EFFECT OF CADMIUM LEVELS ON CHROMOSOME STRUCTURE OF CATFISH (*Clarias batrachus)*

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Thesis
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EFFECT OF CADMIUM LEVELS ON CHROMOSOME STRUCTURE OF CATFISH (*Clarias batrachus)*

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ABSTRACT

This study aimed to determine the level of cadmium (Cd) contamination in various organs of commercial catfish (*Clarias batrachus*). The genotoxic effect of cadmium was determined from frequencies of micronucleus and nuclear abnormalities parameters. For commercial fish Cd contamination, catfish were purchased from six different markets in Bangkok metropolitan area: Wongweanyai, Bangkoknoi, Sainert, Yingcharoen, Khlongtoei, and Ratchawat markets. Cd residues in catfish were determined by the Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS), as 0-36.31, 0-45.27, 0-67.47 and 10.14-185.04 ppb in muscle, skin, gills and liver respectively. The results showed high Cd accumulation in the liver, whereas accumulation in muscle, skin, and gills varied depending on the market. The highest level of Cd was 0.185 ppm (mg/kg wet weight), which was found in the liver tissue of a fish collected from Ratchawat market. This level did not exceed the acceptable standard level set by the Fish Inspection & Quality Control Division of Thailand, which is 1.5 ppm. Therefore, consumption of the commercial catfish may not cause acute Cd toxicity. The genotoxic potential of Cd and Cd uptake in catfish were determined by micronucleus assay and GFAAS, respectively. Young catfish (2 months old) were purchased from a commercial fish farm, cultured in glass aquaria and exposed to various concentrations of Cd (0.005, 0.05, 0.5, 1, 5, 10, and 20 ppm $CdCl_2$) for 96 hours. After the exposure period, the erythrocytes were collected and smeared onto slides. They were observed for micronucleus and nuclear abnormalities and various tissues were collected to determine Cd uptake. The results showed concentration-dependent in increase of micronucleus, which were significantly different from control in 10 and 20 ppm treated groups. For the Cd uptake, the results showed high accumulations in liver, followed by gills, while muscle (including skin), Cd was in the relatively low concentrations. The results were concentration-dependent. Therefore, the increase in Cd uptake due to the increase in Cd concentration correlated well with the increase in genotoxic parameters. The results showed that the Cd uptake level by catfish is a suitable biomarker for Cd exposure. However, micronucleus frequencies assay in catfish erythrocytes induced by Cd was not a suitable biomarker for Cd genotoxic effect, since it required high cadmium concentration to induce genotoxicity.

KEY WORDS: CADMIUM / CATFISH (*Clarias batrachus)* / GENOTOXICITY / GFAAS / MICRONUCLEUS ASSAY

98 pp.

ผลกระทบของระดับแคดเมียมต่อโครงสร้างโครโมโซมของปลาดุก (EFFECT OF CADMIUM LEVELS ON CHROMOSOME STRUCTURE OF CATFISH (*Clarias batrachus*))

