APPLICATION OF MODIFIED ALLIUM TEST USING SHALLOT (*Allium ascalonicum* L.) FOR DETERMINATION OF MERCURY CONTAMINATION IN SEDIMENT AT GOLD MINING SITE

SASILAK TEETHONG

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APPLICATION OF MODIFIED ALLIUM TEST USING SHALLOT (*Allium ascalonicum* L.) FOR DETERMINATION OF MERCURY CONTAMINATION IN SEDIMENT AT GOLD MINING SITE

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ABSTRACT

The objective of this study was to apply the modified Allium test using shallot (*Allium ascalonicum* L.) for determination of mercury (Hg) contamination in sediments at a gold mining site. The toxicity of Hg solution ($HgCl_2$) was initially determined by root growth inhibition of shallot at concentrations of 50, 200, 800, and 3200 µg L^{-1} after 96 hours of exposure. It was shown that the effect of Hg concentration on shallot root growth was significantly different (P<0.001) when compared with the control group. The EC_{30}, EC_{50}, and EC_{70} values of Hg were 163, 440, and 870 µg L^{-1}, respectively.

The genotoxicity of Hg test solutions was evaluated by mitotic index and percentage of chromosome aberration in root meristem cells. The shallot roots were exposed to Hg at concentrations of 163, 440, and 870 µg L^{-1} for 48 hours. The mitotic index was decreased as Hg concentration increased and the chromosome aberration increased as Hg concentration increased. The mitotic index and chromosome aberration obtained from Hg test solution were significantly different from the control group (P<0.001). The types of chromosome aberration were laggard, fragment, bridge, and c-mitosis.

Sediment from the mining site was dissolved in deionized water and its supernatant was used as the one Hg test solution. Another portion the supernatant was spiked with known Hg concentration and used as an alternative Hg test solution. The supernatant had an Hg concentration of 169 µg L^{-1}, the spiked supernatant 620 µg L^{-1}. The mitotic index of both supernatants was not significantly different (P=0.247) whereas the chromosome aberration was significantly different (P<0.001). In addition, chromosome aberration was sensitive to very low concentrations of Hg. Thus, the modified Allium test using shallot has the potential to be applied for determination of Hg contamination in sediment at the concentration range of 169-620 µg L^{-1}.

KEY WORDS : ALLIUM TEST / SHALLOT / MERCURY / MITOTIC INDEX / CHROMOSOME ABERRATION

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การใช้วิธี modified Allium test ด้วยหอมแดง (*Allium ascalonicum* L.) สำหรับตรวจสอบการปนเปื้อนของปรอทในดินตะกอนบริเวณเหมืองแร่ทองคำ

(APPLICATION OF MODIFIED ALLIUM TEST USING SHALLOT (*Allium ascalonicum* L.) FOR DETERMINATION OF MERCURY CONTAMINATION IN SEDIMENT AT GOLD MINING SITE)

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บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อใช้วิธี modified Allium test ด้วยหอมแดง (*Allium ascalonicum* L.) เพื่อตรวจสอบการปนเปื้อนของปรอทในดินตะกอนบริเวณเหมืองทองคำ ความเป็นพิษของสารละลายปรอทวัดได้จากการยับยั้งการเจริญเติบโตของรากหอมแดง เมื่อได้รับสารปรอทที่ความเข้มข้น 50 200 800 และ 3200 ไมโครกรัมต่อลิตร เป็นเวลา 96 ชั่วโมง โดยพบว่าการเจริญเติบโตของรากหอมแดงถูกยับยั้งทุกระดับความเข้มข้นอย่างมีนัยสำคัญ (P<0.001) เมื่อเปรียบเทียบกับกลุ่มควบคุม ความเข้มข้นของปรอทที่ระดับความเป็นพิษ EC_{30} EC_{50} และ EC_{70} มีค่าเท่ากับ 163 440 และ 870 ไมโครกรัมต่อลิตร ตามลำดับ

ความเป็นพิษในระดับยีนของสารละลายปรอทวัดได้จาก ค่าดัชนีการแบ่งเซลล์และความผิดปกติของโครโมโซม(ร้อยละ)ที่ระดับความเข้มข้นปรอท 163 440 และ 870 ไมโครกรัมต่อลิตรนาน 48 ชั่วโมง และพบว่าดัชนีการแบ่งเซลล์มีค่าลดลงและความผิดปกติของโครโมโซมมีค่าเพิ่มขึ้นเมื่อความเข้มข้นของปรอทเพิ่มขึ้น ดัชนีการแบ่งเซลล์และความผิดปกติของโครโมโซมในกลุ่มสารละลายทดสอบแตกต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญ (P<0.001) ทั้งนี้ความผิดปกติของโครโมโซมที่เกิดขึ้นได้แก่ laggard fragment bridge และ c-mitosis

สารละลายดินตะกอนเตรียมได้จากการละลายดินตะกอนจากเหมืองทองคำในน้ำปราศจากไอออนและสารละลายดินตะกอนที่ได้ถูกเติมด้วยปรอทที่ระดับความเข้มข้นเท่ากับ 169 และ 620 ไมโครกรัมต่อลิตร ตามลำดับ พบว่าค่าดัชนีการแบ่งเซลล์ของสารละลายดินตะกอนทั้งสองไม่มีความแตกต่างกันอย่างมีนัยสำคัญ (P=0.247) ในขณะที่ความผิดปกติของโครโมโซมมีความแตกต่างอย่างมีนัยสำคัญ (P<0.001) นอกจากนี้พบว่าความผิดปกติของโครโมโซมมีความไวต่อปรอทที่ความเข้มข้นต่ำ ดังนั้นวิธี modified Allium test ด้วยหอมแดงมีศักยภาพในการนำไปใช้ตรวจสอบการปนเปื้อนของปรอทในดินตะกอนที่มีระดับความเข้มข้นของปรอทระหว่าง 169-620 ไมโครกรัมต่อลิตร

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LIST OF ABBREVIATIONS

CAT	=	Catalase
DMR	=	Department of Mineral Resources
DMSO	=	Dimethyl Sulfoxide
DNA	=	Deoxyribonucleic acid
dw	=	Dry weight
EMS	=	Ethyl Methane Sulfonate
INVITTOX	=	In Vitro Techniques in Toxicology
M	=	Molarlity
MMS	=	Methyl Methane Sulfonate
POD	=	Peroxidase
ROS	=	Reactive oxygen species
rpm	=	Revolutions per minute
SOD	=	Superoxide-dimutase
THg	=	Total mercury
USEPA	=	United States Environmental Protection Agency
WHO	=	World Health Organization
ww	=	Wet weight

CHAPTER I

INTRODUCTION

1.1 Backgrounds and Rationales

Mercury (Hg) poisoning has become a problem of environmental pollution on global scale that cycles between air, water, and soil as a result of natural processes and anthropogenic activities (Patra and Sharma, 2000; Moreno et al., 2005). Mercury is used in various applications including batteries, electric lamps, medical instruments, dental amalgams, paints, and pharmaceuticals. For artisanal gold mining, mercury is used as a tool for the recovery of gold through amalgamation, which is considered a cheap alternative to the usual cyanidation of gold, especially for small-scale mines (Clemete et al., 2004).

The amalgamation method causes one of the major sources of mercury contamination outstandingly in developing countries (Taylor et al., 2005). For instance, mercury is unintentionally spilled onto the ground. Atmospheric transport and deposition at normal temperature is the pathway delivering Hg to receiving water. Furthermore, mercury is often discharged together with other wastes into inadequate tailings ponds, or is disposed of directly into rivers and waterways. The resulting amalgam requires the removal of mercury through vaporization, with the mercury vapor being allowed to escape to the atmosphere with little or no thought of recovery (Clemete et al., 2004; Limbong et al., 2003). The health of the miners and other people living within the area affected through eating fish and other food affected by mercury contamination (Taylor et al., 2005).

Gold mining at Phanom Pha Hill, Phichit Province is formerly the only an artisonal in Thailand since the late 1990s. The shield covers a total area of 48,300 square meters with 0.75-3.0 meter thick and soil density and rock densities are 0.06-

23.2 and 0.05-27.83 gm/m^3, respectively (SPS, 2002). The actual dispute is over the effects of the gold mining process, particularly the mercury used to isolate the gold from the surrounding sediment. Mercury enters the environment during each step involved in acquiring the gold (Pataranawat, 2003).

The modified Allium test is a simple, sensitive, and rapid bioassay that has been widely used as a standard for biomonitoring of environmental contaminants using various genotoxicity parameters. The test can be used to measure both toxicity and genotoxicity. It can be used without any condensation, purification, or sterilization of wastewater or recipient water. The Allium test is easy to handle with low cost, and it shows good correlation with mammalian test system (Nielsen and Rank, 1994). Moreover, the Allium chromosomes are quite large and suitable for detailed analysis. The bulbs can be easily handled, and root grows profusely at regular interval (Sharma and Sharma, 1994). Therefore, the Allium test is suitable for developing countries (Grant, 2006).

The different species of *Allium* are used in genotoxicity test (Sharma and Sharma, 1994). The shallot *(Allium ascalonicum* L.*)* is a local plant in Thailand. It is a low cost material, easy maintenance in the laboratory, adequate biological background, and economic importance. The shallot root and chromosome have been sensitive to heavy metal such as chromium (Liewrungruang, 2003).

From the previous study the Hg concentration of sediment at mining site was found in the range of 159.64 to 401.87 μg kg^{-1}dw. (Pataranawat, 2003). Thus, the purpose of this study is to apply the modified Allium test using shallot to determine the Hg contamination in sediment, particularly in the range of that contamination.

1.2Research Objectives

1.2.1 General objective

To apply the modified Allium test using shallot for determination of Hg contamination in sediment at gold mining site.

1.2.2 Specific objectives

1. To determine Hg concentration of test solution at the toxicity levels of EC_{30}, EC_{50}, and EC_{70} on root elongation of shallot.
2. To determine the effects of Hg at the concentration of EC_{30}, EC_{50}, and EC_{70} of test solution on mitotic index of shallot root cells.
3. To determine the effects of Hg at the concentration of EC_{30}, EC_{50}, and EC_{70} of test solution on chromosomal aberration of shallot root cells.
4. To apply the modified Allium test for determination of Hg contamination in sediment at gold mining site.

1.3 Research Hypotheses

The hypotheses of the study include:

1. The root bundle length of shallot in treated groups will be decreased from that of control.
2. Mitotic index of treated groups will be lower than that of control.
3. Chromosome aberration of treated groups will be increased as Hg concentration increased.
4. Mitotic index and chromosome aberration percentage of shallot in sediment supernatant will be different in each concentration.

1.4 Research Variables

1.4.1 Independent variables

- Concentrations of THg in test solution (μg L^{-1})
- Concentration of THg in supernatant (μg L^{-1})

1.4.2 Dependent variables

- Root bundle length (centimeters)
- Mitotic index
- Percentage of chromosome aberration

1.4.3 Control variables

- Temperature at 30°C±3
- The pH of water (6.9±0.4)
- Source of shallot

1.5 Scope of Study

1. The mercuric chloride ($HgCl_2$) standard solution was used as test solution of total mercury.
2. The sediment was obtained from Khoa Chet Luk reservoir (outlet), Phanom Pha gold mining site. In case of sediment unavailable from the site a similar property of sediment was used.
3. The shallot in used the experiment was bought from Bangchang, Lumphun Province and the age of them was about 3 to 6 months after harvest.
4. Tab water was used as negative control.
5. Methyl Methane Sulfonate (MMS) at 10 mg/L was used as a positive control.
6. Tap water was used as growth medium and dilution for the sample water and positive control (MMS).

7. Root bundle length was measure by using a ruler at time interval of 24 hours to 96 hours. The longest and shortest root lengths were ignored. Then, a mean of root bundle length was calculated for each treatment.

8. Types of chromosome aberration were observed in anaphase and early telophase cells. They were bridges, fragment, vagrant (laggard) chromosome, and combinations of abnormalities.

1.6 Conditions and Limitations of the Study

1. The supernatant was prepared by sediment of Khoa Chet Luk reservoir at Phanom Pha Hills.

2. Tap water was a pH around 7 from storage tank of Faculty of Public Health, Mahidol University.

3. The Hg concentration was determined in term of THg.

1.7 Definition of Keywords

1. Toxicity: Toxicity is the properties of toxicant (mercuric solution) that produced hazardous effects on biological systems (root growth inhibition).

2. Genotoxicity: Genotoxicity is the study of the adverse effects of compounds on the genetic material of cell (DNA) and the subsequent expression of these changes (chromosome).

3. Mean root bundle length: Average of shallot root bundle length in each treatment.

4. EC_{30}, EC_{50}, and EC_{70} at 96 hours: The toxicity levels represent effective concentrations of mercuric chloride solution inhibiting 30%, 50%, and 70% root growth in relation to root growth of control, respectively at 96 hours of exposure.

5. Mitotic cells: Nuclear division cells that are geneally divide in prophase, metaphase, anaphase, and telophase stages.

6. Mitotic index: The index represents the effect of test solution on cell division.

7. Methyl Methane Sulfonate (MMS): The one of alkylating agents has the potential to cause chromosome aberration.

8. Anaphase: The individual chromatids of each pair separate from each other and move to opposite poles of the cell. Chromatids are slender and densely stained.

9. Early telophase: Chromosomes have reached the opposite poles of the cell and the formation of cell plate begins in the center of the two daughter cells.

10. Chromosome aberration: Chromosome aberration is modification of the numerical or structural chromosomes.

11. Bridges: After chromatid breakage, sticky telomere is attached with another chromatid of the same chromosome. Then, chromatids dose not separate after shortening of spindle fiber to the pole. It looks like bridge at the center of the cell.

12. Fragments: A short segment of chromatid or chromosome break has no centromere.

13. Vagrant (laggard) chromosome: Chromosome lies on equatorial plane instead of moving towards the poles at mitotic anaphase.

14. Stickiness: It results from abnomalities of protein in chromatin. Sticky chromosomes indicate highly toxic and usually are not reversible leading to cell death.

1.8 Conceptual Framework

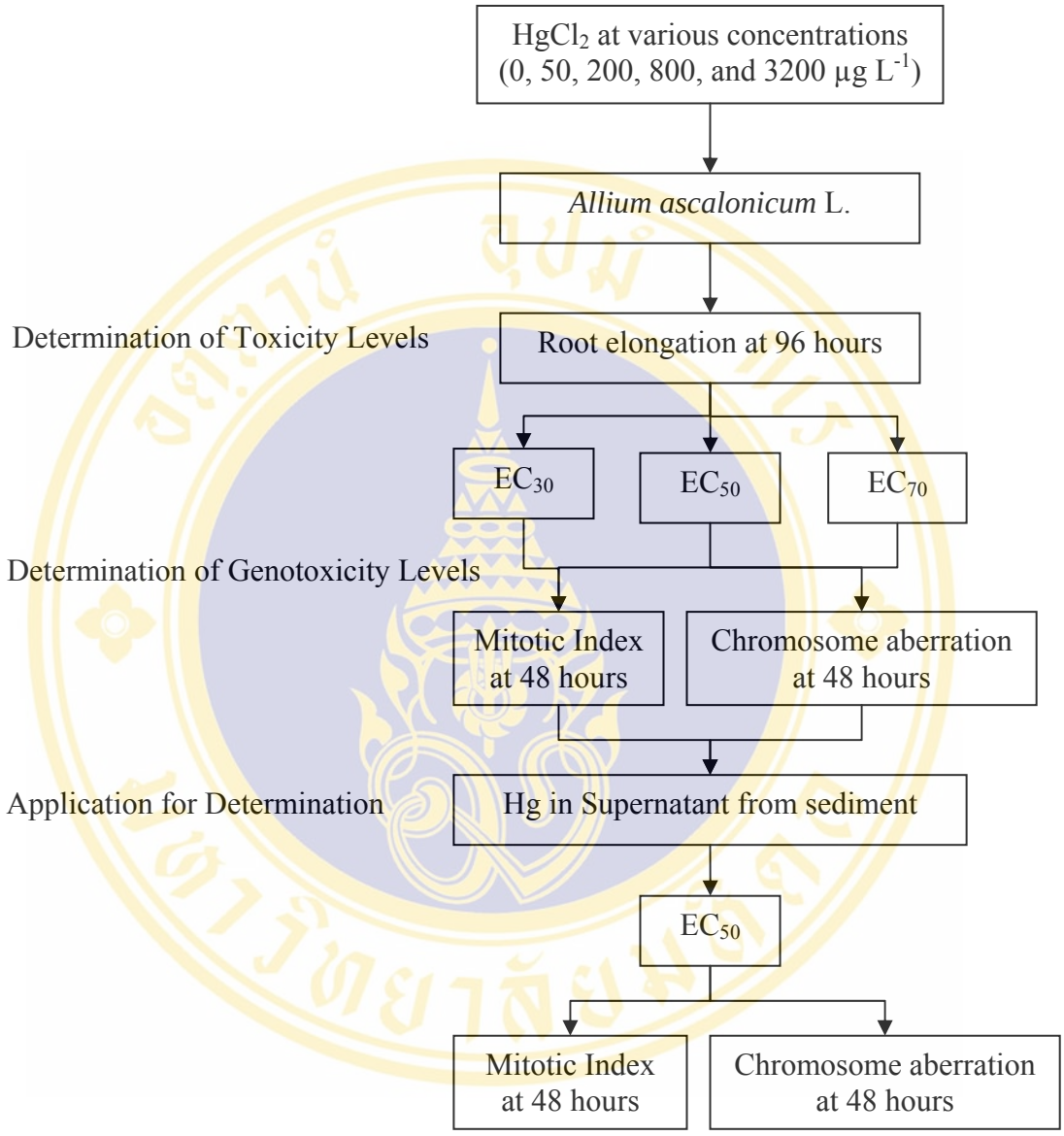

Figure 1.1 Conceptual framework

CHAPTER II
LITERATURE REVIEWS

2.1 Physical, Chemical, and Mechanical Properties of Mercury

Mercury (Hg) is a heavy, silver-white metal, which is in liquid state at normal temperature, and the only metal known which is liquid at 0°C. Mercury falls into group IIB of the periodic table and lists the following properties and characteristics are listed as the following;

Atomic number	80
Atomic weight	200.61
Melting point	-38.9°C
Boiling point	356.9°C
Density	13.5955 g cm^{-3}

Although mercury occurs free in nature, practically all mercury for commercial use is obtained from the ore cinnabar, HgS, which is refined by oxidation. Sulfur dioxide is formed and volatilizes and the free mercury is condensed.

Mercury oxidizes slowly and is insoluble in common solvents. Dilute hydrochloric and sulfuric acids do not attack it. Mercury oxidizes dissolves in dilute nitric acid and hot, concentrated sulfuric acid. It is insoluble in water and alkalis. Two series of compounds, mercurous (Hg_2X_2) and mercuric (HgX_2) are formed and give the characteristic test for mercurous (Hg_2^{2+}) and mercuric (Hg^{2+}) ions (Bidstrup, 1964).

Mercury vaporizes at room temperature, the equivalent vapor pressure being approximately 1.3 μg/Hg or 10 mg/m^3. The threshold limit value for mercury in

atmosphere is set at 0.1 mg/m^3. The facts that the vapor equilibrium at room temperature is above the recommended maximum allowable concentration and that the vapor pressure doubles if the ambient temperature rises by 10 degrees, contribute to the ease with which mercury can be absorbed by inhalation (Bidstrup, 1964).

The biological effects of mercury vary according to the nature of chemical compound administered but in all cases the principal reaction is with thiols, forming mercury mercaptide, and that the variations in distribution and effect are dependent upon this reaction. The lipoid solubility of simple mercurial such as the methyl mercury halides, which are 100 times as soluble in lipoid as in water, would explain the rapid distribution of these compounds to all tissues. The pharmacological behavior of metallic mercury can also be explained in terms of lipoid solubility, which permits universal distribution followed by oxidation to reactive mercuric salts. The solubility of mercury at 40°C is 2.7 mg/L is pentane, 0.6 mg/L in methanol and 0.02 mg/L in water; the solubility in body lipoid is probably intermediate between that of pentane and methanol (Bidstrup, 1964; WHO, 1989).

2.2 Sources and Pathway into the Environment

2.2.1 Natural source

The major natural sources of mercury are the degassing of the earth's crust, emissions from volcanoes and evaporation from natural bodies of water. The most recent estimates indicate that natural emissions are in the order of 2,700-6,000 tons per year. The earth's crust is also an important source of mercury for natural water. Some of this mercury is undoubted natural origin, but some may have been deposited from the atmosphere and may ultimately have been generated by human activities. Thus, it is difficult to assess quantitatively the relative contributions of natural and anthropogenic mercury to run-off from land to natural bodies of water (Pairoj-Boriboon, Kositrat & Inna, 2001; WHO, 1990).

Mercury is also found naturally in a free state or mixed in ores. It is also present in rocks (stated by Department of Mineral Resources (DMR), 2001, correspondence No.1). In general, possible sources of mercury are degassing of the earth's crust through emission and evaporation from the ocean. The amount of mercury globally generated is estimated to be between 25,000 and 125,000 tons per year (IPCS, WHO, 1989). The DMR reported that mercury could be found in Cinnabar (HgS) ore. The properties of Cinnabar are:

- 86.2% of mercury
- Magenta color
- Hardness = 2.5
- Specific gravity = 8.10
- Found in area of geysers
- Transformed to native mercury (Hg), montroydite (HgO), and colonel ($HgCl_2$)
- Found in the form of polymorph of HgS, or isomorph, and ZnS.

Metacinnabar (HgS) is one of the forms of cinnabar, which can be found as a mercury compound. The properties of Metacinnabar are as follows:

- Dark gray color
- Hardness = 3
- Specific gravity = 7.65
- Less than 86.2% of Hg

Additionally, a compound of mercury in ore can be found as impurity in Gold, Silver, and Sulfide or in a compound of Zinc, Lead and Copper (Pairoj-Boriboon, Kositrat & Inna, 2001).

