-0.93) in the rainy season samples that were low compared to samples from other seasons are those associated with the highest average concentrations of Cd and Pb in the rainy season, suggesting an effect of the metals on the freshwater snail tissue. Although, the heavy metal concentrations are under the standard control level, they can have an effect on the genetic material of the freshwater snail, which is an important ingredient in the local food culture. This study suggests that the freshwater snail is a good indicator species, by which to measure the effectiveness of local waste-water treatment systems.

Note that actual conditions in the field are affected by several pollutants, such as fertilizers, heavy metals, chemicals, and insecticides, etc., in addition to Cd and Pb, and these pollutants can all have positive and negative effects on the ecosystem.

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