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บทคัดย่อ

การศึกษานี้เป็นการสำรวจการปนเปื้อนของแคดเมียมในเนื้อเยื่อหลายชนิดของปลาดุกที่มีจำหน่ายในตลาดกรุงเทพมหานคร และทดสอบความเป็นพิษของระดับแคดเมียมที่ตรวจพบต่อสารพันธุกรรมในเซลล์เม็ดเลือดแดงของปลาดุกโดยวิธีไมโครนิวเคลียส รวมทั้งประเมินความเหมาะสมของปลาดุกเพื่อใช้ในการศึกษาความเป็นพิษต่อสารพันธุกรรมโดยตรวจวัดระดับแคดเมียมที่เข้าสู่เนื้อเยื่อต่างๆของปลาดุก และความถี่ของไมโครนิวเคลียสหลังสัมผัสกับแคดเมียมต่างระดับ การเก็บตัวอย่างปลาดุกอาศัยวิธีสุ่มตัวอย่างจากตลาดทั่วกรุงเทพมหานคร 6 ตลาด ได้แก่ ตลาดวงเวียนใหญ่ ตลาดบางกอกน้อย ตลาดสายเนตร ตลาดยิ่งเจริญ ตลาดคลองเตย และ ตลาดราชวัตร จำนวนตลาดละ 6 ตัวอย่าง ระดับแคดเมียมตกค้างในเนื้อเยื่อ ซึ่งตรวจโดยเทคนิคอะตอมมิกแอบซอร์ฟชันสเปกโทรโฟโตมิตรี พบแคดเมียมในเนื้อ หนัง เหงือก และตับปริมาณ 0-36.31, 0-45.27, 0-67.47 และ 10.14-185.04 ppb ตามลำดับ ทั้งนี้เนื้อเยื่อส่วนที่มีการสะสมของแคดเมียมสูงสุดคือ ตับ โดยตับจากตัวอย่างที่เก็บจากตลาดราชวัตรมีปริมาณสูงที่สุด คือ 0.185 ppm ซึ่งเป็นปริมาณที่ยังไม่สูงเกินค่ามาตรฐานที่กำหนดโดยกองตรวจสอบรับรองมาตรฐานคุณภาพสัตว์น้ำและผลิตภัณฑ์สัตว์น้ำ กรมประมง (1.5 ppm) ผลการศึกษาการเหนี่ยวนำให้เกิดไมโครนิวเคลียสโดยระดับแคดเมียมต่างกัน 7 ระดับ (0.005, 0.05, 0.5, 1, 5, 10 และ 20 ppm)ในเซลล์เม็ดเลือดแดงซึ่งได้รับแคดเมียมเป็นเวลา 96 ชั่วโมง พบว่า แคดเมียมระดับสูง (10 และ 20 ppm) เพิ่มความถี่ของการเกิดไมโครนิวเคลียสอย่างมีนัยสำคัญ โดยความถี่ของไมโครนิวเคลียสขึ้นกับระดับของแคดเมียม ในขณะที่แคดเมียมปริมาณต่ำไม่ก่อให้เกิดการเปลี่ยนแปลงความถี่ของไมโครนิวเคลียสเมื่อเทียบกับกลุ่มควบคุมตลอดการศึกษา นอกจากนี้ระดับแคดเมียมซึ่งเข้าสู่เนื้อเยื่อจะขึ้นกับระดับของแคดเมียมในน้ำ และสัมพันธ์กับความถี่ของการเกิดไมโครนิวเคลียส

การศึกษาครั้งนี้แสดงให้เห็นว่าระดับแคดเมียมในเนื้อเยื่อปลาดุกเหมาะสมที่จะใช้เป็นตัวบ่งชี้ระดับการปนเปื้อนของแคดเมียมในน้ำ แต่ปลาดุกมีไวน้อยในการเหนี่ยวนำให้เกิดไมโครนิวเคลียสเมื่อได้รับแคดเมียมในระยะเวลาสั้น จึงไม่เหมาะสมที่จะใช้ในการตรวจวัดคุณสมบัติการก่อกลายพันธุ์ของแคดเมียมโดยวิธีไมโครนิวเคลียส เนื่องจากแคดเมียมที่ตรวจพบในเนื้อเยื่อปลาดุกที่เก็บจากตลาดไม่ก่อให้เกิดการเปลี่ยนแปลงทางโครงสร้างของโครโมโซมปลาดุก ผู้ที่บริโภคปลาอาจไม่เกิดอาการพิษเนื่องจากแคดเมียม แต่ควรศึกษาความเป็นพิษต่อสารพันธุกรรมของแคดเมียมโดยวิธีการอื่น ๆ เพิ่มเติมเพื่อประโยชน์ต่อผู้บริโภค

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LIST OF ABBREVIATIONS

%	percent
°C	degree celsius
Alb	albumin
As	arsenic
Cd	cadmium
$CdCO_3$	cadmium carbonate
$CdCl_2$	cadmium chloride
CdO	cadmium oxide
CdTe	cadmium telluride
cm	centrimeter
DNA	deoxyribonucleic acid
DMT	Duodenal iron transport
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia (for example)
et al.	and colleagues
etc.	et cetera
GFAAS	graphite furnace absorption spectrometer
GM	Geometric mean
g	gram
g/l	gram per liter
HCl	hydrochloric acid
Hg	mercury
HNO_3	nitric acid
h	hour
kg/day	kilogram per day

LIST OF ABBREVIATIONS (cont.)