2.2.2 Anthropogenic Source

For anthropogenic sources, mercury enters the environment from activities of human. The worldwide of mercury mining is estimated to yield about 10,000 tons per year. Mining activities result in losses of mercury through the dumping of mine

tailings and direct discharges to the atmosphere (WHO, 1991). The gold mining was separate gold by mercury. Artisanal gold mining and small-scale mining is using the mercury-based amalgamation process to extract gold from secondary ore bodies or very fine gold particles. Final recovery was always done through heating or burning of the amalgam with high mercury emission to the atmosphere. This cause contaminates mercury at gold mining area (Pataranawat, 2003; Veiga, Maxson and Hylander, 2005). In Thailand, at Phanom-Pha gold mining is a small-scale gold mining. Concentrations of mercury were determined in water, sediment, air, and surface soil at workplace (Pataranawat, 2003).

Mercury and organomercury is used in variety of products and industrial processes. The main use of mercury, though declining, is as the mobile cathode in the chloroalkali industrial, for the production of chlorine and caustic soda from the electrolysis of brine. Caustic soda is product in the last cell, and the mercury recycled back to the first cell. The large quantities of mercury were lost to the environment from these cells. In 1972 UK chloroalkali plants produced 850,000 tons of chlorine and discharged 34 tons of mercury and in 1983 produced 810,000 tons of chlorine and discharged 12 tons of mercury. Also, mercury is used in electrical and measuring apparatus, such as mercury discharge lamps, power rectifiers, mercury batteries, thermometers, barometers, and electrical switches. Mercury salts are used as catalysts in the industrial production of vinyl chloride, vinyl acetate and acetaldehyde from acetylene. In this process Hg(II) is reduced to Hg(0) but regenerated with iron(III). (Pairoj-Boriboon, Kositrat & Inna, 2001; Fergusson, 1991; Vernet, 1994)

Figure 2.1 Amalgam

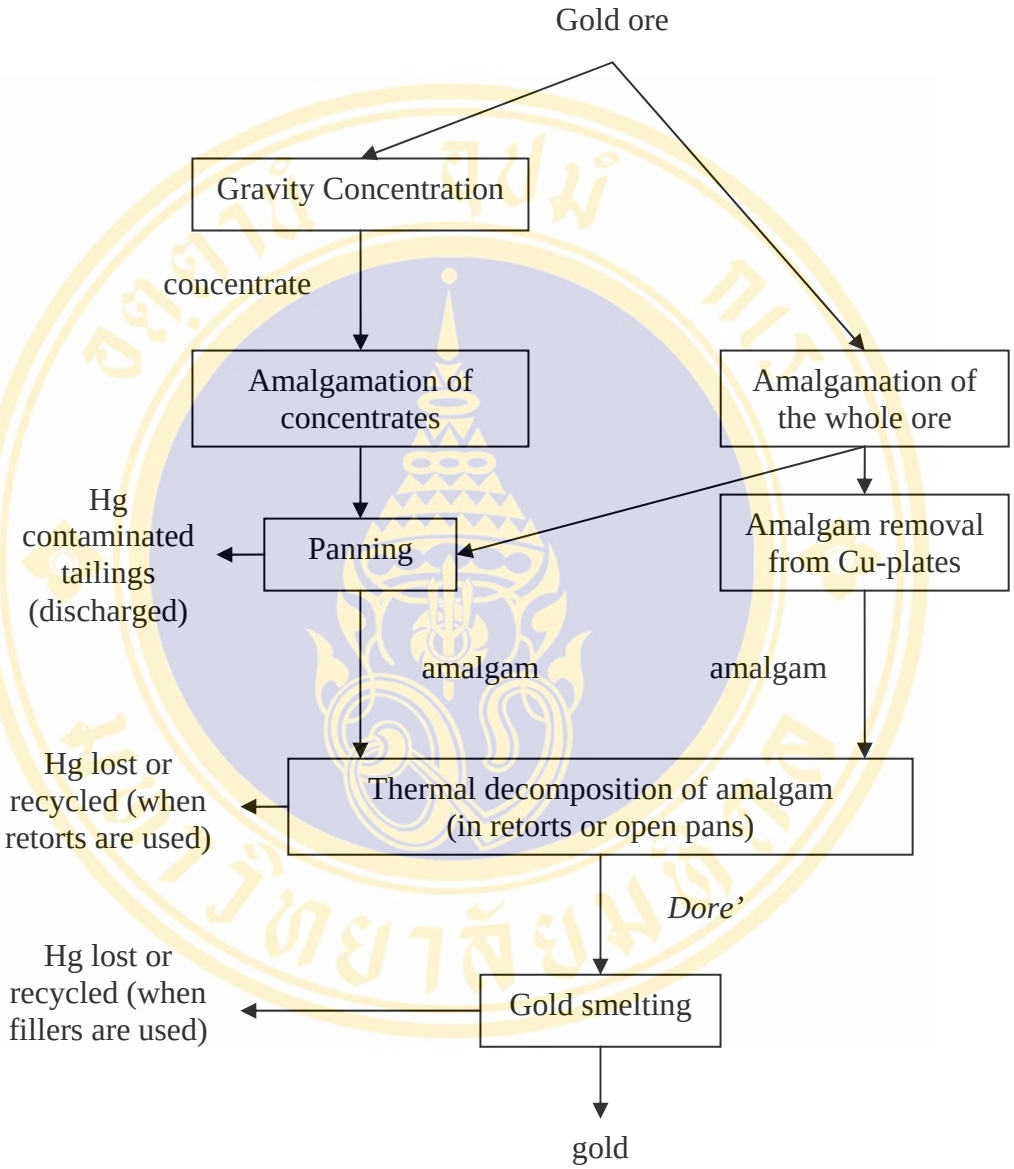

Figure 2.2 Methods used in artisanal mining operation to extract gold from mercury (Veiga, Maxson & Hylander, 2005)

Mercury is also used as dental filling. A dental alloy of Ag, Sn, Cu, and Zn is mixed with elemental mercury to give a dental amalgam, a past, which soon hardens in the tooth cavity. The amalgam is strong, resistant to abrasion, adheres strongly to the tooth, is of very low solubility and impermeable to saliva (Bidstrup, 1964; Pairoj-Boriboon, Kositrat & Inna, 2001; Fergusson, 1991; Vernet, 1994).

Phenyl mercury was used as a slimicide in the pulp and paper industry that has been abandoned in Europe and North America. In agriculture organ mercury are used, mainly phenylmercury and methoxyethylmercury but also small quantities of ethyl mercury. Some mercury compounds ($HgCl_2$, HgO, $Hg(CN)_2$, $HgNH_2Cl$, HgI_2, and Organ mercury compounds) have antiseptic and preservative quantities, which are still used in pharmaceutical and cosmetic. Mercury oxide is used in eye ointments to treat irritation, and HgI_2 is used to treat skin diseases. Organ mercury compounds are also employed as diuretics and in the treatment of syphilis.

Mercury compounds can contaminate the environment resulted from misuse or spillage in both inorganic and organic forms. Moreover, the interconversion of inorganic and organ mercury species can be occurred in the environment, and in some case bacteria. Some of the important environmental interconversions give in Figure 2.3.

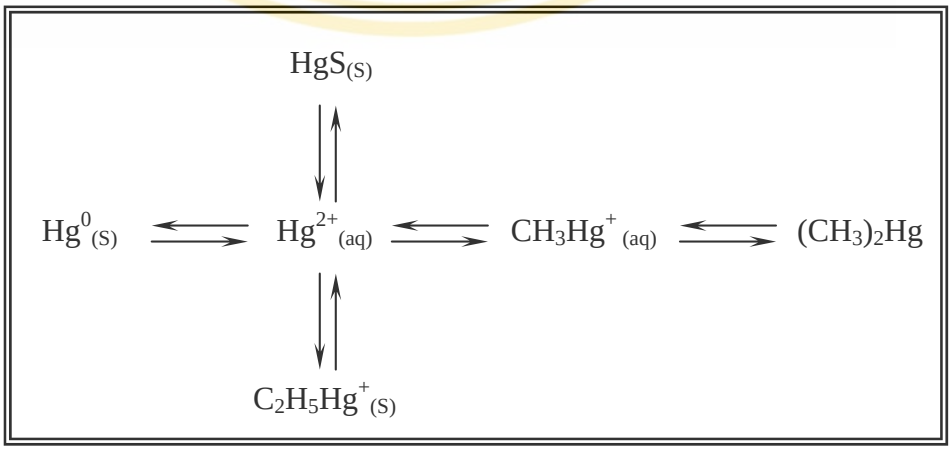

Figure 2.3 The interconversion of mercury species in the environment (Fergusson, 1991)

It is important that the methylation of inorganic mercury can be converted to more toxic methyl mercury and may occur in various environmental matrices, such as in sediment, in the water column, in soil and by humic and fulvic material. Once formed, methyl mercury may be further methylated by methylcobalamine to dimethylmercury. In aqueous solution, the second step is 6,000 times slower than the first. Furthermore, methylation may also occur in the presence of sulfide ion or hydrogen sulfide by a dismutation process (Robert, Melvin & William, 1984).

$$2CH_3Hg^+ + S^{2-} \longrightarrow (CH_3Hg)_2S \xrightarrow{h\nu} (CH_3)_2Hg + HgS \qquad (2.1)$$

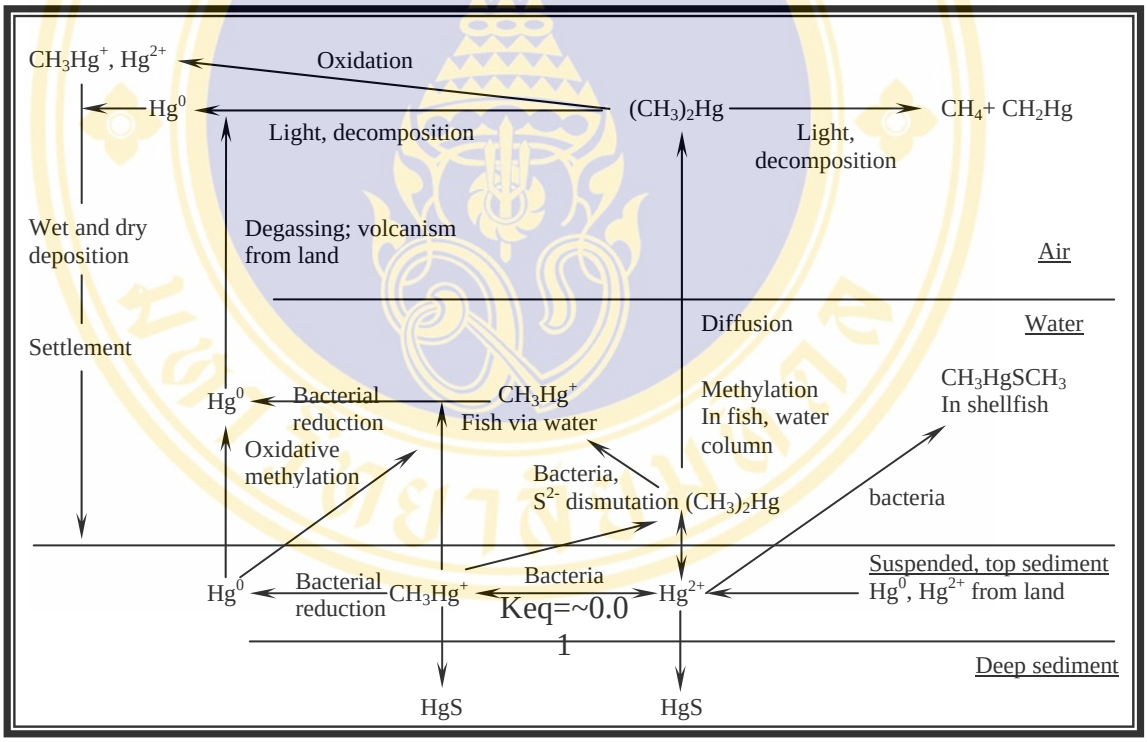

Figure 2.4 An environment mercury cycle (Fergusson, 1991)

Factors affecting methylation extent and rates include total inorganic mercury concentration, organic content, pH, redox potential (E_h), temperature, the nature of the microorganism present, sulfide levels, and the nature of complexation of mercury by natural ligands. Sulfide is a particularly important controlling factor. This

is due to formation of intractable mercuric sulfide, which is hardly methylated and to removal of any methyl mercury by dismutation promoted by sulfide ions. Following methylation, methyl mercury does not usually build up in sediments to more than about 1.5% if the total mercury present. This is an approximate equilibrium level between formation and removal. The demethyation of methyl mercury to Hg(0) and methane may occur in either photolytic decay in the atmospheric or the demethylation by microorganism occurring in water, sediment, soil, or the testine is to Hg(II) then Hg(0). A number of organo-mercury/ inorganic mercury cycles within the sediment/ water/ air system have been proposed in Figure 2.4 (Craig, 1986; Hutchison & Meema, 1987; Newman & McIntosh, 1991).

2.3 The Toxicity of Mercury Compounds to Human Health

The toxicity of mercury occurs at three levels, depending on the chemical form. The order of decreasing toxicity is alkyl mercury (esp. methyl mercury) > Hg metal vapor > Hg(II) salts and phenyl and methoxy mercury salts. Mercury has well characterized toxic effects on both the physiological and the neurological systems of body (Bidstrup, 1964; WHO, 1989; Fergusson, 1991; Newman & McIntosh, 1991).

Mercury metal: The volatility of element mercury, and its use in number of circumstances, means it is a serious toxin. The critical organs are the lungs, kidneys and the brain and in the blood stream. Once oxidized the mercury remains in the brain, whereas the free metal may move out again. The effects of mercury vapor on the respiratory tract are coughing, acute bronchial inflammation, chest pains, and in severe cases respiratory arrest. The exposures of mercury vapor have indicated a range of effects including loss of appetite, tremors, insomnia, shyness, diarrhea, vomiting, and soreness in the oral cavity. Some of these effects indicate disorders of the central and peripheral nervous systems. Renal effects from high levels of mercury vapor exposure are proteinuria.

Inorganic mercury salts: Inorganic mercury in absorbed into the body less than mercury vapor, but the amount depends on the solubility of the species. The critical organ from intake by inhalation or ingestion is the kidney and the effect has been called sublimate nephrosis. Inorganic mercury also has an adverse effect on the central nervous system. Acute ingestion of inorganic mercury can cause precipitation of protein in the gastrointestinal tract and produce gastric pain, vomiting and bloody diarrhea. Renal damage can occur, including oliguria, severe anuria with azotemia and in severe cases renal failure. For chronic exposure, the renal effects recorded are proteinuria, albuminuria, and oedema. The neurological effects are much the same as reported for other chemical forms of mercury. These include tremors, erethism fatigue, loss of memory and self-confidence, and development of idiosyncrasy. In severe case delirium with hallucinations and manic-depressive disorders has been reported.

Methyl mercury: The most serious mercury toxin is methyl mercury because of the solubility and binding ability of methyl mercury to biological ligands results in a large half-life in various organisms for this species, e.g. 60-70 days in man, much longer than for inorganic forms (3-4 days). In addition to the direct toxicity of methyl mercury, a slow decomposition to inorganic mercury may lead to secondary toxic effects as for inorganic mercury. Methyl mercury readily crosses the placental and blood-brain barriers and it causes disintegration (lysis) of cells within the brain. This may involve a CH_3Hg-S interaction. Of the methyl mercury ingested and absorbed into the body, 90-95% became associated with the red cells and 5-10% with the plasma and gets into the brain. The main areas of brain function that are damaged by methyl mercury are those that control sensory, visual, auditory, and coordination. The effects observed on human beings are initially loss of sensation at extremities and around the mouth (paresthesia) followed by loss of coordination in movement, loss of hearing, restricted visual field, blindness coma, and death. Pregnant women and unborn children are most sensitive to methyl mercury toxicity, especially the developing brain system of the child. Infants born to mothers with intakes of mercury have had serious mental disturbances, including retardation of mental and physical development.

2.4 Toxic Effects of Mercury on Plant

Phytotoxic effects of mercury compounds have been reported in several plants, including, and several other grain crops. In general, the degree of impact depends on the concentration, the formulation, the mode of application, and the cultivar.

2.4.1 Higher plant

The absorption of organic mercury from soil by plant is low, and there is a barrier to mercury translocation from plant roots to tops. Thus large increases in soil mercury levels produce only modest increases in plant mercury level by direct uptake from soil. Mercury salts in soil may be reduced by biological and chemical reactions to mercury metal or methylated compounds, which may volatilize and be taken up to leaves, a much more efficient process than via the roots. This is important for plants grown in enclosed spaces. Residues of mercury pesticide or fungicide sprays are, in some case, taken up by plants and translocated to edible portions (Patra & Sharma, 2000).

1) Seed germination, growth, and development

The seed injury caused by organic mercurial to cereals has been characterized by abnormal germination. The effect is characteristic hypertrophy of the root and coleoptile of cereal seedlings, where higher dosages of fungicides are used or when storage conditions are faulty.

The primary effect of mercury may possibly be on the embryo itself, and effects on the endosperm are of secondary importance. Mercury strongly interferes with the –SH system in living cells by causing the formation of –S-Hg-S- bridge. Such a breakdown of the normal –SH system may affect both germination and subsequent growth of the young embryo, because these tissues are particularly rich in –SH groups.

Another effect of mercury induction of thiol-containing compound is able to induce oxidative stress in plants, resulting in lipid peroxidation, K^+ leakage, and alteration of antioxidant enzyme activities. Stress often leads to production of reductive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$). Hg-induced oxidative damages in plant cells have been link to the excess production of ROS. Accumulation of ROS may be the consequence of disruption of the balance between their production and the antioxidative system activity, composed of enzymic antioxidants such as catalase (CAT), peroxidase (POD) and superoxide dismutases (SOD), and non-enzymic scavengers, e.g. glutathione, carotenoids and ascorbate. SOD is the major $O_2^{\bullet-}$ scavenger and its enzymatic action results in H_2O_2 and O_2 formation. CAT and several classes of peroxidases then scavenge the H_2O_2 produced. CAT dismutates H_2O_2 into H_2O and O_2, which is found in peroxidomes, cytosol and mitochodria. POD decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants.

Toxic effects of mercury on seed germination and growth are inhibited elongation of seedling; germination stopped, retarded root-shoot, and decreased root-shoot dry weights. Inhibition was reversed with organic acids, cations, ethylene diamine tetra acetic acid, and hormone. Two peroxidase isozyme bands were observed in seedlings (Patra & Sharma, 2000).

Allium cepa L. showed reduction in the growth of both seedling roots and shoots. The growth and enzyme responses of *Allium cepa* L. to mercury are accelerated growth, particularly at low concentrations. Catalase and peroxidase are respiratory enzymes related to growth and senescence. The toxic phase is that where the concentrations of mercury are phytotoxic and result in reduction in growth and enzyme activities (Patra & Sharma, 2000; Margaret, 1994; Subhadra et al., 1991).

Mercury (II) is able to bind with water channel proteins of root cells causing a physical obstruction to water flow and consequently affect the transportation in plant. There are also reports indicating that mercury accumulation in the root block the uptake and transport of nutrients, and induces excess ethylene production.

2) Biochemical effects

Mercury affects both light and dark reactions in photosynthesis. The extent of toxicity and the mechanism influencing the photosynthetic apparatus depend largely on the way these phenomena are investigated as well as on the age of the plants.

Hg(II) promoted senescence by decreasing chlorophyll, protein, RNA, dry weight, and activities of catalase and protease as well as by increasing free amino acid content, peroxidase activity, and the ratio of acid to alkaline pyrophosphatase activity over control values. Mercury affected functioning of the donor site of photosystem II through the inhibition of catalase activity in the water-photolysis system. It is suggested that due to the effect of mercury on the rate of electron transport on the donor side of photosystem II, the ATP: NADPH ratio was changed, leading to additional changes in physiological processes, such as the accumulation of starch and anthocyanin (Patra & Sharma, 2000).

3) Genetic and related effects

For more than 60 years, numerous experiments have been carried out to study the genetic effects of mercury compounds in experimental test system using a variety of genetic endpoint. In the earliest work on plant test systems, multi-nucleate cells were recorded in the root tips of corn seedlings exposed to solution of New Improved Ceresan (containing ethyl mercury phosphate). Abnormal mitosis led to the formulation of polyploid giant nuclei and micronuclei in these cells. C-mitosis and chromosome doubling were recorded after exposure to the fungicide Gronosan (2% ethyl mercury chloride) of the germinating grain (Patra & Sharma, 2000).

The most noticeable and consistent effect of mercurial was the induction of c-mitosis through disturbance of the spindle activity, resulting in the formation of the polyploid and aneuploid cells, and c-tumors. C-mitosis was induced at similar dosages of all organic mercurial tested, butyl mercury bromide being the most active.

In *Allium* tests, EC_{50} values for mercury were 9.0×10^{-7} M for methyl mercury chloride and 3.3×10^{-6} M for $HgCl_2$. Salts, including mercuric chloride, when

given to *Allium cepa* L. root tips, could, in varying degrees, cause different types of chromosome, nucleus, and nucleolus irregularities.

After exposure to inorganic salts of mercury in *Allium cepa* L. and *Allium sativum* L., the mitotic index in the root-tip cells was reduced and the frequency of chromosome aberrations was increases in degrees directly proportional to the concentration used and to the duration of exposure to the mercurial. The period of recovery after removal of mercury was inversely related to the concentration and duration of exposure. The lowest effective concentration tested was 10 mgL^{-1}. Cytotoxic effects of $HgCl_2$ were greater than were those of Hg_2Cl_2. *Allium sativum* L. was more resistant than was *Allium cepa*, possibly due to the greater amount of heterochromatin in the former and to the presence of lower amounts of sulfur compounds with affinity for mercury in the latter (Patra & Sharma, 2000).

2.4.2 Lower plant

Mercury, at low concentration (3μM), enhanced the intensity of room temperature fluorescence emitted by phycocyanin and induced a blue shift in the emission peak of *Spirulina* cells, indicating alternations in the energy transfer within the phycobilisomes. Selective bleaching of the b-84 chromophore of phycocyanin was inducing by mercury. The differential effect of mercury toward C-phycocyanin and allophycocyanin may be due to the difference in the protein conformation of the two compounds (Patra & Sharma, 2000).

The antimicrobial actions of mercury salts on bacteria may be a result of combination with essential sulphydryl groups. After treatment with mercuric chloride or phenyl mercury nitrate, the bacteria appear dead but are easily revived by active thiol-containing agents. Sulfur compounds without thiol groups do not show similar behavior have published extensive information on the mechanism of microbial resistance to mercury. Organomercurials are more active as bactericides of fungicides than are the inorganic salts. The toxic action of mercurial may also be related to a nonspecific inhibition of a variety of intracellular enzymes and several specific thiol-containing respiratory enzymes in vitro.

In yeast, mercury was inhibitory in the concentration range 10^{-5}-10^{-4} M and increased the lag phage and generation time. Sensitivity to the metal was enhanced by a reduction in pH. The yeast strains showed a dose-related accumulation of mercury over a thousand-fold range of concentrations in the medium.

All mercuric salts had strong inhibitory effects on spore germination and germ tube growth of the pathogen. Neither form of toxicity appeared to be linked with induction of resistance in wheat seedling (Patra & Sharma, 2000).

2.5 Environmental Standard for Mercury

Regards to Water Quality Standard in Thailand, mercury shall not be higher than 0.001 mg/L for drinking water, 0.002 mg/L for fresh water, and 0.005 mg/L for industrial wastewater (Water Quality Management Bureau, 1995). Canadian Fresh Water Sediment Quality Guideline, mercury shall not be higher than 174 μgkg^{-1} dw for sediment (Thongra-ar, 2001).

2.6 Study Area

Phanom Pha gold mining, a small scale gold mining, is located at Moo 7, Nong Pra sub district, Wang Sai Poon district, Phijit Province. In the late 1990s, gold was discovered along the north to the east of hills. The shield covers a total area of 48,300 square meters. The thickness of soil varies from 0.75-3.0 meter with the fertility in soil and fragments of rock of 0.06-23.2 gm/m^3 and 0.05-27.83 gm/m^3, respectively. Soil texture at study area is composed of sand particle 34.3%, silt 23.8%, and clay particle 41.8%. The THg in bivalves from the Khao Chet Luk Reservoir was highly elevated with the value of 3650 μgkg^{-1} ww.

The Phanom Pha Mountain's height is about 105 meters and approximately 50-155 meters from the sea level. Their surrounding areas of the mountain are,

mostly, corn and rice fields. If distinguished by the direction, the four sides have the following feature:

- North Side; adjacent to the unoccupied Phanom Pha Khoa Temple and the local school
- South Side; rice field, 55 meters from the public transport route
- West Side; mixed deciduous forest, close to the dirt road leading to the village and rice field
- East Side; adjacent to public transport route and corn field

The gold ore preparation site is 700 meters south east of the mining site, along the public transport route. According to the public record, the ore preparation site formerly is flat rice and corn field. However, due to the recent inspection of July 2000, this site is not being cultivated and already covered with grasses and weeds, altogether with disperse small bushes. There is the Dai Nam Khun Canal run east-west in the north of this area down to the Khao Chet Luk Reservoir, the receiving stream, which located in the north- west side. The east side is adjacent to the agriculture area and fallow land. (Pataranawat, 2003)

Figure 2.5 Phanom Pha Hill

Figure 2.6 Khoa Chet Luk reservoir at Phanom Pha Hills

2.7 General Background of Toxicity

Toxicology is the study of the nature and mechanism of toxic effects of substances on living organisms and other biologic systems. A toxicant is any substances that cause a deleterious biological effect when organisms are exposed to it (Newman & McIntosh, 1991; Megan, 2001).

2.7.1 Toxic effect

Toxicity results from number of dynamic processes, including absorption, distribution, metabolism of the parent compound, storage, and excretion. To produce a toxic effect on an organism, a toxicant or biotransformation product must be transferred to site of action in a target organ at a sufficiently high concentration and

for a sufficient length of time (Vernet, 1994; Newman & McIntosh, 1991). These processes are generalized in Figure 2.4 (Newman & McIntosh, 1991).

2.7.2 The dose-response relationship

A dose-response relationship describes how a chemical's effects (on people, laboratory animals, wildlife, etc.) change as exposure to the chemical increases. The relationship between dose and the biological effects is the dose-response relationship plotted. The distribution is a typical sigmoid curve, and the area refers to the cumulative response (dead) organisms (Connell, 1997; Wayne & Ming-Ho, 1995).

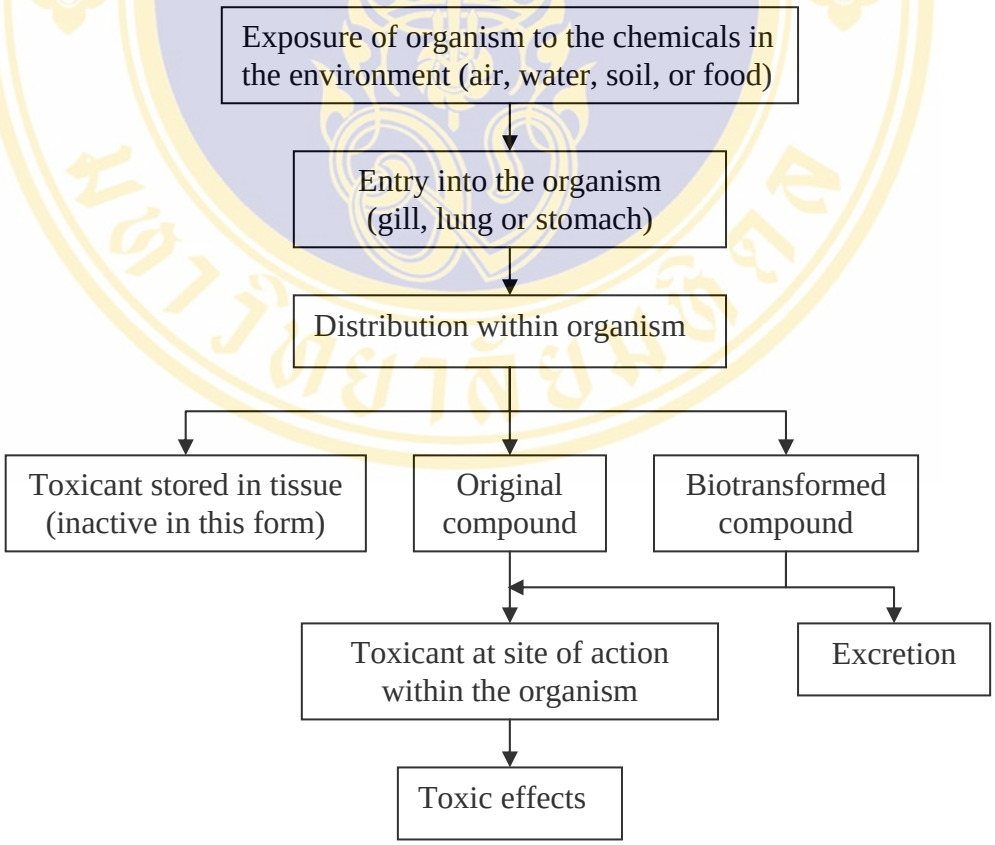

Figure 2.7 Processes for a chemical from the environment to the site of toxic action

2.7.3 Measure of toxicity

The toxicity is measured by exposing test organisms to toxicants. Numerous end points can be measured in toxicity tests such as death, fertility, movement, growth, and mutation. All measures of toxicity refer to either concentration or dose that causes a toxic effect. Concentration-based measured of toxicity state the concentration in the surrounding environment such as water, air, and soil. The dose is usually measured in milligrams per kilogram (mg/kg) or milligrams per liter (mg/L) where milligrams is the amount of chemical present, kilogram refers to the weight of the person or animal exposed and 1 is a liter of air (Newman & McIntosh, 1991; Megan, 2001).

The principle measure of the toxic effects used in toxicity studies is the 50% effect level, where 50% of the individuals are more tolerant and 50% are less tolerant. This represents the average organism in the population and exhibits the greatest consistency in experimental measurements (Newman and McIntosh, 1991). The midpoint is commonly referred to as a LD_{50}, LC_{50}, EC_{50}, and IC_{50}. The definitions are relatively straightforward (Wayne & Ming-Ho, 1995; Connell et al., 1999).

LD_{50}: The dose that causes mortality in 50% of the organisms tested estimated by graphical or computational means.

LC_{50}: The concentration that causes mortality in 50% of the organisms tested estimated by graphical or computational means.

EC_{50}: The concentration that has an effect on 50% of the organisms tested estimated by graphical or computational means. Often this parameter is used for effects that are not death.

IC_{50}: Inhibitory concentration that reduces the normal response of an organism by 50% estimated by graphical or computational means. Growth rates of algae, bacteria, and other organisms are often measured as an IC_{50}.

2.8 Experiment Testing for Toxicity (bioassay)

A bioassay is the experimental use of an organism to evaluate chemical toxicity. Results from bioassay testing are often used to establish environmental quality criteria, to calculate permit limitations for effluent discharge, and to project the potential impacts of chemical accidents. Also, they can determine actual or potential effects of hazardous waste disposal and cleanup (ed. Hare & Atterwill, 1995; Vernet, 1994). The idea behind these bioassays is that the test organism will react in a predictable way to various types of environmental contaminants.

2.8.1 Types of bioassay

The exposure of organisms in variable time period produces four main types of bioassays. First, acute toxicity experiments are conducted for short periods of time from 24 to a maximum of 96 hours. Next, sub cute tests are conducted for 10% of the normal life expectancy of the organisms. Third, multigenerational bioassays are designed to determine effect of a toxicant or toxicants to organisms in several generations. The lastly, chronic tests are conducted for normal expected lifetime of the test organisms. The length of chronic, sub acute and multigenerational bioassays vary extensively, depending on the test organism (Newman & McIntosh, 1991).

2.8.2 Types of control

In the experiment, there are three types of basic control (ed. Watson, 1995).

1) A negative control is the same test medium as the treatment groups without the test substance or solvent carrier.

2) A solvent/carrier control may be required in situations where a poorly soluble test substance is firstly dissolved in a small amount of organic solvent such as acetone, dimethyl sulphoxide-DMSO in preparing the stock solution.

3) A positive control is provided by exposing the test organisms to a toxicant known to produce a well-defined response on them. The aim is to ascertain

the health and sensitivity of the organisms to be used in the test. On this basis, reference toxicants should be toxic at low levels, stable under testing conditions and relatively nonspecific.

2.8.3 Selections of test organism (Wayne & Ming-Ho, 1995)

One of the most crucial aspects of a toxicity test is the suitability and health of the test organisms or in the case of multispecies toxicity tests, the introduced community. It is also important to clearly define the goals of the toxicity test. Toxicity tests are performed to gain an overall picture of the toxicity of a compound to a variety of species. The criteria for choosing a test species for use in toxicity test are listed below.

1) The test organism should be widely available through laboratory culture, procurement from a hatchery or other culture facility, or collection from the field.

2) The organism should be successfully maintained in the laboratory environment and available in sufficient quantities.

3) The genetics, genetic composition, and history of the culture should be known.

4) The relative sensitivities to various classes of toxicants of the test species should be known relative to the endpoints to be measured.

5) The sensitivity of the test species should be representative of the particular class or phyla that the species represents.

6) In multi-species toxicity tests the interactions among the component species should be understood.

2.8.4 The exposure systems

Apart from selection of suitable test organisms, the exposure systems used can also have an important influence on toxicity results. Four basic techniques are commonly used (ed. Watson, 1995).

1) Static tests involve exposing the test organisms in still test medium without any change of medium for the duration of the test.

2) Test medium in a recirculation test is usually pumped into the test chamber from a reservoir, returned, and circulated with in the closed system.

3) A renewable test is essentially similar to a static test except that the test medium is completely or partially renewed at fixed intervals such as every 24 hours.

4) For flow-through test, the test solution is passed though the test chambers, usually from a large reservoir, and the medium is not returned after it has passed through the test chamber.

2.8.5 Conducting a bioassay

The first step is to conduct a range-finding bioassay to find the approximate toxicity. Secondly, more accurate bioassay, termed the definitive bioassay, is the conducted in which the test organisms are exposed to five or six concentrations determined from the range-finding bioassay. The number of test organisms exposed at each concentration and replications are decided according to the objectives (Newman & McIntosh, 1991).

2.8.6 Calculating toxicological data

The simplest way to estimate toxicity is to plot the logarithm of concentration or dose of the toxicant (x-axis) against the biological response of the test organisms (y-axis). The effective concentration (EC_{50}) to estimate from this graph, a horizontal line is drawn from the point of 50% effect to where it intersects to the toxicity curve. Point is the concentration of toxicant that affects 50% of the test organisms exposed to the toxicant under the stated experimental conditions (Newman & McIntosh, 1991).

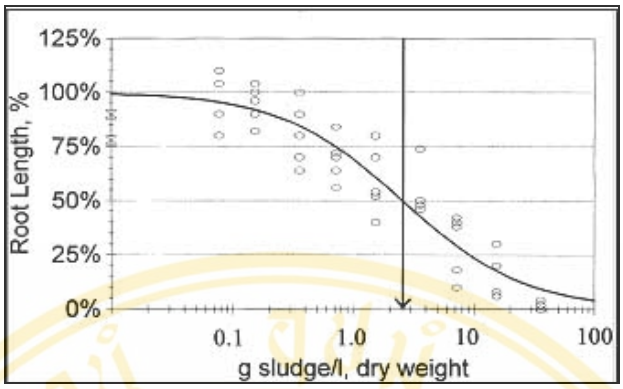

Figure 2.8 Decreasing percent of root length giving a response against the log toxicant concentration (Rank & Nielsen, 1998)

2.9 General Background of Genotoxicity

Genotoxicity is the study of the adverse effects of compounds on the genetic material of cells (DNA) and the subsequent expression of this changed (Connell, 1997). The genotoxicity is of concern because the induction of genetic damage may cause an increased incidence of genetic disease in future generations and contribute to somatic cell (other cells except sex cell) diseases including cancer in the present generation. Therefore, it is significantly important to detect compounds that affect the genetic material and to avoid human exposure to them (Connell, 1997; Wayne & Ming-Ho, 1995).

2.9.1 Basic terms using in genotoxicity

There are many terms using in genotoxicity such as genotoxic, mutagenic, and clastogenic effect. These terms are related to different endpoints used to detect such effects. Genotoxic effect is any defined damage of the DNA helix and may also include the organization of DNA such as Chromosomes and chromosome movement. Mutagenic effect is mostly an alternative term for genotoxicity, but it is sometimes meant for mutations in single or multiple genes only. Clastogenic effect is the effects of chromosome structure including the chromosomes or the chromatid (Walum,

Stenberg & Jenssen, 1990). Moreover, other terms are used in this field. For example, genotoxics are agents, which alter or rearrange genes. Such alternations are usually initiated by DNA damage and the rearrangement commonly called mutation. Any agent that causes mutation is a mutagen (ed. William, James & Roberts, 2002).

2.9.2 Basic fundamentals of genetics

2.9.2.1 Deoxyribonucleic acid (DNA)

Deoxyribonucleic acid (DNA) is the genetic material that codes for all characteristics of life. DNA consists of two long strands of nucleotides that spiral around each other, forming a helix. A nucleotide consists of a pentose (five-carbon) sugar, a phosphate group and a nitrogenous base. The nitrogenous bases are of two types: pyrimidines and purines. The pyrimidines consist of cytosine (C) and thymine (T), while the purines are adenine (A) and guanine (G). The two stands are held together by hydrogen bonds between the bases. The nitrogenous bases are always paired. For instance, a cytosine on stand will always be paired with guanine on the other stand. Likewise an adenine will always be paired with thymine. These matched pairs of bases are called DNA base pairs. There are two different DNA base pairs: AT and GC; also, chemical forms of the components of DNA are illustrated in Figure 2.9 (Connell, 1997).

DNA occurs in either a single-stranded or double-stranded form at various times in the life cycle of a cell. The double helix structure of complementary stands has the capacity to reproduce itself very accurately by using the strands as templates. During DNA replication, the strands are separated and from each strand a new double-stranded DNA is synthesized in Figure 2.10.

The other function of DNA, besides simply replicating itself, is the synthesis of proteins. Proteins may have a structural, enzymatic, or regulatory function in the organisms (Connell, 1997; ed. William, James & Roberts, 2002).

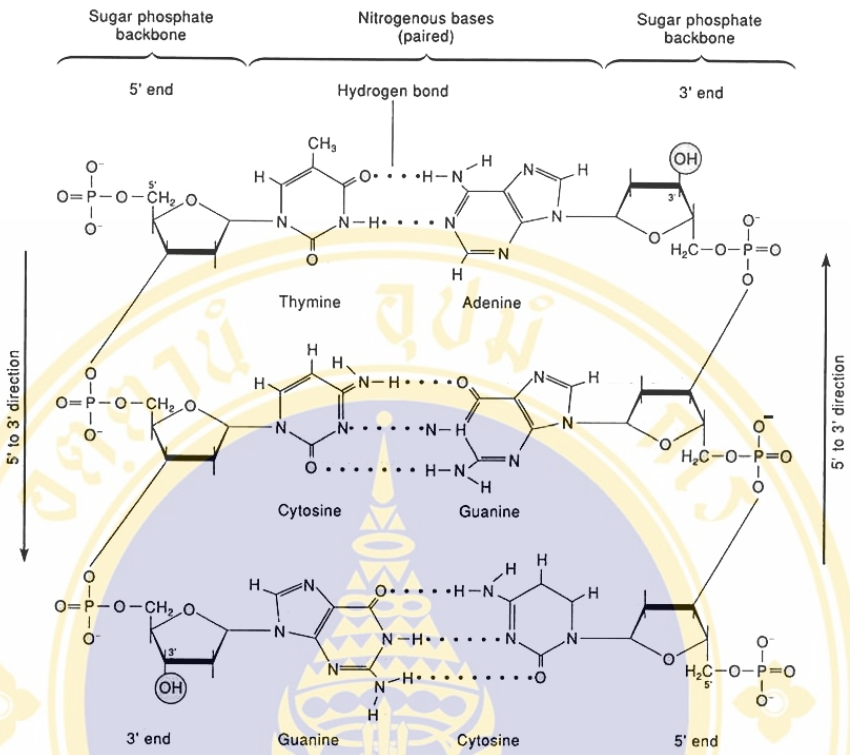

Figure 2.9 The chemical forms of the component of DNA (Moore et al., 1995)

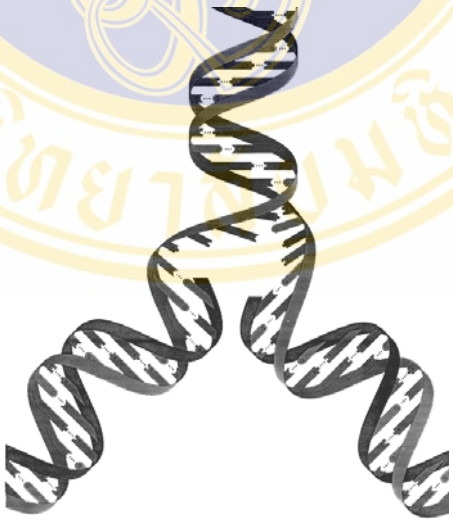

Figure 2.10 DNA stands divide and bases attach themselves to the new stand (Moore et al., 1995)

2.9.2.2 Chromosome structure

Chromosomes found in the nucleus of the cell consist of DNA and specialized proteins (predominantly histones). One chromosome is composed of two chromatids. Chromosomes also have another structural feature, the centromere, which is usually located near the center of the chromosome. Spindle fibers are made of microtubule called the spindle apparatus. The mature spindle apparatus is elongated, with its ends pointing to opposite poles of the cell. Fibers from opposite poles of the spindle attach to each chromosome on either side of its centromere, to a disc-shaped structure called the kinetochore, a complex protein that binds to the centromere. The structural chromosome is shown in Figure 2.11 (Parker, 2000).

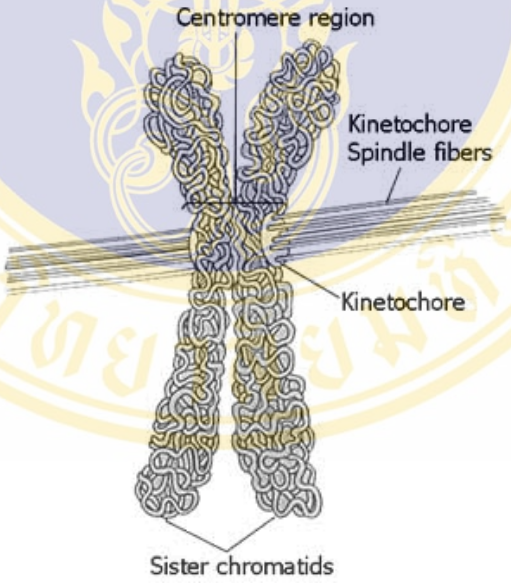

Figure 2.11 Structure of chromosome (James, 1995)

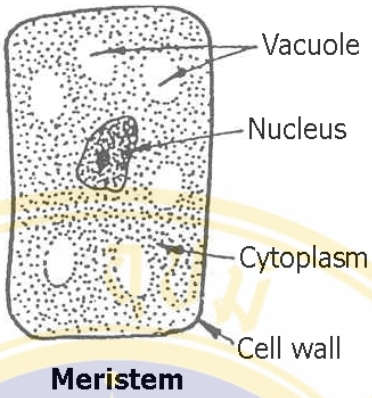

Figure 2.12 Meristem cell (Mathew & Derek, 1985)

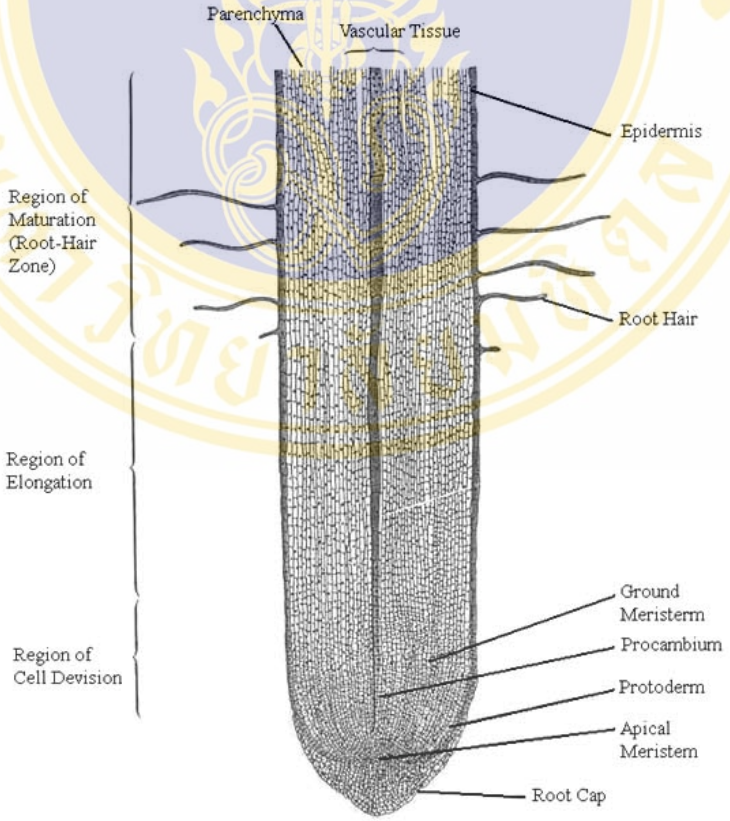

Figure 2.13 Longitudinal section through root tip (Mathew and Derek, 1985)

2.9.2.3 Cell cycle

Cells must progress through a series of steps called the cell cycle when they divide. One cell cycle is the interval of time between the formation of a cell and its division to form two new cells (Moore et al., 1995).