LD_{50}	the mean lethal dose
m	meter
mg/day	milligram per day
mg/kg BW	milligram per kilogram body weight
mg/ml	milligram per milliliter
min	minute
ml	milliliter
mm	millimeter
mmHg	Millimeters of mercury
mM	millimolar
MN	micronuclei
MT	metallothionein
N	number of sample
Na_2HPO_4	sodium phosphate dibasic
NaH_2PO_4	sodium phosphate monobasic
n.d.	not detectable
ng/ml	nanogram per milliliter
Ni	nickle
nm	nanometer
Pb	lead
ppb	part per billion
ppm	part per million
SD	standard deviation
SEM	standard error of mean
Zn	zinc
ZnS	zinc sulfide
μm	micrometer

LIST OF ABBREVIATIONS (cont.)

µg/kg	microgram per kilogram
µg/L	microgram per liter
µg/ml	microgram per milliliter

CHAPTER I
INTRODUCTION

Over the past decade, Thailand has undergone through rapid urbanization and industrial development. The intensive development in industries and infrastructures, as well as the immense increasing in waterway transportation and other activities resulted in the deposition of diverse quantities of chemicals into the aquatic environment. Heavy metals, the important group of pollutants, are ubiquitous in polluted aquatic environment. Their presence in the environment has increased in some areas to the level which threaten the health of aquatic and terrestrial organisms. Moreover, some of these metals find their way into human through food and drinking water, which causes serious threat to health.

Cadmium (Cd) is one of the heavy metal pollutants. It is naturally present in zinc ores, and is generally produced as a by-product of zinc smelting (ATSDR, 1999). Although cadmium is a rare element, its concentration in the lithosphere ranges from 0.15-0.2 mg/kg (Fleischer, 1974). It was estimated that about 25,000 to 30,000 tons of cadmium are released into the environment each year, about half from the weathering of rocks into water resource (ATSDR, 1999). Forest fires and volcanoes also release some cadmium into the air. Cadmium released from human activities was estimated to be about 4,000 to 13,000 tons per year with major contributions from mining activities and burning of fossil fuels, e.g., coal fired electrical plants (ATSDR, 1999). It is also released into the environment through application of phosphate fertilizer in the agriculture and disposal of cadmium-containing wastewater by industrial activities. As a no degradable cumulative pollutant, cadmium contamination is an important problem due to its persistent nature in the environment (Sorensen, 1991).

It is well known that the disposal of pollutants into aqueous ecosystems can lead to their accumulation both in sediments and through bioaccumulation up the food chain, directly from contaminated water or indirectly from feeding on living organisms in the contaminated water. Human can be exposed throughout lifetime to low levels of

toxicants present in both the water and aquatic food (Al-Sabti, 1991). Thus, the study of water contamination is important to determine human exposure to the aquatic environmental pollutants.

For the assessment of the toxic effects of different pollutants in the aqueous environment, fish are proven to be very important indicator organisms, due both to their top position in the food chain and their requirement for great volumes of water in respiration, making their exposure to pollutants very intensive (Al-Sabti, 1991). Since it responses to toxicants in a similar way to higher vertebrates, the distribution and effects of chemical contaminations in the aquatic environment can be determined in fish (Al-Sabti and Metcalfe, 1995). Fish can accumulate cadmium from the polluted environment in their tissues (Bryan and Langston, 1992; Kargin, 1998; Kalay *et al*., 1999; Rashed, 2001; Yilmaz, 2003; Ashraf, 2005), resulting in adverse effects, especially in genotoxicity (Sanchez-Galan *et al*., 2001; Chandra and Khuda-Bukhsh, 2004; Cavas *et al.*, 2005).