In plant, the process of division occurs in special regions called meristems. Meristems are usually found at the tips of roots and shoots. For example, the meristem cell and the root tip in Figure 2.12 and Figure 2.13 (Rost, 1998; Nitsri, 1998).

Traditionally, the G1, S, and G2 phases of the cycle are grouped together and called interphase. The remaining phase, cell division (M) is actually composed of two parts: mitosis (nuclear division) and cytokinesis (cytoplasmic division). Specific metabolic events occur in each cell cycle phase. The cell cycle is shown in Figure 2.15. The time required for each stage various with cell type and species. Most cells spend about 90 percent of their cell cycle in interphase (Linda, 1997).

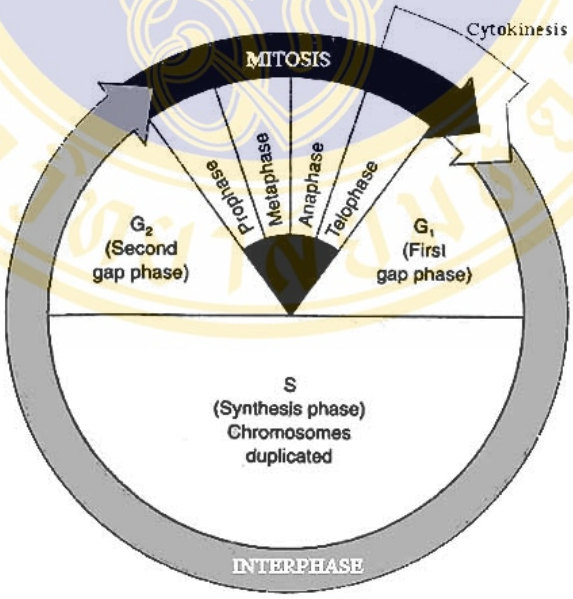

Figure 2.14 The cell cycle

1) Interphase

G1: During G1, the cell prepares itself metabolically for DNA synthesis in S phase. These preparations include both the accumulation and synthesis of specific enzymes to control DNA synthesis and the production of the DNA sub-units or nucleotides. Also, cell enlarges, organelles multiply and proteins are made during this phase.

S: S phase stands for synthesis of DNA. During this cycle, the cell duplicates its DNA molecules. The DNA synthesis is called self-replications because DNA makes exact copies of itself. In the process of DNA replication, the normal double-stranded form of DNA has the two strands separated. Therefore, enzymes commence using the original DNA strands as the template to synthesize the new replicated strand of DNA. DNA is replicated, and histones are synthesized. Each chromosome has two identical threads of DNA.

G2: The G2 phase begins after DNA synthesis is complete, when newly replicated chromatin gradually begins to coil and condense into a compact form. Cell manufactures more proteins and other substances for mitosis.

During G1, S, and G2, the DNA molecules are long and apparently tangled in the nucleus usually called chromatin.

2) Cell division (M)

a. Mitosis (nuclear division)

Mitosis occurs in somatic (non-reproductive) cell, and it plays an important role in the development of an organism. Mitosis refers to the separation of chromosome and the formation of two genetically identical daughter nuclei. It is the shortest phase of the cell cycle. Thus, it is usually followed by Cytokinesis.

Prophase: The spindle is formed and the chromatin material (DNA and protein) of the nucleus becomes shortened into well-defined chromosomes. During late prophase, each chromosome is composed of two chromatids connected by a constricted region called a kineochore; also, the nuclear membrane breaks down.

Metaphase: The chromosomes now arrange themselves on the equatorial plane of the cell. The spindle fibers compose of microtubule bundles. They extend from the poles near the ends of the cell to their attachment point on each chromosome.

The chromosomes are now distinct bodies of two closely associated halves (two chromatids). Because each gene is replicated, when the chromosomes split longitudinally, each chromatid contains a full set of genes.

Anaphase: The chromosomes do not remain long in the equatorial plane. The individual chromatids of each pair soon separate from each other and move to opposite poles of the cell. Chromatids are more slender and densely stained. Figure illustrates the mitotic chromosome separation. The kinetochores separate to give identical chromatids as shown in Figure 2.15.

Telophase: When the divided chromosomes have reached the opposite poles of the cell, telophase begins. The chromosome aggregates and begins to uncoil into long, thin chromatin strand. The nuclear enveloped and the nucleolus reformed.

b. Cytokinesis

Cytokinesis begins before telophase is finished. The partition of the cytoplasm occurs by the formation of the cell plate in the center of the two daughter cells. Then, the cells return once again to G1 of the cell cycle (Moore et al., 1995; Rost, 1998; Singh, 1993).

The cell cycles in root tip of Allium are shown in Figure 2.16.

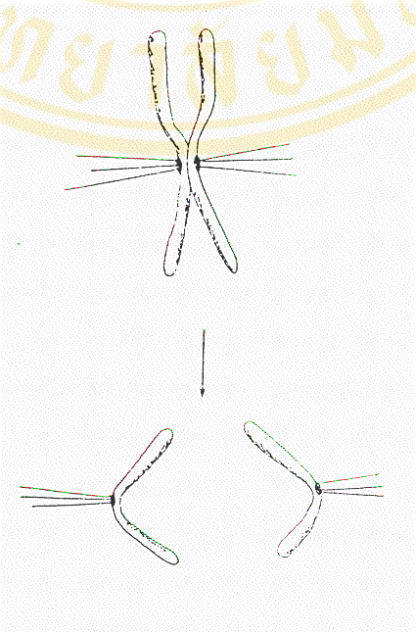

Figure 2.15 Mitotic chromosome separations (Burgess, 1989)

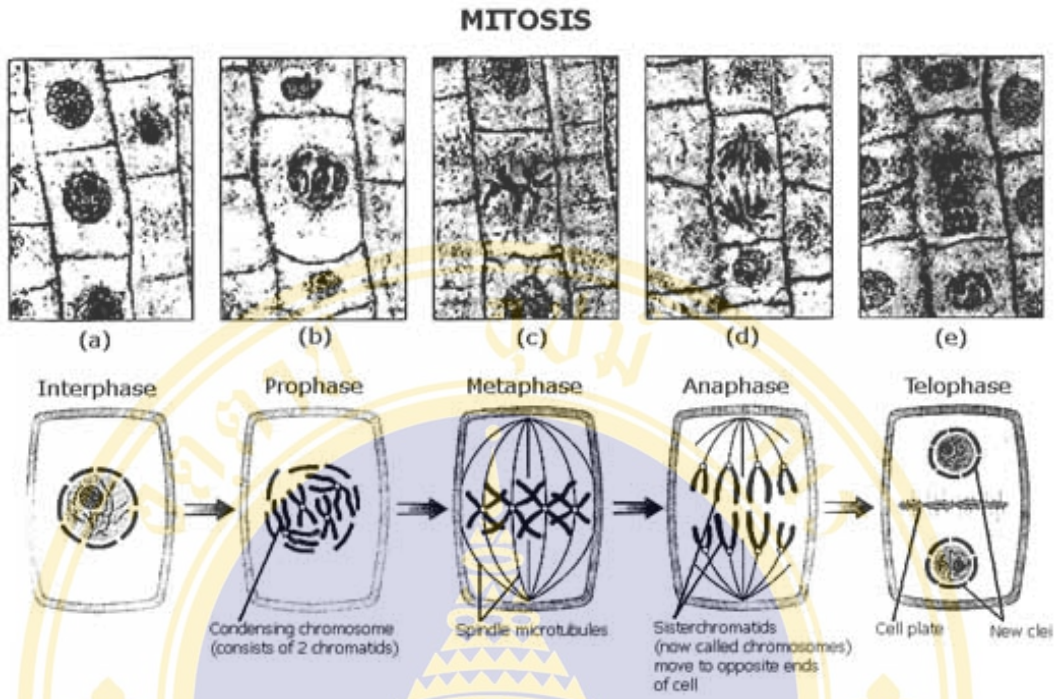

Figure 2.16 Interphase and the stage of mitosis in onion (*Allium cepa* L.) root tip cells prepared with stains (Linda, 1997)

2.9.3 Classification of Genetic Alterations

DNA damage consists of two broad categories: visible detectable through cytogenic analysis of chromosomes (macrolesions), and non-visible changes which occur at the nucleotide level (Micronesians) are shown in Figure 2.17 (Brusick, 1980).

Macrolesion is referred to the genetic lesion visualized by microscopy. Abnormal chromosome numbers result in daughter cells and may be recognized as a change in the number (gain or loss chromosomes or set of chromosomes). Changing in chromosome structure (clastogenic effects) is categorized by the abnormal chromosome morphology (ed. William, James & Roberts, 2002).

For microlesion, gene or point mutation results from the addition or deletion of nucleotides or from the substitution of one nucleotide for another during DNA replication or repair. Such molecular changes may exert their effects by

changing the function of single gene and lead to subsequent genetic alteration (ed. William, James & Roberts, 2002; ed. Brusick, 1994).

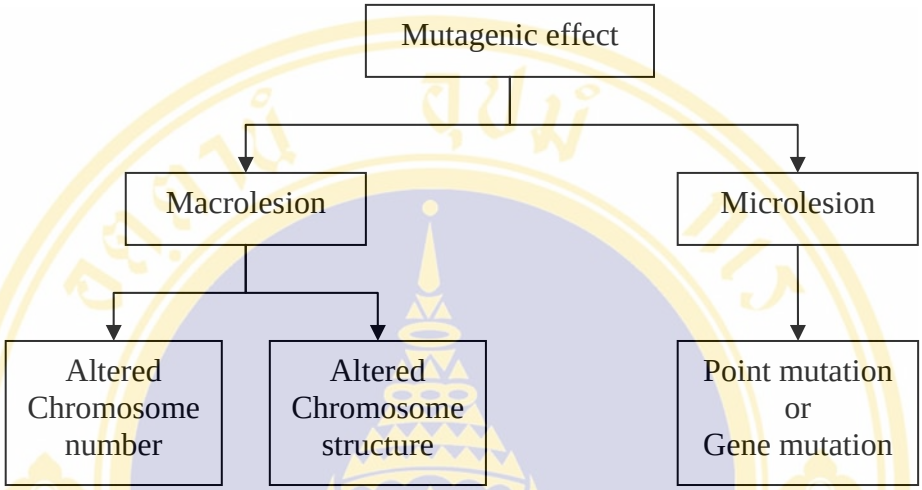

Figure 2.17 Classification of DNA change (Brusick, 1980)

2.10 Chromosome Aberration

Chromosome aberration or alteration in chromosome results from the genetic change. This change is much more DNA damage than the point mutations (Robert, Melvin & William, 1984). Chromosome aberration is defined as a modification of the genetic material and is detected by light microscope in appropriately prepared cells.

2.10.1 Mechanisms of chromosome aberration

Two theories are currently available to explain the mechanism of chromosome aberration. One is the classic “breakage-first” hypothesis. This theory assumes that the initial lesion is a break in the chromosomal backbone that is indicative of a broken DNA strand. Several possibilities exist following such an event: (1) the ends may repair normally and rejoin to form a normal chromosome; (2) the

ends may not be repaired, resulting in a permanent break; or (3) they may be mis-repaired or join with another chromosome to cause a translocation of genetic material. A second theory is the “chromatid exchange” hypothesis. If the exchange occurs with a chromatid from another chromosome, an “exchange figure” results. This theory assumes that the initial lesion is not a break and that the lesions can either be repaired directly or may interact with another lesion by process called exchange initiation (ed. William, James & Roberts, 2002).

2.10.2 Classification of chromosome aberrations

Chromosomal aberrations are classified into two major groups: structural aberration and numerical aberration. Some abnormality chromosome structure or number can occur naturally but mutagenic agents can cause the frequency of these events increasingly.

2.10.2.1 Structural chromosome aberrations

Structural aberrations are the abnormal chromosome morphology. The structural aberrations are further divided into two; chromatid-typed and chromosome-typed aberrations.

The production of both chromosome and chromatid aberration by an agent depend on the nature of the clastogen (chromosome-breaking agent) and cell cycle stage of target cells. For example, the chromosome type occurs when DNA strand is broken in G1. In contrast, the chromatid type occurs when cells are exposed in S or G2 phase (Brusick, 1980). The chromatid typed and chromosome typed aberration are shown in Figure 2.19.

Structural chromosome aberration is mainly the result of breaks in the chromatid arms shown in Figure 2.19. The definitions of each structural chromosome aberration types are shown in Table 2.1 (ed. Watson, 1995; Brusick, 1980).

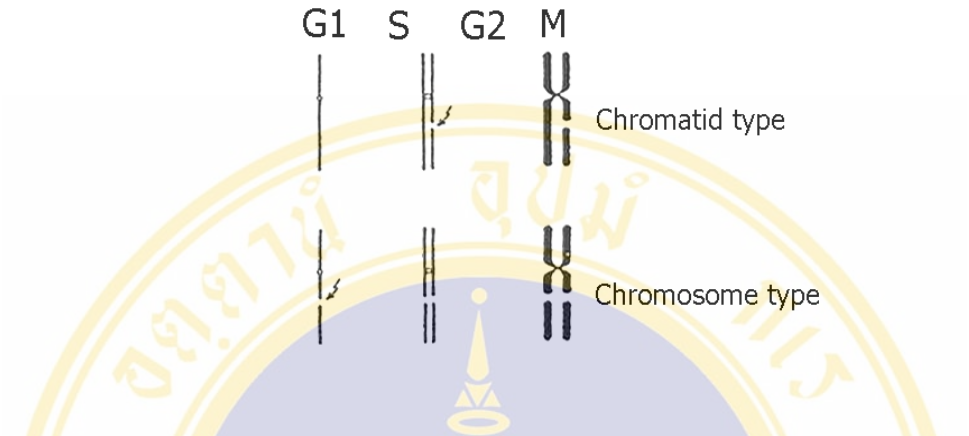

Figure 2.18 The chromatid typed and chromosome typed aberration (Amnach, 1991)

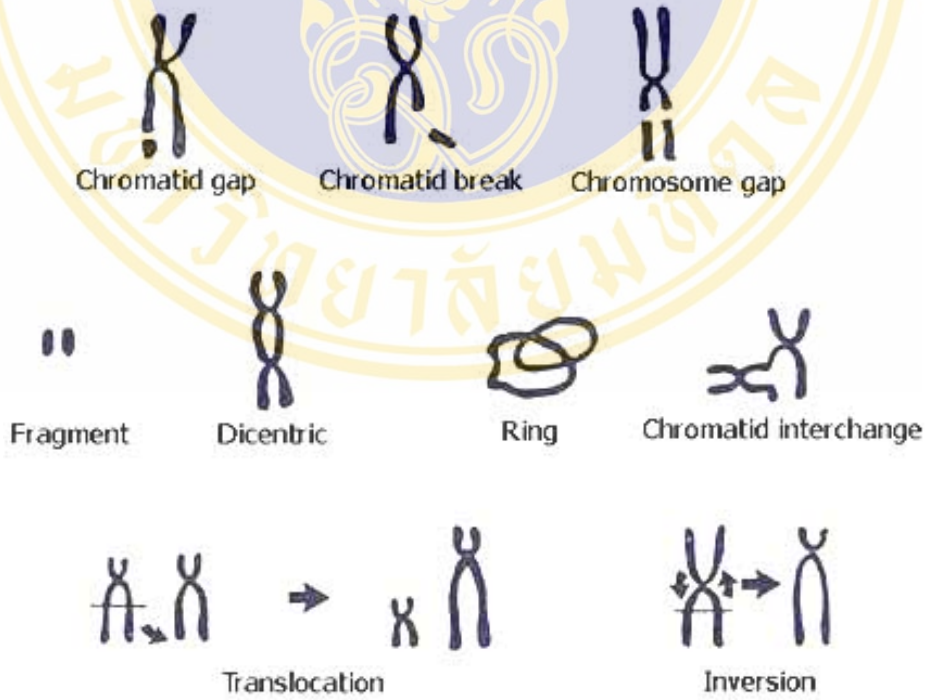

Figure 2.19 The types of structural chromosome aberration (Anderson & Conning, 1990)

Table 2.1 Definitions of Aberrations (Liewrungruang, 2003)

Term	Definition
Chromatid gap	A nonstaining region in one chromatid, the size is equal to or smaller than width the chromatids.
Chromatid break	A nonstaining region in one chromatid larger than the width of chromatid.
Chromatid deletion	Deleted material at the end of one chromatid.
Chromatid exchange	
- Chromatid interchange	Exchange some parts of chromatid between chromosomes.
- Chromatid intrachange	Exchange some parts of chromatid within the same chromosomes.
Chromosome gap	A nonstaining region in sister chromatids, the size is equal to or smaller than width the chromatids.
Chromosome break	A nonstaining region in two sister chromatid larger than the width of chromatid.
Fragment	A short segment of chromatid without a centromere.
Acentic- chromosome-fragment	Two parallel chromatid segments without a centromere.
Dicentric chromosome	A chromosome containing two centromeres.
Translocation	Obvious transfer to material between two or more chromosomes
Inversion	The alteration of a DNA molecule by removing a fragment, reversing its orientation, and putting it back into its place in the same chromosome.
Ring chromosome	Deletion at both the ends of chromosome and joining of the ends of the two chromosome arms.

2.10.2.2 Numerical chromosome aberrations

Numerical chromosome aberrations caused by an incomplete separation of replicated chromosomes during cell division are categorized into two types; aneuploidy and polyploidy. Aneuploid and polyploid cells have chromosome numbers that differ from the normal number for the species. In aneuploidy, the deviation in chromosome number involves one or a few chromosomes while in polyploidy, the alteration involves complete sets of chromosomes especially haploid (n). For instance, in humans, where the normally diploid (2n) chromosome number is 46, cell with 45 or 47 chromosome would be described as aneuploid. In contrast, cells with 69 chromosomes would be described as polyploid, in this case triploid (3n) (Amdur, Doull & Klassen, 1992).

2.11 Genotoxicity Tests by Phytoassays

Higher plants provide valuable genetic assay systems for screening and monitoring environmental pollutants. Plant cells can be investigated under a wide range of environmental conditions such as pH, water content, and temperature. Chromosomal organization is similar to the human system (Li & Hefich, 1991). The results from higher plant genetic assays contribute to protect the public from agents that cause mutation and cancer. Moreover, higher plant genetic assays are inexpensive and easy to handle and ideal for use in developing countries (Grant, 2006). The criteria from Gene-Tox Program of U.S.EPA are listed in Table 2.2 (Ecobichon, 1997).

Table 2.2 Selection criteria for study on higher plant genetic systems (Liewrungruang, 2003)

Selection criteria
1) Ease of use
2) Well-developed methodology
3) Used by number of investigation
4) A large data base on chemical mutagens
5) Adaptability of protocols to different climatic conditions
6) Ease of distribution of source material

2.12 The Allium Test

The first, Levan used the Allium test system using onion (*Allium cepa* L.) in 1938. Since that time, the basic test system has been developed to use as environmental monitoring. Root growth inhibition and adverse effects on chromosomes provide toxicity and genotoxicity (ed. Hare & Atterwill, 1995). The positive results from the Allium test should be considered as warning or an indicator that the tested chemicals might be risk to human health and to the environment (Fiskesjo, 1985).

The Allium test is one of the most useful and convenient methods to monitor toxicity and genotoxicity. The root tip is often the first part of any plant that is likely to come into contact with chemicals and pollutants found in soil and water supplies. Observation of the root tip system is particularly sensitive to the harmful effects of such environmental contaminants. Gross effects can be quantified by measurement of root growth inhibition, whereas examination of the chromosomes in the root tip cells can indicate mutagenic effect (ed. Hare & Atterwill, 1995). Also, the root cells posses certain enzymes, the mixed function oxidases, which activate many promutagens to mutagens. This activating system will improve the detection of those chemicals, which exert their toxic effect in a reactive metabolite (Fiskesjo, 1989). The simple growth test method may be performed by anybody without any specific training. The results demonstrated as short or long root bundles are easy to interpret as environmental damage. The *Allium* chromosome being quite large and allows a detailed analysis. The bulbs can be easily stored and handled. In addition, root with meristems can grow profusely at regular intervals (Sharma & Sharma, 1999).

Because of many advantages of the Allium test, it is gradually in the parts of genotoxicity testing in various organizations such as National Swedish Environmental Protection Board, the Ergatt/FRAME Data bank of In Vitro Techniques in Toxicology (INVITTOX) and U.S. EPA in Gene-Tox Program (Ecobichon, 1997; Fiskesjo, 1994a).

2.12.1 Technical materials and methods of the Allium test

1) The test organism

Bulbs of *Allium cepa* L. should be stored under dry conditions at 10-20°C. Bulbs may be kept more than a year until material from the next season is available. However, some 20% bulbs may dry up or be destroyed by mold. Thus, the number of onions should be stored about 3 or 4 times the number needed for experiments. The variation within the population is compensated for the use of a series of equal-size onion (Fiskesjo, 1985). Fiskesjo recommended that the Allium test of river water or industrial wastewater should be placed the series of onion bulb directly in the test solutions without previous germination of the root tips (Linda, 1997). Twelve onions are use in each series, and the best 10 are selected for measuring root length. When small amounts of samples are available, five to six onions are set up in each series (Fiskesjo, 1994b).

2) Growth medium for the Allium test

- *Growth medium*

a. Nutrient solution for plant growth

Nutrient	Stock	Final concentration
$Ca(NO_3)_2.4H_2O$	1.0 mM	0.1 mM
KNO_3	2.0 mM	0.2 mM
$MgSO_4.7H_2O$	1.0 mM	0.1 mM
KH_2PO_4	1.0 mM	0.1 mM
Fe-EDTA.$3H_2O$	0.2 mM	0.02 mM

b. Trace elements

$MnSo_4$	3.6 μM	0.364 μM
$CuCl_2$	0.48 μM	0.048 μM
Na_2MoO_4	0.0078 μM	0.00078 μM
$ZnSO_4$	0.0042 μM	0.00042 μM
H_3BO_3	3.7 μM	0.37 μM

The stock solution should be ten-fold dilution with distilled water, and the pH adjusted to 7 before test start (Fiskesjo, 1994b).

- *Tap water as growth medium*

The most convenient growth medium is to use tap water for control and for the dilution of tested chemicals. The tap water must be good quality; with aluminium in water from private well with low pH. If copper pipes are used the transport of drinking water, health authorities in Sweden recommend running the water for three minutes before sampling. Because copper concentration in higher than 0.05 mg/L. It will inhibit the Allium roots (Fiskesjo, 1994b).

3) Preparation of the orcein stain (2% aceto-orcein)

Acetic acid (150 ml) in a flask is heated and does not boiled on a hotplate. The orcein (5 g) are added carefully to acetic acid because the foam will come up. Then, the orcein solution is placed at room temperature and covered with aluminium foil for 2 or 4 days. During that time, the flasks should be shaking several times a day. Next, distilled water (150 ml) is added to the flask. The orcein solution is filtered and stored in a dark bottle at room temperature (Fiskesjo, 1994b).

2.12.2 Test procedures

2.12.2.1 Root growth inhibition

The inhibition of root growth is one of the most rapid responses to toxic concentrations of a heavy metal and has been frequently used in many tolerance tests (Wong & Bradshaw, 1982). The purpose of this part is to study the effects of tested chemicals on the root growth of the Allium by measuring the root growth as percent of control to estimate the toxicity levels (Fiskesjo, 1994b).

1) Prior to test start, the outer scales of the bulbs and the brownish bottom plate should be removed. If many onions are to be the same time, the peeled bulbs should be put into fresh water during the continued cleansing procedure to protect the

root primordial from drying. The bottom side of bulb is shown in Figure 2.20 (Fiskesjo, 1994b).

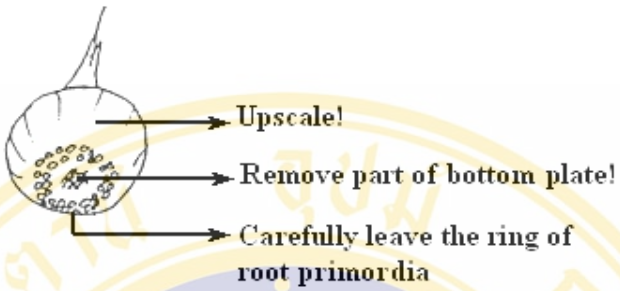

Figure 2.20 Bulb from bottom side

2) Bulbs from this pool are placed on a soft layer of paper and directly put on test tubes, filled with the test liquid. The experiments should be performed in relatively constant room temperature at about 20°C and protected against direct sun light. The test procedure may follow either the original form or the modified form of the method. In original form, the root growth is started in tap water of good quality. When the roots have reached the length of 1-2 cm, the onions are placed directly in the test liquids without the previous germination of the root tips.

3) Twelve onions are placed in test in each concentration for 4 days (96 hours), and the liquid is changed every 24 hours. After 4 days, the ten onions that appear to be developing the best in each series are selected for measuring root length (Fiskesjo, 1985).

2.12.2.2 Mitotic index and chromosome aberration

The purpose of this part is to assess chromosome damage and cell division disturbances providing additional information about genotoxicity (ed. Hare & Atterwill, 1995).

1) Three or five onions are sufficient for this part (Fiskesjo, 1994b). The prepared onions for the experiment are the same as the toxicity test. The different is to place the onions in the control water for two days or to begin root germination about 2 cm. Then, the onions are change to test samples and negative and positive controls.

MMS is recommended as positive control in 10 mg/L (Nielsen & Rank, 1994). The test solutions should be changed every 24 hours for 48 hours. Growing root tips are cut and fixed for the later preparation of slides after the growth period of 48 hours.

2) Fixing the roots is mix 6 cm^3 absolute alcohol with 2 cm^3 glacial acetic acid. This mixture is called Farmer's fluid and must be freshly prepared. The tips can sit in the fixative up to 24 hours .Once added to the Farmer's fluid; the root tips can be stored into a solution of 70% ethyl alcohol for many months in a refrigerator (Hill, 2007; Science & plants of schools, 1999).

3) The root tips are washed in the cold water for 4-5 minutes, and then dry them on filter paper. There are transferred to pre-heated 1M hydrochloric acid at 60°C for 5 minutes, and washed cold water again. The root tip is taken out of water and placed on a micro slide. Cut each root tip about 1-2 mm from the growing tip. Add a drop of 2% acetoorcein onto the tip. Cover with a cover slip, and apply pressure to the cover slip with a pencil eraser until the cells in the tip spread out in a single layer. Then, the nail vanish is sealed along the cover glass edges. This slide will keep a few months in refrigerator without the loss of color and contrast within the cells (Carolina, 2007; Science & plants of schools, 1999; Fiskesjo, 1994b).

2.12.3 Expression of result

2.12.3.1 Macroscopic parameters

As standard observations the following macroscopic parameters may be used (ed. Hare & Atterwill, 1995; Fiskesjo, 1985).

- *Root form*:

Crochet hooks is the bending of the roots that may occur especially after treatment with certain metal salts.

C-tumor formation is observed as a swelling of the root tips. This may be observed after 3-5 days of cultivation after various types of treatment, but it is more obvious after a longer time period.

- *Root length*:

A special comment on the procedure for measuring of the root lengths may be needed. Normally, a ruler measures the length of the whole root bundle outside the test tube. A method gives one value for each bulb. A more accurate way to measuring would be to measure each root from each bulb, requiring the removal of the roots and the termination. In comparison of the two different ways of root length measuring, the mean values of bundle length are somewhat lower than the mean value of all roots. However, the relationship of both results is nearly the same (about 40%). Therefore, this whole-bundle measuring is to be preferred because it is reliability, time saving and continuity in observation (Fiskesjo, 1985).

The root lengths are plotted in a diagram between log concentration of treatment and mean root length in % of control to find the toxicity levels such as the EC_{10}, EC_{50}, and EC_{90}, respectively. They mean effective concentration inhibiting 10, 50, and 90 % root growth in relation to control of these values, EC_{50} is the most commonly used parameter showing the degree of toxicity.

The root growth inhibited over 45% strongly indicates that the presence of toxic substances (Kincl et al., 1996). The green leaves sprouting from the growing onions may also be observed and photograph to indicate toxicity between the tested chemicals and the control (Fiskesjo, 1985).

2.12.3.2 Microscopic parameters

The following parameters are suggested for genotoxicity test (Fiskesjo, 1985; Fiskesjo, 1994b).

- *Mitotic index* (MI): The number of dividing cells per 300 observed cells in each slide or 2,000 cells in each concentration is sufficient.

- *Chromosome aberration*: Chromosome aberration cells are observed and classified in 100 cells of normal metaphase and anaphase.

1) Stickiness Sticky chromosomes indicate highly toxic and usually are not reversible leading to cell death.

2) Chromosome bridges and/or fragments: These effects result from chromosome and/or chromatid breaks. They refer to clastogenic effects and are used as the indicator of mutagenicity.

3) Vagrant (laggard) chromosome (weak c-mitosis): It indicates the risk of aneuploidy.

4) C-mitosis: It indicates weak toxic effect that may be reversible. It is described as inactivation of the spindle followed by a random scattering of the condensed chromosomes in the cell.

5) Micronuclei: Small nuclei are found in the cytoplasm of cells.

6) Multipolar: There are many polars in one nucleus (Liu Jiang & Li, 1992; ed. Wang, Gorusuch & Hughes, 1997).

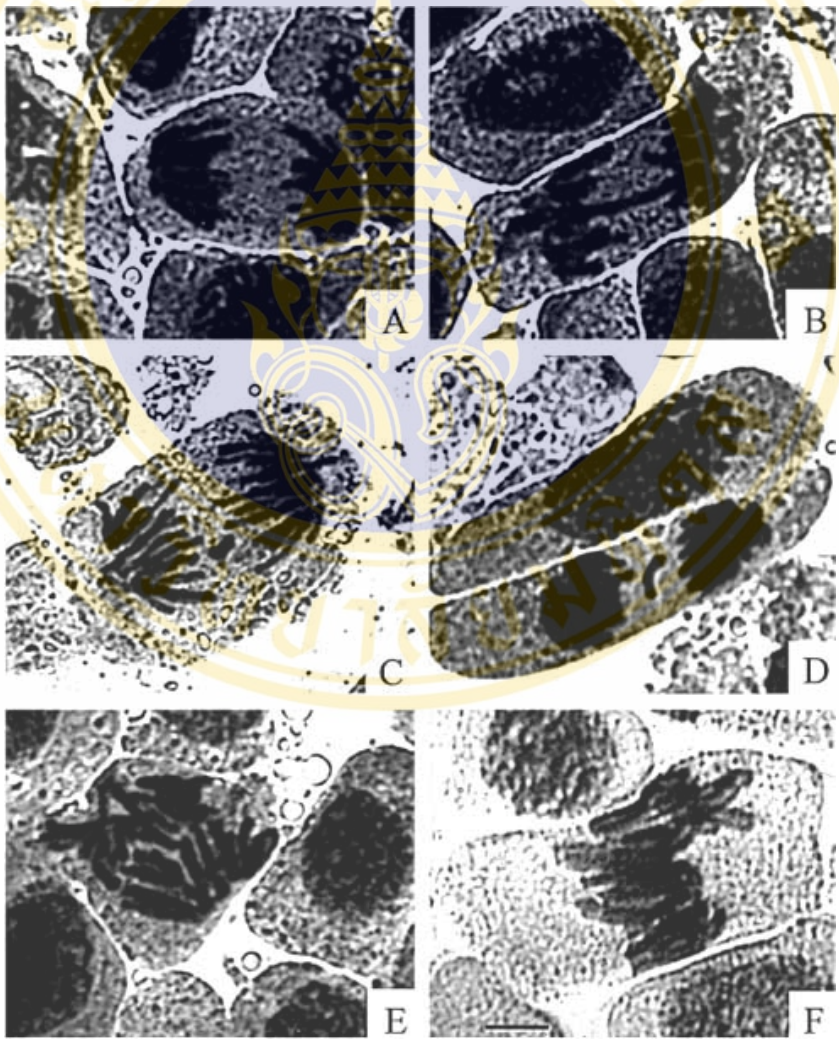

Figure 2.21 Chromosomal aberrations observed in root tip cells of *Allium cepa*: A—normal anaphase; B—cell with a bridge; C—anaphase cellwith a fragment; D—telophase cell with a fragment; E—vagrant chromosomes; F—c-mitosis. Scales10 mm. (Kovalchuk et al., 1998)

2.13 Shallot (*Allium ascalonicum* L.)

Shallots are grown extensively in the humid regions of the tropic. In Thailand, it is economical importance, high domestic consumption and potential for export. The main planting areas are three in the north: Chaing Mai, Lampoon, and Uttraradit and the Northeast: Srisaket (Rabinowitch & Brewster, 1990).

2.13.1 Taxonomic classification (Rabinowitch & Brewster, 1990)

Class Monocotyledones
Superorder Liliiflorae
Order Asparagus
Family Amaryllidaceae
Genus *Allium*
Species *Allium ascalonicum* L.

2.13.2 Botany

Shallot belongs to the genus Allium, which includes several cultivated crops such as onion, garlic, and leek. Generally, shallot bulb are pear-shaped, narrowed in the upper part into a rather long point and covered with a russet colored skin of a coppery red color in the lower part. Shallot is distinguished from normal bulb onions by their habit of multiplying. A single shallot bulb usually contains several initial shoots, and after the bulb is planted, several leafy shoots grow out from it. Each shoot rapidly produces a small bulb; thus a cluster form is attached to the original base plate. The bulbs can be separated and the process repeated during the next growing season. Each shallot bulb produces a cluster of 6 or more bulbs. The basic chromosome numbers (n) of shallots is 8 or 2n=16 (Ecobichon, 1997; ed. Hare & Atterwill, 1995). The shallot bulb and a cluster form are shown in Figure 2.22.

Figure 2.22 Shallot bulb

2.13.3 Production

2.13.3.1 Soil and climate

Shallots are grown in different kinds of soils, but they favor a well structured of sandy loam or loamy soil with proper drainage and absence of persistent weeds. The pH interval from 5.5 to 6.5 is the major requirement for the successful production of shallots. The size and the quality of bulb depend on the soil type, fertility, and cultivate. Soils that are too alkaline or acidic are not suitable for bulb growth. The optimum temperature is around 13-25°C, and photoperiod is 8-10 hours per day (Botany.com, 2004).

2.13.3.2 Propagation and cultural management

Shallots can be grown from seed and sets (bulb division). After three months of dormancy the shallot bulbs can be planted. The “mother” bulbs divide forming several bulbs. For uniformity in production, planting similar size bulbs is essential. The weak clumps and the smallest bulbs are discarded. To save bulbs for the following year, only the highest quality bulbs from the highest quality clumps are stored. These clumps should be as free from diseases as possible. Shallots are shallowing rooted; thus, they should be irrigated frequently. Green shallots can be harvested in 30 to 60 days, mature bulbs in 90 to 120 days (Botany.com, 2004).

2.13.3.3 Storage

Because of their small size, shallots tent to pack closely; so they should not be placed into deep piles. Shallots should be stored in good aeration. This is important to remove excessive moisture and to minimize diseases. Low relative humidity and low temperatures are important to keep high quality of shallot and free from sprouting and root growth (Botany.com, 2004; Sunee, 1988).

2.14 Application of the Allium Test

2.14.1 Test of tap water and distilled water

Fiskesjo, G. studied a comparison of tap water and distilled water using as control water for 10 days. The results indicated that the root growth in tap water at pH 7.8 (copper less than 0.05 mg/L) increased nearly linear to around 12 cm in 10 days, whereas the growth in distilled water at pH 5.6 stopped completely after the fourth day (2.5 cm). Therefore, tap water was recommended as control water, because the distilled water usually lacks of nutritious for root growth (Fiskesjo, 1985).

2.14.2 Test of pure chemicals

- HCl and NaOH

Fiskesjo (1985) studies the effect of pH on the root growth. The pH values were between 2 and 12 for 5 days to determine and were set by addition of HCl and NaOH to tap water (pH 7.3). The results shown that the pH interval from 3.5 to 11 and did not cause growth restrictions because root cells were able to buffer the test sample to a pH of about 6.7 for permitting growth.

- MMS and EMS

Rank and Nielson (1997) studied about Methyl Methane Sulfonate (MMS) and Ethyl Methane Sulfonate (EMS) are used as positive controls in mutagenicity

testing. However, they recommend that MMS (10 mg/L) be found to be about ten times more potent inducing chromosome aberrations than EMS (100 mg/L). Record of micronuclei in interphase cells showed, that this endpoint dose not give more information of clastogenicity than recording of chromosome aberration in anaphase-telophase cells.

- $HgCl_2$ and $MeHgCl_2$

In this research, Subhadra et al. (1991) have evaluated sensitivity of *Lemna minor* and roots of *Allium cepa* L. to low levels of inorganic and organic mercury, as indicated by growth and the activity of two growth-related enzymes, namely catalase and peroxidase. The EC_{50} for $HgCl_2$, base on root growth in *A. cepa*, was 2 mg/L, but the EC_{50} for $MeHgCl_2$ was not found within the range of concentration tested (0.0001-0.1 mg/L). The concentration of $HgCl_2$ and $MeHgCl_2$ that induced the highest enzymic activity in *Allium cepa* L. root were 0.05 mg/L and 0.001 mg/L, respectively.

- $HgCl_2$

Agar and Uysal (1997) studied the cytogenetic effects of mercuric chloride on root tip of *Allium cepa* L. at different concentrations. The roots were treated with 0.5,1.0, and 5.0 mg of the mercuric chloride for 6 and 24 hours and then allowed to recover for 12, 24, and 48 hours. It was found that, the mercuric chloride has a marked mitodepressive action on mitosis. Mitotic abnormalities were increased and mitotic indexes were decreased depending on the concentration of mercuric.

2.14.3 Test of natural water

- Braan River, Sweden

The classic case for using the Allium test to monitor source of pollutant is Braan River, Sweden case. The BT Kemi AB, a herbicide-producing factory, released the wastewater to the Braan River and its surrounding in southern Sweden. Root tips of the common onion, *Allium cepa*, were exposed to samples of the river water both upstream and downstream of suspected source of contaminant. The degree of toxicity was estimated by the length of the root bundle relative to the root length of control.

The results from the Allium test were admitted as evidence in lawsuit against the contaminating factory and helped the claimants to win the case (Fiskesjo, 1994a).

2.14.4 Test of waste water

In 1994, Nielsen and Rank applied Allium test to screening of toxicity and genotoxicity in wastewater. Wastewater was collected from two municipal wastewater treatment plants and twelve different industries representing five lines of business (chemical, metallic, petrochemical, pulp- and paper, and textile dye industries). Effect on the growth of Allium roots was measured after five days of exposure. Growth inhibition values, EC_{50} and EC_{30}, showed no toxic effect for eight of the fourteen plants. The most toxic effect was found in wastewater from one of the pulp- and paper plants. Allium root tip cells were analyzed for chromosome aberrations after 24 h of exposure. Wastewater from nine of the fourteen plants was able to induce chromosome aberrations at a statistically significant level. The textile dye industry was the only line of business, which did not show any genotoxic effect. Three of the plants (municipal wastewater, metallic, and pulp- and paper) showed genotoxicity in spite of being nontoxic in the growth inhibition experiment (Nielsen & Rank, 1994).

Smaka-Kincl et al. (1996) applied Allium test for evaluated water quality. Test liquids are divided into 8 quality classes: the first class is the least polluted surface water, the second and the third classes are more polluted surface water, the forth and the fifth classes are biological treatment plant output waters, the sixth till the eighth quality classes are untreated waste waters.

Liewrungruang (2003) studies the modified Allium test-using shallot *(Allium ascalonicum* L.*)* for screening toxicity and genotoxicity of chrome plating wastewater. The modified Allium test detected total chromium concentration of the chrome plating wastewater in the range of 5-6 mg/L at EC_{50} value by root growth inhibition. The mitotic index of treat groups (EC_{30}, EC_{50}, and EC_{70}) was significantly decreased from that of the control group. The toxicity of wastewater at EC_{50} and EC_{70} caused chromosome abnormality.

Grisolia et al. (2005) evaluated the genotoxicity of domestic sewage in muniplal wastewater treatment plant. The study was used *Allium cepa* L. root tip cells through cytological parameters such as aberrant cells in anaphase-telophase and mitotic index. In Allium test, each of four stages of the wastewater treatment routine was analyzed, i.e., crude sewage, primary effluent, secondary effluent, and tertiary effluent. The number of aberrant cells did not differ among the four stages tested, nor when compared wit the control. At all stages, the most concentrated sample were more toxic than the respective diluted samples, as demonstrated by the decreased mitotic index.

2.14.5 Test of soil

Kong and Ma (199) used three major plant bioassays: the *Allium* root anaphase aberration (*Allium*-AA), the *Tradescantia*-micronucleus (Trad-MCN), and the *Tradescantia* stamen hair mutation (Trad-SHM tests) for detect genotoxicity of contaminated soil and shallow well water. Shallow well water samples were collected from five different farms and soil solution were extracted with distrilled water or dimethyl sulfoxide (DMSO) from pesticide-contaminated and pesticide-free soil samples. Soil solutions of DMSO extracts was higher genotoxicity than that of distrilled water extracts. Among these three plant bioassays, the trad-MCN test has the highest efficiency.

Kovalchuk et al. (1998) applied the *Allium cepa* L. chromosome aberration test for measure genotoxicity of soil. The soil in Ukraine became contaminated by the accident on Chernobyl Nuclear Power Plant reactor IV in April 1986. The accident released radioactive material into the biosphere and to the formation of a complex pattern of nuclear contamination over a large area. They observed a strong, significant correlation of ^{137}Cs activity of soil sample. The results showed high toxicity and genotoxicity of radioactively polluted soil.

2.14.6 Test of solid waste

In 1998, Rank and Nielsen were applied *Allium cepa* L. genotoxicity analyses wastewater sludge. They were sampled during three winter periods from three Danish municipal wastewater treatment plants differing in size and industrial load. The toxicity of the sludge wastes in the *Allium* root inhibition assay, and the results expressed as EC_{30} and EC_{50} values showed that the toxicity could be positive correlated to the industrial load. However, when genotoxicity was tested at concentrations corresponding to the EC_{30} and EC_{50} values in the *Allium cepa* L. anaphase-telophase assay, only two sludge samples from the smallest plant with the lowest industrial load induced significant chromosome aberrations. Concentrations of the heavy metal's Pb, Ni, Cr, Zn, Cu, and Cd were also determined and could partly be correlated with the toxicity of the sludge and the industrial load of the treatment plants.

Chandra et al. (2005) studied the possible genotoxicity effects of leachates from solid waste of metal and dye industry using the *Allium cepa* L. chromosome aberration assay. The results revealed that both metal waste leachate and dye waste leachate contained high concentrations of chromium, nickel, and iron that significantly induced cytogenetic alterations. The investigation inferred that abnormalities caused by metal waste leachate were higher than dye waste leachate both in soil and aqueous media. There toxic responses may have relied on raised heavy metal concentrations metal-based than dye industrial waste.