The cytogenetic investigations in fish were carried out under actual field and laboratory conditions to detect genotoxic activity in an aquatic environment. Cytogenetic studies of water pollutants in fish have shown increase in frequency of chromosomal aberration (Al-Sabti 1984; Al-Sabti 1985; Al-Sabti 1986a). Cytogenetic observations were primarily based on chromosomal aberration analysis, even though such test exhibits some disadvantages such as low mitotic activity, difficulty in finding a sufficient number of metaphases for scoring chromosome aberrations and the limitation of a suitable fish karyotype (Kligerman *et al*., 1975; Hooftman and Raat, 1982). Micronucleus (MN) test applied in genotoxic biomonitoring of clastogenic effects of pollutants has proven the most practical tool in environmental mutagenesis. This endpoint is widely used in aquatic organisms such as mussels, bivalve mollusks, tadpoles and fishes (Metcalfe, 1988; Rodriguez-Ariza *et al*., 1992; Al-Sabti, 1994; Al-Sabti and Metclafe, 1995; Vernier, *et al*., 1997).

As a preliminary step in monitoring the levels of genotoxic pollutants in water, the native fish species was used. In this study walking catfish (*Clarias batrachus*), a scaleless-skin fish, was chosen because it is a commonly found fish with considerable economical importance. It is a widely distributed species, around South and South East Asia countries and farmed in Cambodia, Laos, Guam, Indonesia, India, Malaysia,

Philippines, China, Taiwan, and Thailand (Axelrod *et al*., 1971). Within areas of its native range, the walking catfish is valued as a food fish and is the focus of both subsistence fishers and commercial farming operations (Piumsombun, 2000). Although an omnivore, it is a voracious predator with major preys include insect larvae, insects such as beetles, mayflies, dragonflies, fish eggs and occasionally they may take plant material (Courtenay *et al.,* 1974), thus making them accumulate aquatic contaminants via consumption, particularly cadmium.

The efficiency and suitability of the catfish as biomarker of cadmium pollution have proven. The purposes of this study were to determine the extent of cadmium contamination in various tissues of catfish sampled from markets in Bangkok metropolitan area and to explore the correlations that may exist between cadmium levels and the degree of genetic damage in catfish erythrocytes *in vivo* exposed to cadmium by micronucleus assay. Finally, the results from this study will provide information on water pollution and food safety.

CHAPTER II
OBJECTIVES

1. To survey cadmium contents in commercial catfish (*Clarias batrachus*), covering Bangkok metropolitan.
2. To evaluate the suitability of catfish as a model for genotoxicity study.
   2.1 Determination of cadmium uptake by catfish
3. To evaluate the genotoxic potential of detected cadmium levels in catfish erythrocytes.
   3.1 Determination of micronucleus induction by cadmium
   3.1 Determination the relationship between micronucleus frequencies and cadmium uptake levels.

CHAPTER III
LITERATURE REVIEWS

1. Cadmium

1.1 Introduction

Itai-Itai disease has been an endemic among the elderly women in the Jinzu River basin in Toyama, Japan, since the World War II and up to the early 1970s. In 1955, Dr. Noboru Hagino first reported his studies to an academic society and called this syndrome Itai-Itai disease (Chang *et al.*, 1996). The Japanese word "itai" means "ouch" or "painful" in English. It was the most well known event of toxic effects of cadmium on man, people suffered from a disease that, under much pain and suffering, resulted in severe bone deformation and, in many cases, death. It was due to the river water being polluted by cadmium-containing waste from the Kamioka mine, which produced mainly zinc, as well as relatively small amounts of lead and cadmium (Chang *et al.*, 1996). The river water was used for irrigation of rice fields, which resulted in cadmium-contaminated rice. The consumers, women in particular, then suffered from decalcification of the skeleton (osteomalacia), which led to skeletal deformation and frequent bone fractures. Even the slightest exertion, such as coughing, could result in, for example, broken ribs.

1.2 Physical and chemical properties of cadmium

Cadmium (Cd) is a nonessential trace element, with atomic number of 48 and molecular weight (MW) of 112.4. It is a silvery-white, lustrous, but tarnishable metal. It is ductile and has a relatively high vapor pressure (Sorensen, 1991). The most remarkable characteristics of cadmium are its great resistance to corrosion, its low melting-point and excellent electrical conduction. Therefore, cadmium compounds exhibit excellent resistance to chemicals and to high temperatures (International Cadmium Association, 2004).