2.14.7 Test of pesticide

The presence of pesticide in vegetables and fruit is a source of human exposure to toxic and genetoxic chemicals. Biscardi et al. (2003) monitored concurrently the presence of pesticides and genotoxic compounds extracted from vegetable and fruits from the markets of region are Southern Italy. The extracts were analysed for genotoxicity with two plant tests in *Allium cepa* L. roots; the micronucleus test and the chromosomal abberation test: *Allium cepa* L. tests were

sensitive for monitoring genotoxicity in food extracts. The micronucleus test in interphase cells gave much higher mutagenicity than the chromosomal aberration test in anaphase-telophase cells (Biscardi et al., 2003).

Atrazine is used as pesticide; its release into the environment. Bolle et al. (2004) presented study new data on the genotoxic potential of antrazine using the *Allium cepa* L. chromosome aberration test. This assay detected the clastogenicity of antrazine at the two highest test concentrations (1 and 5 μg/L) that are likely to be encountered in water, a common site of antrazine contamination (Bolle et al., 2004).

2.14.8 Test of plant toxic

Soliman (2001) studied the genotoxic of aqueous extracts of neem (*Azadirachta indica* A. Juss.). Meliaceae leaves, kernels and seed coats were evaluated using *Allium cepa* L. chromosome aberration assay. Neem seed coat extract was the least effective in its ability to inhibit cell devision, where as kernel extract was the most effective. Bridges chromosome was the most frequent kind of aberration in dividing cells. Neem seed kernel extract was more chromosomal aberrations than neem leaf and neem seed coat extract.

CHAPTER III

MATERIALS AND METHODS

3.1 Introduction

This study was achieved to determine the toxicity and genotoxicity of mercury (Hg) in mercuric chloride on the shallot root (*Allium ascalonicum* L.). The toxicity was measured by root growth inhibition and calculated in terms of toxicity levels. The genotoxicity was determined by mitotic index and chromosome aberrations. Description of procedures and experimental techniques are presented in this chapter.

3.2 Experimental Set-Up

3.2.1 Tap water preparation using as growth medium

The tap water was used as growth medium for control and dilution of mercuric chloride solutions and MMS. The criteria of tap water was used in the experiments are a pH around 7 and without any toxic ions especially copper (not over 0.5 mgL^{-1}).

3.2.2 Mercury stock solution preparation

Add 0.1354 gram mercuric chloride to 75 mL of distilled water. Then add nitric acid to this solution. Adjust volume to 100 mL with distilled water.

3.2.3 MMS preparation for experiment

Add 0.077 mL of MMS to 1 L of tap water in volumetric flask. Adjust volume to 10 L with tap water.

3.2.4 Supernatant preparation

Sediment sample was collected manually in Khoa Chet Luk reservoir (outlet) at Phanom Pha Hills. A sediment sample was sieved to remove coarse particles greater than those which were passing through 2 mm screen. Sediment was added with water at ratio 1:2.5 and mixing the simple by rotary agitator with 30 rpm for 18 hours at 25°C. The supernatant solution was precipitated sand for 30 minutes.

3.2.5 Spiked supernatant preparation

Spiked supernatant was prepared by add Hg standard solution at the level of EC_{50} (440 µg L^{-1}) into supernatant solution.

3.2.6 Shallot bulb preparation

Shallot bulbs obtained from Lumphun Province and were stored in a cool, good airflow and dry area after they were harvested about three months. There was no root growth during this period which was called as dormancy stage. Shallot bulbs, 3.0-5.0 grams by weight as a test organism, had not been treated with any kind of growth regulation and fungicide. Dried and mould-attacked bulbs should be discarded. Also, the shallots should not have started shooting of green leaves because restriction of leaf growth may be used as a parameter for the effects of test chemicals. The loose outer scale was carefully removed, and the dry bottom plate was scraped away without destroying the root primodia prior the experiment. There may be some poorly growing shallots. Therefore, the series of onions should be used in the experiment.

3.3 Material, Chemical Reagent, and Equipment

3.3.1 Experimental Materials

1) Shallot bulbs
2) Circular acrylic plastics
3) Test tubes
4) Plastic containers 700 ml
5) Storage tanks 80 and 200 L

3.3.2 Chemical reagent

1) Concentrate nitric acid (conc. HNO_3)
2) Sodium hydroxide (1M NaOH)
3) 2% Acetoorcein
4) Hydrochloric acid (1M HCl)
5) McClintock Solution for fixative (absolute ethyl alcohol 3 parts: glacial acetic acid 1 part)
6) 70% ethyl alcohol
7) Methyl Methane Sulfonate (MMS)
8) Mercuric Chloride

3.3.3 Laboratory equipment and analysis

1) pH meter
2) Electronic balance
3) Volumetric flasks
4) Beakers
5) Pipettes
6) Filtration paper
7) Hot plate
8) Atomic Absorption Spectrophotometer
9) Light microscope
10) Slide and cover slips
11) Stainless steel stick
12) Ruler
13) Dropper bottles
14) Forceps
15) Razor-blades
16) Petridishes
17) Nail varnish
18) Graduated cylinders
19) Thermometer with humidity

3.4 Experimental Strategies

3.4.1 Phase I To determine the effects of Hg solution on root elongation of shallot

3.4.1.1 Experimental procedure

In each concentration, eighteen samples of shallot bulbs were prepared and placed on the test tubes. The mercuric chloride solution was prepared with four definitive concentrations (50, 200, 800, and 3200 μgL^{-1}). The test solution and control (tap water) were changed every day for 4 days (96 hours). The lengths of root bundles were measured at time interval 24 hours. The mean root bundle lengths of 18 samples in each concentration were expressed as percentage of control. The toxicity levels on root growth inhibition; EC_{30}, EC_{50}, and EC_{70} were calculated from a plot of root bundle lengths as percent of control against the concentrations.

3.4.1.2 Statistical analysis

The toxicity levels (EC_{30}, EC_{50}, and EC_{70}) were estimated from the root growth curve using regression models; linear, exponential, and polynomial. Then the equation with maximum coefficient of multiple determinate (R^2) was selected. From the equation, the effective concentrations at 30, 50, and 70% were calculated.

3.4.2 Phase II To determine the effects of Hg solution on mitotic index and chromosome aberration

3.4.2.1 Experimental procedure

At concentration of EC_{30}, EC_{50}, and EC_{70} values, the genotoxicity test was carried out with tap water as negative control and MMS as positive control at 10 mg/L. Six-shallot bulbs were exposed to each concentration. The first 48 hours the shallot bulbs were grown in tap water. Then, they are exposed to test solution for 48 hours, and the test solutions were changed every day. After 48 hours the shallot roots were fixed and macerated with fixative reagents for one day at room temperature.

3.4.2.2 Data analysis

One slide prepared for every shallot bulb. Shallot root cut off the last 6 mm and add 2 ml Farmer's fluid. One root tip cut and placed on a slide. The root tip was rinsed with cold water, and the excess of liquid was removed by filter paper. There are transferred to pre-heated 1M hydrochloric acid at 60°C for 5 minutes, and washed cold water again. The root tip is taken out of water and placed on a micro slide. Cut each root tip about 1-2 mm from the growing tip. One drop of 2% Acetoorcein was added Cover with a cover slip, and apply pressure to the cover slip with a pencil eraser until the cells in the tip spread out in a single layer. The cover slip was fixed carefully with nail varnish. The slides can be kept fresh for month in a freezer.

From each slide, the mitotic index was determined by counting all of stages of mitotic cell out of 400 cells per slide, six slides per concentration following this equation.

$$\text{Mitotic index} = \frac{\text{Number of mitotic cells x 100}}{\text{Total number of cells}} \quad (3.1)$$

The chromosome aberration was determined by examination of the first 100 normal anaphase and early telophase cells per slide, six slides per concentration. Bridges, fragments, bridges and fragments, stickiness, vagrant (laggard) chromosome and c-mitosis were scored. Then, the total chromosome aberration in each concentration was calculated as percentage following the equation.

$$\text{\% Chromosome aberration} = \frac{\text{Chromosome aberrant cells x 100}}{\text{100 cells in anaphase and early telophase}} \quad (3.2)$$

If all toxicity levels do not cause chromosome aberration, the experiment was set up with higher toxicity levels. Then the experiment was conducted the same procedures following 3.4.2.1 for genotoxicity again to find effective concentration, that cause chromosome aberration with statistical significance

3.4.2.3 Statistical analysis

Oneway ANOVA was used for comparing the mitotic index of Hg concentration. To detect the significant of difference ($P<0.05$) of variables, a multiple comparison (Bonferronni) test was performed. Chi-square was used for comparing the chromosome aberration of shallot root cells in various Hg concentrations. It is used to determine whether two variables with multiple categories are homogenous. In case, the study has three or more treatments and the test of homogeneity yields a P-value less than or equal to 0.05, that the treatment are not identical. It cannot to conclude which treatments differ without further testing. Then, partial Chi-square was calculated three or more treatment.

The significant level in this study was determined at $\alpha = 0.05$.

3.4.3 Phase III Application of Allium test for Hg contaminated sediment monitoring at gold mining site

At toxicity levels of EC_{50}, the genotoxicity test was carried out with supernatant of Hg contaminated sediment. Tab water and MMS at 10 mgL^{-1} were used as negative and positive control, respectively. Mercuric chloride solution was added into supernatant at Hg concentration level of EC_{50}. This concentration was used for toxicity test in application for biomonitoring of Hg contamination sediment. All test supernatant and sediment were digested for THg analysis by the method used at the Wetland Biogeochemistry Institute, Louisiana State University (Appendix C). The Hg concentration was determined by Atomic Absorption using CV-AAS (Cold Vapor–Atomic Absorption Spectrophotometry. The shallot bulbs were prior grown in tap water for 48 hours and then they were exposed to test contamination for another 48 hours.

Phase I To determine the effects of Hg on root growth of shallot

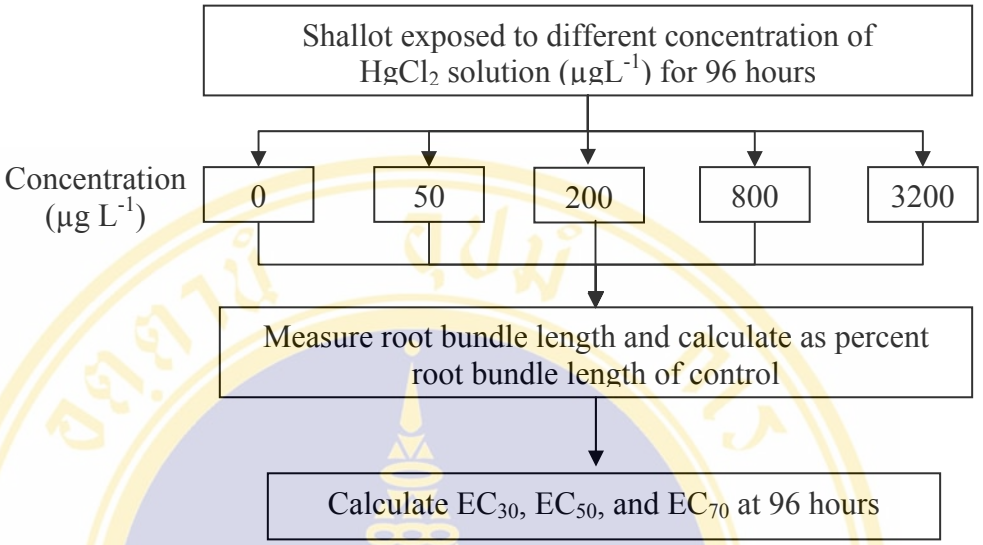

Figure 3.1 Experimental set up for modified Allium test to determine the growth toxicity

Phase II To determine the effects of Hg on mitotic index and chromosome aberration

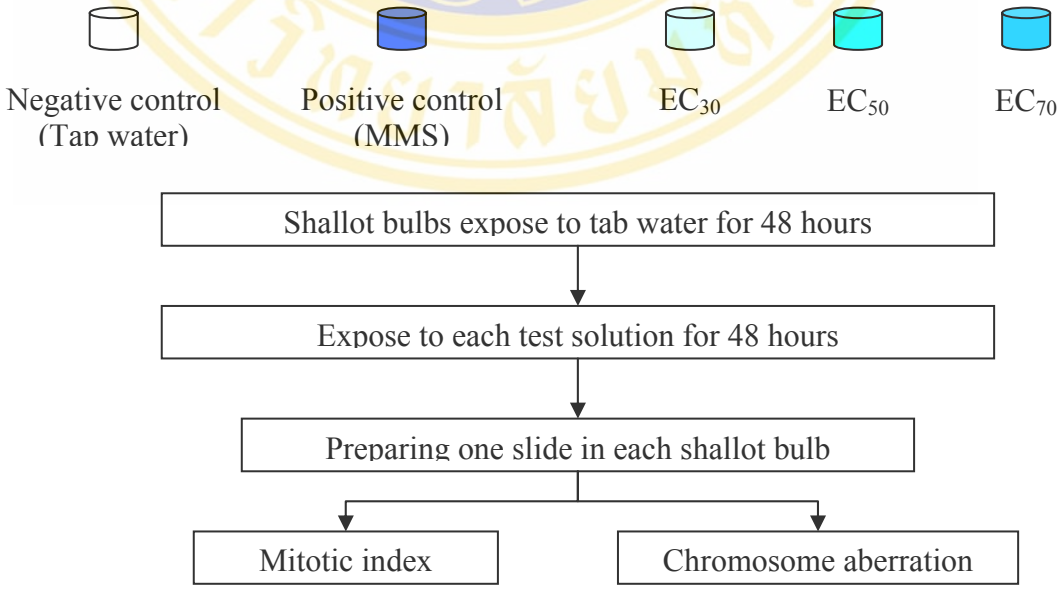

Figure 3.2 Experimental set up for modified Allium test to determine the genotoxicity

Phase III Application of Allium test for Hg contaminated determining

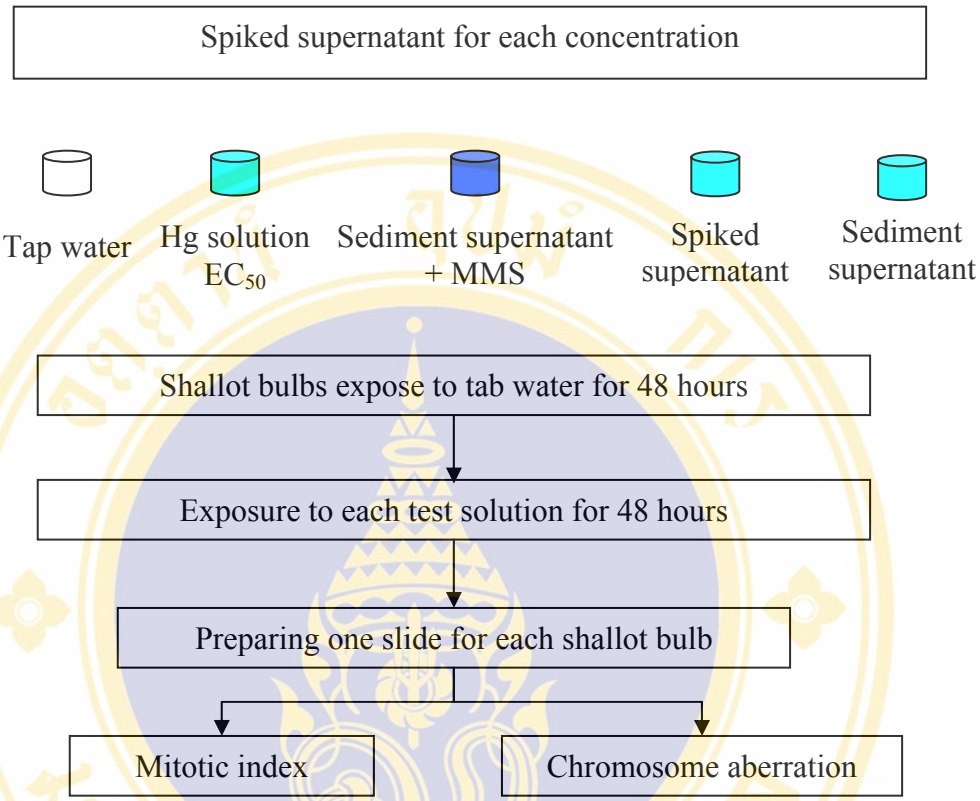

Figure 3.3 Experimental set up for modified Allium test to determine the genotoxicity of Hg contaminated in sediment

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Modified Allium Test Using Shallot (*Allium ascalonicum* L.)

The modified Allium test using shallot (*Allium ascalonicum* L.) was applied for screening toxicity estimated by the root growth inhibition and genotoxicity of mitotic index and chromosome aberration in meristematic root cells.

4.1.1 Root growth inhibition test (root elongation of shallot)

The toxicity of Hg solution using the modified Allium test was performed using the root elongation or root bundle length as end point.

The Hg concentration of 0, 50, 200, 800, and 3200 μgL^{-1} were used as test solution in the experiment. The concentrations were ranged to find out the lowest concentration that has no effects as well as the highest concentration that totally inhibit root elongation of shallots. The effects of THg on shallot root length with various concentrations at the levels of 0, 50, 200, 800, and 3200 μgL^{-1} were shown in Figure 4.1 and Figure 4.2.

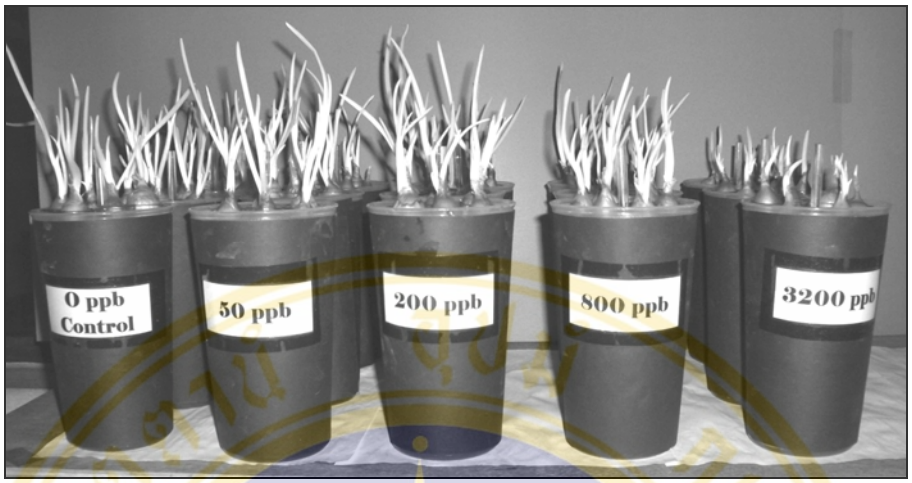

Figure 4.1 Growth of shallot at 96 hour

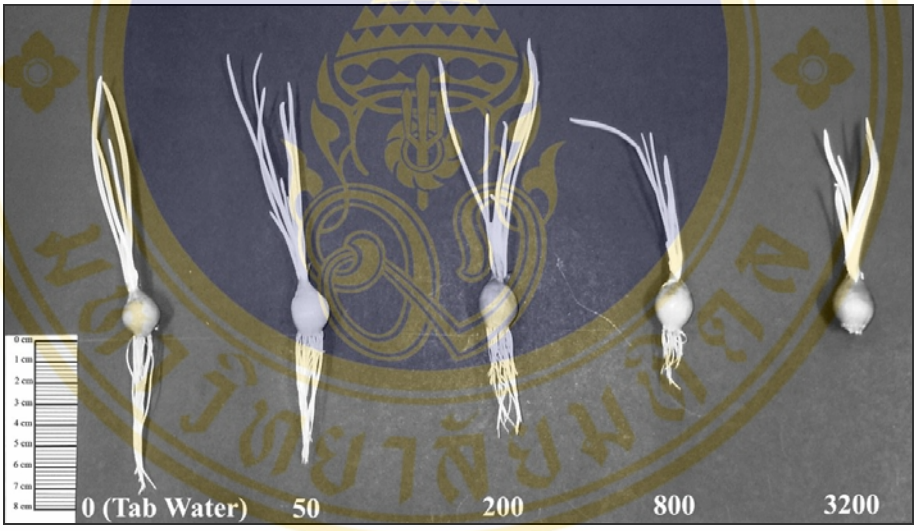

Figure 4.2 Root elongation of shallot at different Hg concentration (0-3200 μgL^{-1} Hg)

The mean root bundle length at different concentration of Hg solution on root elongation of shallot for 24, 48, 72, and 96 hours was presented in Figure 4.3. The root elongation slightly increased in all test solutions at 24 hours. After that the lower concentrations (0-200 μgL^{-1} Hg) were increased rapidly while root growth at the higher concentrations (more than 800 μgL^{-1} Hg) were slightly inhibited and after 48 hours there was no root growth at concentration of 3200 μgL^{-1} Hg.

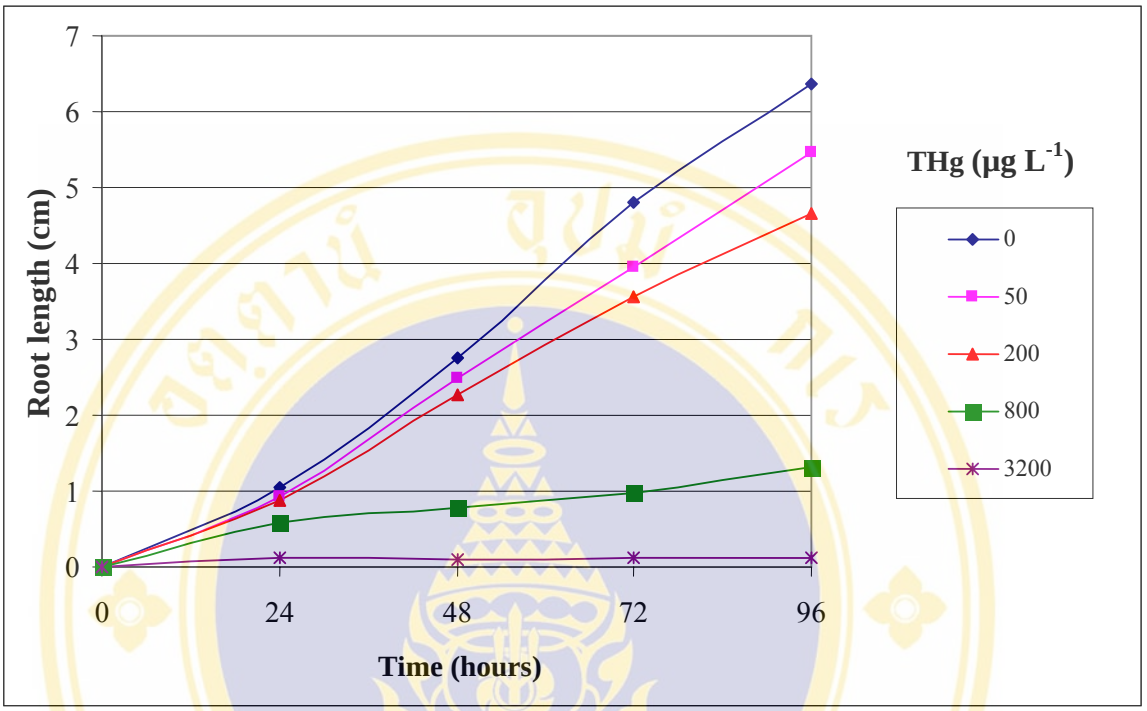

Figure 4.3 The effect of THg on root elongation of shallot

The effects of THg at lower concentration of 0-200 on root elongation inhibition were gradually increased. At the first period of 24 hours, the shallot might be in the period of acclimatization itself. However, at the highest concentration of 3200 μgL^{-1} Hg, the root growth was almost inhibited within 48 hours, the growth of shallot roots was almost inhibited with the length of root at about 0.1 cm (root length less than 0.5 cm means no seed germination/root elongation) (AAHA et al., 1998).

The shallot root growth at the Hg concentrations of 50, 200, 800, and 3200 μgL^{-1} was different from the control group as shown in Table 4.1, Table 4.2, Table 4.3, and Table 4.4 for exposure period of 24, 48, 72, and 96 hours, respectively.

Table 4.1 The root bundle length at various Hg concentrations at 24 hours

Hg concentration μgL^{-1}	Replicated	Root length (cm) $\bar{X} \pm$ SEM*	% Root growth as control
0	1	1.01 ± 0.05	100.00
	2	1.00 ± 0.10	100.00
	3	1.12 ± 0.06	100.00
50	1	0.87 ± 0.04	86.07
	2	0.96 ± 0.08	96.47
	3	0.94 ± 0.04	83.54
200	1	0.87 ± 0.05	86.07
	2	0.82 ± 0.08	81.67
	3	0.93 ± 0.04	82.57
800	1	0.58 ± 0.06	57.02
	2	0.53 ± 0.06	52.78
	3	0.67 ± 0.05	59.41
3200	1	0.10	9.87
	2	0.10	10.00
	3	0.13 ± 0.01	11.39

SEM* = Standard error of the mean

Table 4.2 The root length at various Hg concentrations at 48 hours

Hg concentration μgL^{-1}	Replicated	Root length (cm) $\bar{X} \pm$ SEM*	% Root growth as control
0	1	2.71 ± 0.17	100.00
	2	2.72 ± 0.09	100.00
	3	2.87 ± 0.10	100.00
50	1	2.64 ± 0.14	97.62
	2	2.44 ± 0.15	89.80
	3	2.41 ± 0.12	83.75
200	1	2.38 ± 0.11	88.09
	2	2.20 ± 0.11	80.82
	3	2.22 ± 0.11	77.18
800	1	0.84 ± 0.07	31.21
	2	0.81 ± 0.09	29.59
	3	0.72 ± 0.08	25.15
3200	1	0.10	3.70
	2	0.10	3.67
	3	0.10	3.48

SEM* = Standard error of the mean

Table 4.3 The root bundle length at various Hg concentrations at 72 hours

Hg concentration μgL^{-1}	Replicated	Root length (cm) $\bar{X} \pm$ SEM*	% Root growth as control
0	1	4.86 ± 0.10	100.00
	2	4.82 ± 0.11	100.00
	3	4.76 ± 0.11	100.00
50	1	3.99 ± 0.19	95.36
	2	3.93 ± 0.14	95.84
	3	3.93 ± 0.13	96.72
200	1	3.72 ± 0.11	88.78
	2	3.36 ± 0.18	81.98
	3	3.59 ± 0.09	88.37
800	1	1.09 ± 0.11	25.99
	2	0.93 ± 0.05	22.67
	3	0.91 ± 0.04	22.28
3200	1	0.10	2.39
	2	0.13 ± 0.01	3.12
	3	0.10	2.46

SEM* = Standard error of the mean

Table 4.4 The root bundle length at various Hg concentrations at 96 hours

Hg concentration μgL^{-1}	Replicated	Root length (cm) $\bar{X} \pm$ SEM*	% Root growth as control
0	1	5.89 ± 0.22	100.00
	2	6.18 ± 0.14	100.00
	3	7.03 ± 0.13	100.00
50	1	5.04 ± 0.15	85.46
	2	5.34 ± 0.10	86.44
	3	6.01 ± 0.22	86.84
200	1	4.33 ± 0.23	73.52
	2	4.48 ± 0.08	72.50
	3	5.15 ± 0.06	71.11
800	1	1.29 ± 0.15	21.96
	2	1.06 ± 0.07	17.22
	3	1.58 ± 0.06	22.79
3200	1	0.1	1.46
	2	0.1	1.62
	3	0.16 ± 0.01	2.17

SEM* = Standard error of the mean

At 96 hours period of time, the root elongation of treated shallot roots at the levels of concentration of 50, 200, 800, and 3200 μgL^{-1} Hg was significantly different from the control group with the values of relative root growth (%) at about 86.25 %, 72.38 %, 20.66 %, and 1.75%, respectively (P<0.001).

The dose-response curve of the THg and Log of relative root growth was shown in Figure 4.4. The linear equation was determined for calculation of the effective concentrations (EC) at the toxicity levels of EC_{30}, EC_{50}, and EC_{70}, which retarded 30%, 50%, and 70% of root growth, respectively. The toxicity concentrations at various levels were then estimated from the following linear equation:

$$\hat{y} = 85.112e^{-0.0012x} \quad (4.1)$$

and $R^2 = 0.9767$

and they were calculated to be 163, 440, and 870 $\mu g\ L^{-1}$, respectively.

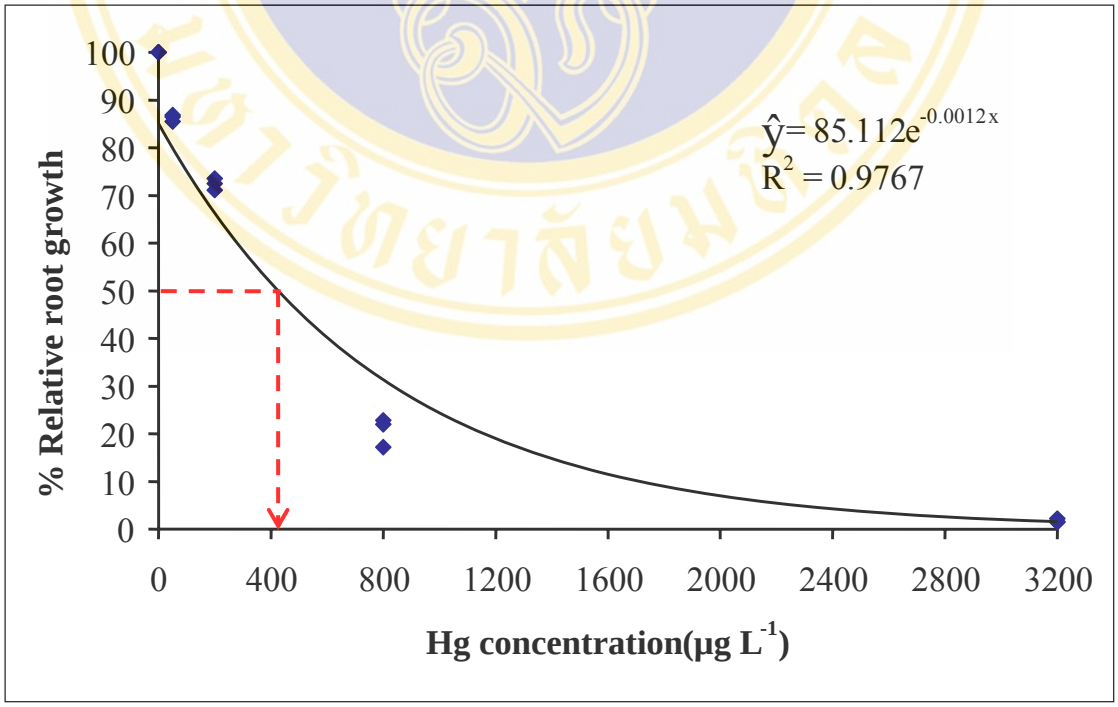

Figure 4.4 Dose response of relative root growth of shallot

The shallots showed a reduction in the root growth when the higher concentrations of Hg were applied. The results were agreed with those obtained from *Allium cepa* L. that the EC_{50} of $HgCl_2$ was 2 mgL^{-1} (base on root growth), which indicated that the growth of *Allium cepa* L. was accelerated induced by the low Hg concentration regards to the assay. The growth was increased until the increase of concentration was up to a limit, beyond which the growth rate decreased. In *Allium cepa* L., the catalase activity and peroxidase activity decreased with increase of Hg concentration (Patra and Sharma, 2000; Subhadra et al., 1991). The results corresponded to the hypothesis that the root bundle length of shallot in treated groups were significantly decreased from that of control.

In higher plants, mercuric ion is able to induce oxidative stress in plants by generation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\bullet$), and singlet oxygen ($^1O_2$), collectively termed reactive oxygen species (ROS). ROS can rapidly attack all types of biomolecules such as nucleic acid, proteins, lipids, and amino acids, leading to irreparable metabolic dysfunction and cell death. The antioxidant enzymes, such as superoxide-dimutase (SOD), catalase (CAT), and peroxidase (POD) constitute a system that keeps ROS at low steady state concentrations in cells and tissue. The highest concentration of heavy metals, the activity of CAT, POD, and SOD in root decreased sharply. The oxygen scavenging function of SOD was impaired (Hung, Yu and Lin, 2005; Patra and Sharma, 2000; Patra et al., 2004; Subhadra et al., 1991; Zhang et al., 2007; Zhou et al., 2007).

Regards to the study in cucumber (*Cucumis sativus* L.) (Cargnelutti et al., 2006), alfalfa (*Medicago sativa* L.) (Zhou et al., 2007), rice (*Oryza sativa* L.), lettuce (*Luctuca sativa* L.), and garden pea (*Pisum sativa* L.) (Patra and Sharma, 2000) growth retarded when exposed to heavy metal, e.g. Hg, as well as the growth of both seedling roots and shoots.

4.1.2 Genotoxicity test (mitotic index and chromosome aberration)

The shallot roots were exposed to THg at the three levels of concentration obtained from the previous study; they were 163, 440, and 870 μgL^{-1} for EC_{30}, EC_{50}, and EC_{70}, respectively. The mitotic index and chromosome aberration in anaphase and early telophase in shallot were investigated.

The shallots were exposed to tap water 48 hours prior to THg (48 hours). Chromosome aberration in root meristem cells was induced by Hg concentration at the levels of 163 (EC_{30}), 440 (EC_{50}), and 870 (EC_{70}) μgL^{-1}. The effect of Hg on the mitotic index and chromosome aberration of *Allium ascalonicum* L. meristem cells was summarized in Table 4.5 and Table 4.6.

The mitotic index of the treated shallot root cells was in the range of 4.75-9.83% and significantly different from those of control group at the value of 12.20% ($P<0.001$). The lowest percentage of mitotic index (4.75%) was observed at the Hg concentration of 870 μgL^{-1} (EC_{70}).

Table 4.5 The effects of THg solution on mitotic index of shallot

THg ($\mu g\ L^{-1}$)	Mitotic cells (400 cells/slide)						Total mitotic cells	Mitotic Index (%) $\bar{X}$±SEM*
	1	2	3	4	5	6		
Tap water (negative control)	44	48	50	55	52	44	293	12.21±0.45
163 (EC_{30})	36	35	36	47	41	41	236	9.83±0.47
440 (EC_{50})	25	27	29	24	19	22	146	6.08±0.36
870 (EC_{70})	22	18	13	22	20	19	114	4.75±0.34
MMS (positive control)	31	49	39	32	46	36	233	9.71±0.75

SEM* = standard error of the mean

Table 4.6 The effects of THg on chromosome aberration of shallot

THg ($\mu g\ L^{-1}$)	Chromosome aberration (cells)								Total 600	% aberration cell $\bar{X} \pm$ SEM*
	Vagrant/ Laggard	Fragment	Bridge	C-mitosis	BF[1]	BL[2]	FL[3]	BFL[4]		
0	58	47	20	1	3	14	4	1	148	24.67 ± 0.61
163 (EC_{30})	160	85	69	6	12	10	0	0	342	57.00 ± 1.32
440 (EC_{50})	168	111	59	12	10	13	5	0	378	63.00 ± 1.18
870 (EC_{70})	156	124	48	20	8	24	1	0	381	66.50 ± 4.64
MMS	117	101	67	0	7	17	2	0	311	51.83 ± 4.17

1 = Bridge+Fragment, 2 = Bridge+Laggard, 3 = Fragment+Laggard, 4 = Bridge+Fragment+Laggard

SEM* = standard error of the mean

The chromosome aberration significantly increased as Hg concentration increased ($P<0.001$). The types of chromosome aberration were laggards, fragment, bridges, and c-mitosis. Furthermore, combinations of bridge and laggard, bridge and fragment, fragment and laggard, and bridge, fragment and laggard were also found (Figure 4.5).

The effects of THg on chromosome aberration of shallot were shown in Figure 4.6. The percentage of chromosome aberration, 24.67% and 51.83% for the negative and positive control, respectively, were higher than those obtained from the study in the year 2003 (Liewrungruang, 2003) with the values of 4.3 % and 27.67% for the negative and the positive control, respectively. It might be the variation of shallots in different crops and the quality of tap water used that had influence on chromosome aberration of shallot.

The results corresponded to the hypotheses that the mitotic index of treated shallot root cells was significantly lower than of that of control group and the percentage of chromosome aberration were significantly increased as Hg concentration increased at the 95% confidence interval.

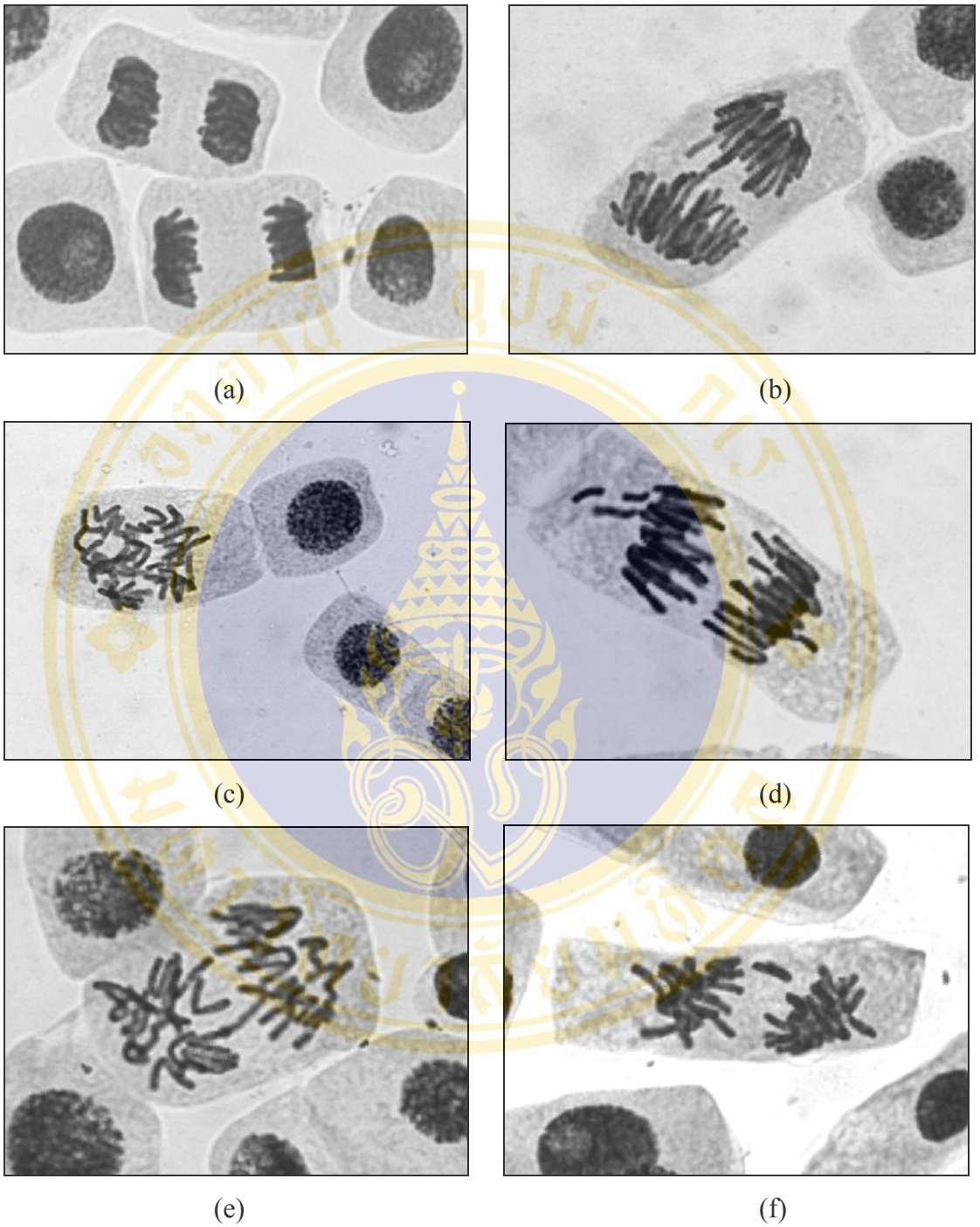

Figure 4.5 Chromosome aberrations observed in root tip cells of shallot (*Allium ascalonicum* L.)
(a) normal anaphase; (b) cell with a bridge; (c) c-mitosis; (d) cell with fragment; (e) cell with laggard; (f) cell with laggard and vagrant chromosomes

Exposure to $HgCl_2$ in *Allium cepa* L. and *Allium sativum* L. reduced mitotic index in the root tip cells and increased the frequency of chromosomal aberration in degrees directly proportional to the concentrations used and to the duration exposed. (Agar and Uysal, 1997; Patra et al., 2004). Hg strongly interact with the sulfhydryl groups (-SH), impairing spindle function and leadind to chromosomal aberration and polyploidy. Another important mechanism of Hg genotoxicity is its ability to produce free radicals that can cause DNA damage (Zhou et al., 2007; Silva-Pereira et al., 2005)

4.2 Allium Genotoxicity Test for Hg Contaminated Sediment at Gold Mining Site

The sediment of Khoa Chet Luk reservoir in mining area used for supernatant preparation was composed of sand, silt, and clay in the proportional percentage of 30.0%, 14.5%, and 55.5%, respectively (S.P.S., 2000). The concentration of Hg contamination in sediment was ranged from 158 to 896 μgkg^{-1} dw. The Hg concentration used in sediment and spiked supernatant at the toxic level of EC_{50} were 169 and 620 μgL^{-1}, respectively.

4.2.1 Mitotic index and chromosome aberration

The effects of THg in supernatant on mitotic index and chromosome aberration of shallot root cells were investigated and the results were shown in Table 4.7 and Table 4.8. The mitotic index in meristems of roots exposed to contaminated supernatant with the Hg concentration range at the level of 169-620μgL^{-1} Hg was not significantly different from the sediment supernatant (169 μgL^{-1}) and sediment added MMS supernatant (169 μgL^{-1}). However, there was significantly different between Hg contaminated group and control (P<0.001). The reason why there was not significantly different in mitotic index was that the sediment prepared for supernatant was initially contaminated of Hg. The THg had an adverse effect on cell division of shallot root cells, leading to the reduced of mitotic index.

The shallot roots exposed to spiked supernatant with the Hg concentration of 620 μgL^{-1} was significantly higher different in the frequency of chromosome aberrations as compared with the sediment supernatant (P =0.001). The chromosome aberration in root shallot cells was induced by THg in spiked supernatant and the most prominent aberrations were vagrant/laggard, fragment, and c-mitosis. The results did not correspond to the hypothesis that the mitotic index of shallot in sediment supernatant was not significantly different in each concentration. Nevertheless, the chromosome aberration corresponded to the hypothesis which the percentage of chromosome aberration of shallot in sediment supernatant was significantly different.

Table 4.7 The effect of Hg contaminated in supernatant on mitotic index of shallot

Supernatant	THg ($\mu g\ L^{-1}$)	Mitotic cells (400 cells/slide)						Total mitotic cells	Mitotic Index (%) $\overline{X}$±SEM*
		1	2	3	4	5	6		
Sediment	169	22	22	28	20	26	21	139	5.79 ± 0.32
Spiked sediment (EC_{50})	620	17	13	25	18	15	27	115	4.79 ± 0.57
Sediment +MMS	169	18	25	22	21	20	19	125	5.21 ± 0.25
Hg solution (EC_{50})	440	22	19	21	24	20	20	126	5.51 ± 0.48
Tap water		40	40	41	44	44	42	251	11.04 ± 0.21

SEM* = standard error of the mean

Table 4.8 The effect of Hg contaminated in supernatant on chromosome aberration of shallot

Supernatant	THg ($\mu g\ L^{-1}$)	Chromosome aberration (cells)								Total (600)	% aberration cell $\overline{X}$ ± SEM*
		Vagrant/ Laggard	Fragment	Bridge	C-mitosis	BF[1]	BL[2]	FL[3]	BFL[4]		
Sediment	169	62	24	23	8	0	11	1	0	129	22.00±0.97
Spiked sediment (EC_{50})	620	213	80	54	11	0	45	5	0	408	68.50±1.18
MMS + sediment	169	126	87	70	15	2	21	3	1	325	54.17±0.95
Hg solution (EC_{50})	440	165	87	37	14	45	3	6	9	357	59.50±0.43
Tap water		66	34	23	7	10	3	2	0	145	24.17±0.87

1 = Bridge+Fragment, 2 = Bridge+Laggard, 3 = Fragment+Laggard, 4 = Bridge+Fragment+Laggard

SEM* = standard error of the mean

4.2.2 Genotoxicity comparation between THg in solution and contaminated supernatant

The effect of Hg on chromosome aberration of shallot root cells exposed to the spiked Hg supernatant (620 μgL^{-1}) and the Hg solution (440 μgL^{-1}) were significantly higher different (P<0.001). Eventhough the same level of Hg concentration (440 μgL^{-1}) was spiked into both supernatant and test solution, the higher concentration was found in supernatant (620 μgL^{-1}) due to the initially Hg contaminated in sediment used. Thus the higher chromosome aberration was found significant higher than exposed to the spiked supernatant. Then we can assume that Hg in supernatant presented in bioavailable form (exchangeable form). Hg(II) sorbed by the mineral and organic surfaces was in a exchangeable form, which is highly available for plant uptake (Iwegbue et al.,2007, Cruz-Guzman et al., 2003).