Cadmium is a metallic, transition element with two electrons in the outermost shell and the penultimate shell filled with eighteen electrons, as a member of Group 2B of the periodic table (Sorensen, 1991). Cadmium has possible valences of 0, +1, and +2, but it forms most of its compounds in the +2 oxidation state. Cadmium metal is therefore slowly oxidized in moisted air, and when heated in air, it rapidly forms cadmium oxide (Carr, 1992). Because cadmium is nearly always divalent, chemically, it closely resembles zinc and occurs by isomorphous replacement in almost all zinc ores (Cotton and Wilkinson, 1972).

Cadmium exists in many forms. The most common forms are elemental cadmium, cadmium carbonate, cadmium chloride, cadmium oxide, cadmium sulphate and cadmium sulphide. Their physical and chemical properties are summarized in Table 1.

Table 1. Physical and chemical properties of cadmium and its compounds (ATSDR, 1999)

Characteristics	Cadmium	Cadmium carbonate	Cadmium chloride	Cadmium oxide
Formula	Cd	$CdCO_3$	$CdCl_2$	CdO
Valence	0	+2	+2	+2
Molecular weight	112.40	172.42	183.32	128.41
Color	Silver-white	White	Colorless	Red or brown
Physical state	Solid	Solid	Solid	Solid
Melting point	320.9^{o}C	Decomposes at <500 ^{o}C	568 ^{o}C	1426 ^{o}C
Boiling point	767^{o}C	No data	960^{o}C	1426^{o}C
Solubility in water	Insoluble	Practically insoluble	1400 g/L at 20^{o}C	Insoluble
Vapor pressure	1 mmHg at 394^{o}C	No data	10 mmHg at 656^{o}C	1 mmHg at 1000^{o}C

1.3 Cadmium sources

The metal cadmium is a relatively rare element and is not found in the pure state in nature (Friberg, *et al.*, 1992). Cadmium is naturally found as the cadmium sulphide, also known as greenockite or cadmium blende, which is often associated with the zinc ore, sphalerite (ZnS) (Chang *et al.*, 1996). It is economically recoverable only when found in this form or associated with other non-ferrous metal ores, such as those of lead and copper, as a by-product during the roasting, smelting, or other processing of these ores (ASTDR, 1999).

Cadmium is a commonly occurring environmental contaminant in food, water, and air. There are two major emission sources of cadmium that are significant and widespread, anthropogenic and natural source (Chang *et al*., 1996) , the data was shown in Table 2. Anthropogenic sources of cadmium to the environment are refining and use, which include copper and nickel smelting and fossil fuel combustion. Natural sources of cadmium to the atmosphere are volcanic activity, forest fires and windblown transport of soil particles. It is important to note that the anthropogenic sources of cadmium add 3–10 times more cadmium to the atmosphere than natural sources (Irwin *et al.*, 1997). Other sources of concern are phosphate fertilizers, which may contain high concentration of cadmium depending on the origin of the rock, and the application of contaminated sewage sludge as a soil amendment (Thornton, 1992).

Table 2. Global emission of trace metals to the atmosphere in 1983 (in 10^3 tons) (Nriagu and Pacyna, 1988).

Source category	As	Cd	Hg	Pb	Zn
Fossil fuel combustion	0.4-3.7	0.2-1.1	0.6-3.5	2.7-18.4	3.1-23.4
Gasoline combustion	-	-	-	248.0	-
Nonferrous metal industry	9.6-15.1	2.6-8.2	0-0.2	30.0-69.6	51.0-93.8
Other anthropogenic sources	2.0-6.8	0.3-2.7	0.3-2.5	8.0-40.0	15.9-76.3
Total anthropogenic emissions	18.8	7.6	3.6	332.0	132.0
Natural emissions	7.8	1.0	6.0	19.0	4.0

1.4 Applications of cadmium

Cadmium is a modern toxic metal and was discovered as an element only in 1817 in Germany by F. Stromeyer (Stoeppler, 1991). Unlike most metals, cadmium use began fairly late with its large-scale application dating from 1940s (Klaasen, 2001). Because of its non-corrosive properties, the principal uses of cadmium metal in the early date are the coating and electroplating of metals to prevent corrosion.