4.2.3 Application of modified Allium test using shallot for monitoring Hg contamination in sediment at gold mining site

The modified Allium test using shallot can be applied for monitoring Hg contamination in sediment. The active range for Hg concentration from the experiment was 169-620 μgL^{-1}. It was possibly to apply for monitoring sediment at mining site because the range of Hg concentration for contaminated sediment was 160 and 402 μgkg^{-1} dw (Pataranawat, 2003) and the standard of Hg for sediment of Canadian Fresh Water Sediment Quality Guideline is 174 μgkg^{-1}dw (Thongra-ar, 2001). The chromosome aberration was the only endpoint to measure the toxicity of Hg due to no significantly different was found in mitotic index. This result can be used as base line data to develop a protocol for monitoring Hg possibly.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The modified Allium test using shallot (*Allium ascalonicum* L.) was applied for screening toxicity estimation by the inhibition of the root growth and genotoxicity assessment by microscopic studies of the chromosome aberration in meristematic root cells of THg. These results were compared by statistical analysis and concluded as follows:

1) Root bundle length in Hg test solutions significantly differed from that of control. Root growth of shallot (*Allium ascalonicum* L.) was inhibited by Hg at the concentration of 440 μgL^{-1} at EC_{50}.

2) Hg solution at the concentration of 163, 440, and 870 μgL^{-1} was able to inhibited the root cell division of shallot. Mitotic index of root cells in Hg test solutions was significantly different from that of control.

3) Hg induced chromosome aberration in root meristem cells of shallot at the Hg concentration of of 163, 440, and 870 μgL^{-1}. Chromosome aberrations significantly increased as Hg concentration increased.

4) Mitotic index of root cells in sediment supernatant at various Hg concentrations was not significantly different.

5) The chromosome aberration in supernatant spiked with Hg and MMS was significantly higher than control and natural sediment supernatant.

6) The modified Allium test using shallot could be approached to develop for determination of Hg contamination in sediment at gold mining site and the chromosome aberration was the endpoint of measurement.

5.2 Recommendations

1) Using growth nutrient instead a tap water, it can get rid of the factor that causes chromosome damage.

2) Calculating genotoxicity by planted shallot, applying, sediment directly was suggesting recommend. Using the method, modified Allium test, screen the Hg contamination in sediment.

3) Find various ranges of EC levels and toxicity test in supernatant, in needed to apply with the procedure.

4) Compare sensitivity of Micro-Tox assay to modified Allium test, for the purpose of appropriated method, applying to economical country.

5) Finding the concentration of Hg in roots, bulbs, and leave that lead to translocation of Hg into every functional parts of shallot.

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APPENDIX

APPENDIX A

1. DATA OF THE EXPERIMENT

Table A-1 The effect of THg at 0, 50, 200, 800, and 3200 μgL^{-1} on root length of shallot (*Allium ascalonicum* L.)

THg (μg L^{-1})	Time (hours)	Replicated	Root length (cm)								Mean	S.D.
			1	2	3	4	.	.	.	18		
0	**24**	**1**	0.7	0.7	0.9	0.9	.	.	.	.	1.01	0.3085
		2	0.2	0.3	0.6	0.7	.	.	.	.	1.00	0.4256
		3	0.6	0.8	0.8	0.9	.	.	.	.	1.12	0.2734
	48	**1**	2.5	3.0	3.7	3.0	.	.	.	.	2.71	0.7116
		2	2.0	2.0	2.5	2.5	.	.	.	.	2.72	0.3859
		3	2.0	2.5	2.5	2.5	.	.	.	.	2.87	0.4127
	72	**1**	4.0	4.2	4.5	4.5	.	.	.	.	4.86	0.9833
		2	3.8	4.0	4.2	4.6	.	.	.	.	4.82	0.4728
		3	3.8	4.1	4.2	4.2	.	.	.	.	4.76	0.5821
	96	**1**	4.0	4.5	4.5	5.1	.	.	.	.	5.89	0.9533
		2	5.0	5.3	5.7	5.7	.	.	.	.	6.18	0.9792
		3	6.1	6.2	6.5	6.5	.	.	.	.	7.03	0.7059
50	**24**	**1**	0.5	0.6	0.6	0.7	.	.	.	.	0.87	0.1904
		2	0.4	0.4	0.5	0.8	.	.	.	.	0.96	0.3590
		3	0.6	0.7	0.8	0.8	.	.	.	.	0.94	0.2058
	48	**1**	1.5	1.7	2.0	2.3	.	.	.	.	2.64	0.6491
		2	1.0	1.8	2.0	2.0	.	.	.	.	2.44	0.6289
		3	1.5	2.0	2.0	2.0	.	.	.	.	2.41	0.5070
	72	**1**	2.1	3.0	3.5	3.5	.	.	.	.	3.99	0.8127
		2	2.8	2.9	3.5	3.6	.	.	.	.	3.93	0.668
		3	2.9	3.2	3.2	3.5	.	.	.	.	3.93	0.5636
	96	**1**	4.1	4.2	4.5	4.7	.	.	.	.	5.04	0.8342
		2	4.5	4.9	5.0	5.1	.	.	.	.	5.34	1.021
		3	4.2	4.2	4.7	4.9	.	.	.	.	6.01	0.9411
200	**24**	**1**	0.5	0.5	0.6	0.6	.	.	.	.	0.87	0.2218
		2	0.2	0.3	0.3	0.4	.	.	.	.	0.82	0.3434
		3	0.6	0.7	0.8	0.8	.	.	.	.	0.93	0.2301
	48	**1**	1.4	1.5	1.8	2.0	.	.	.	.	2.38	0.4643
		2	1.5	1.5	1.5	1.5	.	.	.	.	2.20	0.4602
		3	1.2	1.7	1.8	1.8	.	.	.	.	2.22	0.4579

Table A-1 The effect of THg at 0, 50, 200, 800, and 3200 µgL^{-1} on root length of shallot (*Allium ascalonicum* L.) (continued)

THg (µg L^{-1})	Time (hours)	Replicated	Root length (cm)								Mean	S.D.
			1	2	3	4	.	.	.	18		
200	**72**	**1**	3.0	3.2	3.3	3.5	.	.	.	.	3.72	0.623
		2	2.0	2.0	2.3	2.4	.	.	.	.	3.36	0.7477
		3	2.9	3.0	3.3	3.4	.	.	.	.	3.59	0.5135
	96	**1**	2.1	2.6	3.1	3.5	.	.	.	.	4.33	0.9579
		2	3.8	4.0	4.1	4.3	.	.	.	.	4.48	0.5721
		3	4.7	4.8	4.9	5.0	.	.	.	.	5.15	1.0044
800	**24**	**1**	0.2	0.3	0.3	0.4	.	.	.	.	0.58	0.2487
		2	0.2	0.2	0.3	0.3	.	.	.	.	0.53	0.2516
		3	0.3	0.4	0.4	0.4	.	.	.	.	0.67	0.2169
	48	**1**	0.5	0.5	0.5	0.5	.	.	.	.	0.84	0.2995
		2	0.3	0.3	0.3	0.4	.	.	.	.	0.81	0.3654
		3	0.3	0.3	0.3	0.4	.	.	.	.	0.72	0.3590
	72	**1**	0.3	0.5	0.5	0.7	.	.	.	.	1.09	0.4639
		2	0.8	0.6	0.7	0.7	.	.	.	.	0.93	0.2701
		3	0.6	0.7	0.8	0.8	.	.	.	.	0.91	0.1886
	96	**1**	0.5	0.5	0.7	0.8	.	.	.	.	1.29	0.6421
		2	0.7	0.7	0.8	0.8	.	.	.	.	1.06	0.3606
		3	1.2	1.3	1.3	1.3	.	.	.	.	1.58	0.2625
3200	**24**	**1**	0.1	0.1	0.1	0.1	.	.	.	.	0.10	0.2200
		2	0.1	0.1	0.1	0.1	.	.	.	.	0.10	0.0549
		3	0.1	0.1	0.1	0.1	.	.	.	.	0.13	0.0546
	48	**1**	0.1	0.1	0.1	0.1	.	.	.	.	0.10	0.0530
		2	0.1	0.1	0.1	0.1	.	.	.	.	0.10	0.0436
		3	0.1	0.1	0.1	0.1	.	.	.	.	0.10	0.0482
	72	**1**	0.1	0.1	0.1	0.1	.	.	.	.	0.10	0.0485
		2	0.1	0.1	0.1	0.1	.	.	.	.	0.13	0.0546
		3	0.1	0.1	0.1	0.1	.	.	.	.	0.10	0.042
	96	**1**	0.1	0.1	0.1	0.1	.	.	.	.	0.10	0.023
		2	0.1	0.1	0.1	0.1	.	.	.	.	0.10	0.0383
		3	0.1	0.1	0.1	0.1	.	.	.	.	0.16	0.0686

2. DATA OF THE MERCURY CONCENTRATION

Table A-2 The Hg concentration of sediment, sediment supernatant, and spiked supernatant

Sample	No.	THg (μg L^{-1})
Sediment	1	157.64
	2	627.43
	3	664.38
	4	760.02
	5	895.78
	Average	621.05
Sediment supernatant	1	223.79
	2	137.19
	3	168.51
	4	147.49
	Average	169.25
Spiked supernatant	1	745.81
	2	495.05
	Average	620.43

APPENDIX B
STATISTICAL

Table B-1 Statistical analysis of % root length of shallot at the levels of Hg concentration 0, 50, 200, 800, and 3200 μgL^{-1}

Tests of Normality

		Shapiro-Wilk		
HgConc		Statistic	Degree of freedom	P-value
%Root growth	50	.944	3	.545
	200	.992	3	.831
	800	.859	3	.265
	3200	.909	3	.413

ANOVA

%Root growth

Source of variation	Sum of Squares	Degree of freedom	Mean Square	F-ratio	P-value
Between Groups	21933.173	4	5483.293	2461.857	.000
Within Groups	22.273	10	2.227		
Total	21955.446	14			

Table B-1 Statistical analysis of % root length of shallot at the levels of Hg concentration 0, 50, 200, 800, and 3200 μgL^{-1} (continued)

Multiple Comparisons

Dependent Variable: %Root growth
Bonferroni

Dependent Variable	Concentration	Difference of % root growth	P-value	95% Confidence Interval	
				Lower Bound	Upper Bound
0	50	13.75333(*)	<0.001	9.3892	18.1175
	200	27.62333(*)	<0.001	23.2592	31.9875
	800	79.34333(*)	<0.001	74.9792	83.7075
	3200	98.25000(*)	<0.001	93.8859	102.6141
50	0	-13.75333(*)	<0.001	-18.1175	-9.3892
	200	13.87000(*)	<0.001	9.5059	18.2341
	800	65.59000(*)	<0.001	61.2259	69.9541
	3200	84.49667(*)	<0.001	80.1325	88.8608
200	0	-27.62333(*)	<0.001	-31.9875	-23.2592
	50	-13.87000(*)	<0.001	-18.2341	-9.5059
	800	51.72000(*)	<0.001	47.3559	56.0841
	3200	70.62667(*)	<0.001	66.2625	74.9908
800	0	-79.34333(*)	<0.001	-83.7075	-74.9792
	50	-65.59000(*)	<0.001	-69.9541	-61.2259
	200	-51.72000(*)	<0.001	-56.0841	-47.3559
	3200	18.90667(*)	<0.001	14.5425	23.2708
3200	0	-98.25000(*)	<0.001	-102.6141	-93.8859
	50	-84.49667(*)	<0.001	-88.8608	-80.1325
	200	-70.62667(*)	<0.001	-74.9908	-66.2625
	800	-18.90667(*)	<0.001	-23.2708	-14.5425

* The mean difference is significant at the 0.05 level.

Table B-2 Statistical analysis of mitotic index of THg at toxicity levels 163, 440, and 870 μgL^{-1} at 96 hours

Tests of Normality

	Shapiro-Wilk		
	Statistic	Degree of freedom	P-value
Mitotic Index	0.954	30	0.215

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

ANOVA

MitoticIndex

	Sum of Squares	Degree of freedom	Mean Square	F-ratio	P-value
Between Groups	221.346	4	55.336	37.368	<0.001
Within Groups	37.021	25	1.481		
Total	258.367	29			

Table B-2 Statistical analysis of mitotic index of THg at toxicity levels 163, 440, and 870 μgL^{-1} at 96 hours (continued)

Multiple Comparisons

Dependent Variable: MitoticIndex
Bonferroni

Test Solution	Test Solution	Difference of mitotic index	P-value	95% Confidence Interval	
				Lower Bound	Upper Bound
0	170	2.37500(*)	0.024	0.2123	4.5377
	460	6.12500(*)	<0.001	3.9623	8.2877
	905	7.45833(*)	<0.001	5.2957	9.6210
	MMS	2.50000(*)	0.015	0.3373	4.6627
170	0	-2.37500(*)	0.024	-4.5377	-0.2123
	460	3.75000(*)	<0.001	1.5873	5.9127
	905	5.08333(*)	<0.001	2.9207	7.2460
	MMS	0.12500	1.000	-2.0377	2.2877
460	0	-6.12500(*)	<0.001	-8.2877	-3.9623
	170	-3.75000(*)	<0.001	-5.9127	-1.5873
	905	1.33333	0.693	-0.8293	3.4960
	MMS	-3.62500(*)	<0.001	-5.7877	-1.4623
905	0	-7.45833(*)	<0.001	-9.6210	-5.2957
	170	-5.08333(*)	<0.001	-7.2460	-2.9207
	460	-1.33333	0.693	-3.4960	0.8293
	MMS	-4.95833(*)	<0.001	-7.1210	-2.7957
MMS	0	-2.50000(*)	0.015	-4.6627	-0.3373
	170	-0.12500	1.000	-2.2877	2.0377
	460	3.62500(*)	<0.001	1.4623	5.7877
	905	4.95833(*)	<0.001	2.7957	7.1210

* The mean difference is significant at the 0.05 level.

Table B-3 Statistical analysis of chromosome aberration of THg at toxicity levels 163, 440, and 870 μgL^{-1} at 96 hours

TestSolution * CA Crosstabulation

		Chromosome aberration								Total
		Laggard	Fragment	Bridge	BL[1]	C-Mitosis	BF[2]	FL[3]	BFL[4]	
Test Solution	0	58	47	20	14	1	3	4	1	148
	170	160	85	69	10	6	12	0	0	342
	460	168	111	59	13	12	10	5	0	378
	850	156	124	48	24	20	8	1	0	381
	MMS	117	101	67	17	0	7	2	0	311
Total		659	468	263	78	39	40	12	1	1560

1 = Bridge+Laggard, 2 = Bridge+Fragment, 3 = Fragment+Laggard, 4 = Bridge+Fragment+Laggard

*Pearson Chi-Squre (P-value < 0.001)

Table B-4 Statistical analysis of mitotic index of THg in control and spiked supernatant at 96 hours

Tests of Normality

TestSolution		Shapiro-Wilk		
		Statistic	Degree of freedom	P-value
MitoticIndex	Sediment	0.878	6	0.259
	Spiked	0.902	6	0.389
	Sediment+MMS	0.957	6	0.794
	460	0.980	6	0.952
	Tap water	0.900	6	0.371

ANOVA

MitoticIndex

	Sum of Squares	Degree of freedom	Mean Square	F-ratio	P-value
Between Groups	159.922	4	39.980	58.115	<0.001
Within Groups	17.199	25	0.688		
Total	177.121	29			

Table B-4 Statistical analysis of mitotic index of THg in control and spiked supernatant at 96 hours (continued)

Multiple Comparisons

Dependent Variable: MitoticIndex
Bonferroni

TestSolution	TestSolution	Difference of mitotic index	P-value	95% Confidence Interval	
				Lower Bound	Upper Bound
Sediment	Spiked	1.00000	0.471	-0.4741	2.4741
	Sediment+MMS	0.58333	1.000	-0.8907	2.0574
	460	0.27833	1.000	-1.1957	1.7524
	Control	-5.24667(*)	<0.001	-6.7207	-3.7726
Spiked	Sediment	-1.00000	0.471	-2.4741	0.4741
	Sediment+MMS	-0.41667	1.000	-1.8907	1.0574
	460	-0.72167	1.000	-2.1957	0.7524
	Control	-6.24667(*)	<0.001	-7.7207	-4.7726
Sediment +MMS	Sediment	-0.58333	1.000	-2.0574	0.8907
	Spiked	0.41667	1.000	-1.0574	1.8907
	460	-0.30500	1.000	-1.7791	1.1691
	Control	-5.83000(*)	<0.001	-7.3041	-4.3559
440	Sediment	-0.27833	1.000	-1.7524	1.1957
	Spiked	0.72167	1.000	-0.7524	2.1957
	Sediment+MMS	0.30500	1.000	-1.1691	1.7791
	Control	-5.52500(*)	<0.001	-6.9991	-4.0509
Control	Sediment	5.24667(*)	<0.001	3.7726	6.7207
	Spiked	6.24667(*)	<0.001	4.7726	7.7207
	Sediment+MMS	5.83000(*)	<0.001	4.3559	7.3041
	460	5.52500(*)	<0.001	4.0509	6.9991

* The mean difference is significant at the 0.05 level.

Table B-5 Statistical analysis of chromosome aberration of THg in control and spiked supernatant at 96 hours

TestSupernatant and CA

Count

		CA								Total
		Laggard	Fragment	Bridge	BL[1]	CMitosis	BF[2]	FL[3]	BFL[4]	
Test Supernatant	Sediment	62	24	23	11	8	0	0	0	128
	Spike	213	80	54	45	11	0	5	0	408
	Sediment +MMS	126	87	70	21	15	2	3	1	325
	460	165	87	37	14	45	3	6	9	366
	Control	66	34	23	7	10	3	2	0	145
Total		632	312	207	98	89	8	16	10	1372

1 = Bridge+Laggard, 2 = Bridge+Fragment, 3 = Fragment+Laggard, 4 = Bridge+Fragment+Laggard

*Pearson Chi-Squre (P-value < 0.001)

APPENDIX C
MERCURY ANALYSIS

All samples in this study were analyzed in the laboratory for total mercury by the method developed at Wetland Biogeochemistry Institute, Louisiana State University. This method was the most accurate, reliable and use widely for the mercury analysis. Mercury is determined using the cold vapor atomic absorption spectrophotometer technique (CV-AAS) (Gambrell, 1991).

1. Reagent preparation

1) **Sulfuric acid conc.(H_2SO_4)**: reagent grade

2) **Nitric acid conc.(HNO_3)**: reagent grade

3) **Sodium chloride-hydroxylamine hydrochloride solution (NH_2OH-NaCl)**: dissolved 60 g NaCl and 60 g of NH_2OH-HCl in a 500 ml volume metric flask and dilute with distill water to 500 ml.

4) **Potassium permanganate ($KMnO_4$)**: 5 % solution: dissolved 50 g $KMnO_4$ in a 1000 ml volume metric flask and dilute with distill water to 1000 ml.

5) **Potassium per sulfate ($K_2S_2O_8$)**: 5 percent solution: dissolved 25 g $K_2S_2O_8$ in a 500 ml volume metric flask and dilute with distill water to 500 ml.

2. Procedures for soil samples

1) Homogenized the soil samples and weight out a 0.2-2 g in the BOD bottle.

2) The BOD bottles along with the samples have to be place in the freezer about 60 minutes before adding reagent. Another method to cool samples was to put the BOD bottles with samples in the ice bath prior to adding reagent. The cooling process was counteracted the heating of the sample that can result from the addition of the acid and the potential volatilization of mercury that may result.

3) Added 5 ml concentrated sulfuric acid and 5 ml concentrated nitric acid to each sample. All process should be done under the hood so that the vapor from the acid will be vented outside.

4) Add 15 ml 5 percent potassium permanganate and wait 15 minutes. If the pink permanganate color does not persist for 15 minutes add additional permanganate. Repeat this step as many times as necessary until the pink color persist. Record the volume of potassium permanganate need for each sample.

5) Placed all samples in the hot air oven and set the oven at 60^{o}C and digest the unstopped samples for 2 hours. Record the 2 hours period from time the blank sample reached 60^{o}C.

6) Cool the samples at room temperature and add 5 ml potassium persulfate solution. Stopped the samples and allow standing overnight.

7) Add 6 ml or sufficient Sodium chloride-hydroxylamine hydrochloride solution until the brown color of manganese oxides and excess potassium permanganate were dissipated. One addition was usually sufficient. Allow 5 minutes to reduce any excess permanganate.

8) Add 136 ml to empty BOD weight and recorded the new number (BOD weight + 136 g = bottle sample weight). Add distill water as closed to new weight as possible. If not exact be sure to record new number for calculation.

3. Calculation

Calculated the mercury concentration in the soil as follow:

Hg ug/kg (wet weight) = 1000X/g

Hg ug/kg (dry weight) = 1000X/g(%S)

Where

X = weight of mercury in the samples, ug

g = wet weight of samples used, g

%S = percent solids in the samples as a decimal fraction.

BIOGRAPHY

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