Cadmium has a limited number of applications but it is used for a variety of consumer and industrial materials. The major intentional uses of cadmium are Ni-Cd batteries, cadmium pigments, cadmium stabilizers, cadmium coatings, cadmium alloys and cadmium electronic compounds such as cadmium telluride (CdTe). The major classes of products where cadmium is present as an impurity are non-ferrous metals (zinc, lead and copper), iron and steel, fossil fuels (coal, oil, gas, peat and wood), cement, and phosphate fertilisers (Cook and Morrow, 1995).

In recent years, the consumption pattern of cadmium in its various end use applications has increasingly shifted away from the traditional market areas of pigments, stabilisers and coatings to rapidly growing applications in Ni-Cd batteries (International Cadmium Association, 2004), as shown in Figure 1.

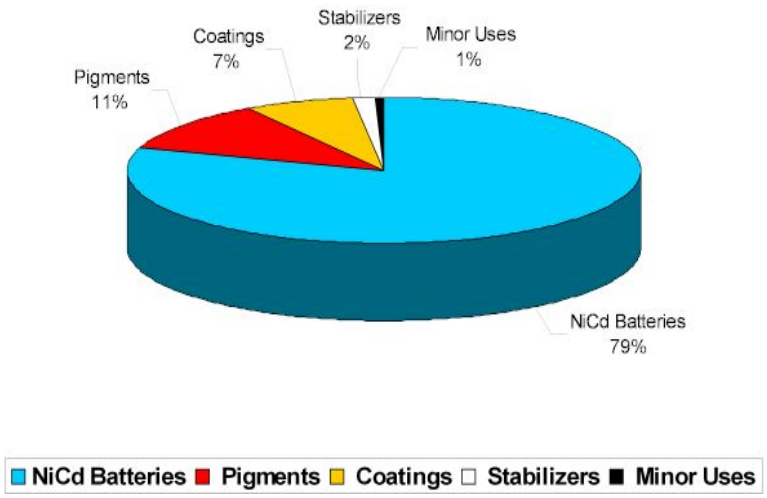


Figure 1. Consumption pattern of cadmium in 2003 (International Cadmium Association, 2004)

1.5 Cadmium exposure

Human cadmium exposure occurs both in occupational and general environment. For general population, food and cigarette smoke are the major sources of human exposure. Cadmium exposure is mainly from cigarette smoke which contains relatively high concentration of this element. For non-smokers in general population, diet is the main route of exposure to cadmium, while intake via drinking water is negligible (WHO, 1992).

1.5.1 Airborne cadmium

In air, cadmium exists as fine particulate (less than 10 mm). The main chemical species in the atmosphere is cadmium oxide although other cadmium salts may also be present (ASTDR, 1999). The cadmium compounds in air are stable and undergo little atmospheric transformation. Cadmium particulate is dispersed by wind and eventually either settled out by rain or snow or dry deposited on land or surface water. The fine particulate containing cadmium can remain airborne for days to weeks and travel hundreds to thousands of kilometers.

Workplace exposure to cadmium is particularly hazardous in the presence of cadmium fumes or airborne cadmium. Major occupational exposure occurs in electrolytic refining of lead and zinc, and other industries that employ thermal processes; e.g., iron production, fossil fuel combustion, and cement manufacture (Klaasen, 2001). A major non-occupational source of respirable cadmium is cigarettes. Smokers may double their daily intake of cadmium compared with nonsmokers. The smokers may take in an additional 1-3 μg of cadmium into their body per day from each pack of cigarettes smoked, while passive smoking does not appear to increase exposure to cadmium appreciably (ASTDR, 1999). Aside from tobacco smokers, people who live near hazardous waste sites or factories that release cadmium into the air have the potential for airborne exposure of cadmium.

1.5.2 Waterborne cadmium

Cadmium occurs naturally in aquatic systems, it exists either dissolved or as part of insoluble complexes and is more readily found in the free ionic (ASTDR, 1999). Solubility is promoted by acidic conditions; dissolution of suspended or

sediment-bound cadmium may result when there is an increase in acidity (Fleischer, 1974). Soluble cadmium is quite mobile in water and in soil, partitioning into the sediment is enhanced by precipitation and sorption to mineral surfaces and organic materials as well as by action of sediment bacteria. The levels in the sediment tend to be at least an order of magnitude higher than in the overlying water column. High levels of organic material in the water promote formation of organic complexes with cadmium which are poorly soluble. Also in reducing environment, cadmium may precipitate out as cadmium sulfide.

Cadmium is found less than 0.001 mg/L in unpolluted freshwater, but can excess in water contaminated by industrial wastes or by effluents from sewage treatment plants (Fleischer, 1974), as showed in Table 3.

Table 3. Cadmium concentrations in water and sediment of Chao Phraya River and associated waterways (McLaren *et al*., 2004)

Locations	Cd in water (μg/l)		Cd in sediment (μg/kg)	
	Range	Mean	Range	Mean
Chao Phraya River, Ayutthaya	0.02-0.54	0.23	184-265	208
Chao Phraya River, Nonthaburi	0.02-0.12	0.07	198-387	226
Chao Phraya tributary, Nonthaburi	0.011-0.016	0.013	36-394	188
Sum-Rong Canal, Samutprakarn				
(a) Dry season	n.d.-0.62	0.34	226-953	493
(b) Wet season	n.d.	n.d.	95-197	147
Phraya Bunleur Canal, Ayutthaya	n.d.-0.009	0.005	77-379	278

n.d. = Not detectable

1.5.3 Cadmium pollution in soils

Cadmium gets into soil from the use of fertilizers and as a result of zinc-mining processes, in which cadmium is a discarded impurity (Järup, 2002). For example, cadmium concentration in Swedish soil has increased continuously during

the last century and high-fiber diets have been shown to result in increased dietary cadmium (Järup *et al*., 1998).

Cadmium in soils may leach into water, especially under acidic conditions. Transformation processes for cadmium in soil are mediated by sorption from and desorption to water, and include precipitation, dissolution, complexation, and ion exchange (ATSDR, 1999).

1.6 Cadmium in Food

In general non-smoking population, dietary intake represents the largest source of cadmium exposure. Cadmium can be taken up and retained by aquatic and terrestrial plants and can substantially bioconcentrated in aquatic invertebrates and fish. In terrestrial animals, cadmium is particularly concentrated in the liver and kidney of animals that eat the contaminated plants. There are many way that cadmium can enter into human food (Figure 2).

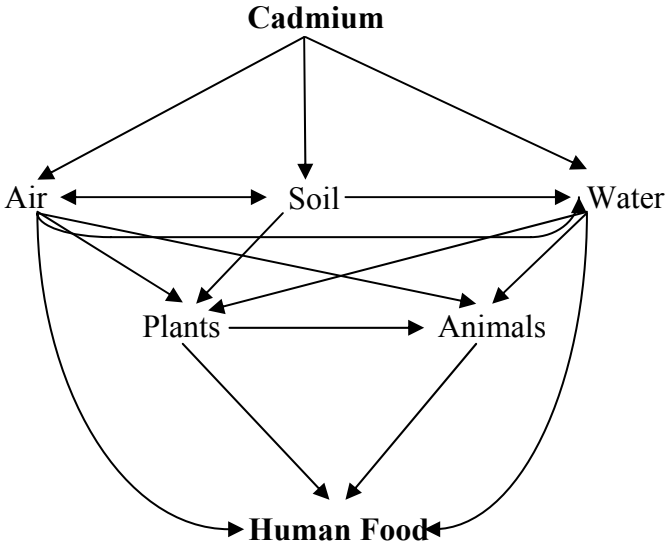


Figure 2. Routes of pollutant cadmium flow into human food.

Food, fiber, and many products essential to sustain human life come directly or indirectly from plants which are grown in soil. The soil is the main source of metal found in plants both of nutrients and some toxic heavy metals. Elemental compositions in biota are influenced by many respects, including, some species of plants such as rice

and wheat have an activity to accumulate cadmium naturally by absorption from soil; low soil pH tends to increase the availability of cadmium. The presence of potentially toxic substances in the soil could result in disorders. Since ancient times, the trace element deposition onto soil has been the cause of many human ailments. For example, the etiology of Itai-Itai disease is attributed to consumption of rice grown on soil contaminated by cadmium-containing wastes (Chang, *et al*., 1996).

Concentration of cadmium in drinking water is generally below 2.0 µg/l, implying that it is not an important source of cadmium exposure for most people (WHO, 1992). On the other hand, pollution of cadmium in the surface water can be transferred to the aquatic food sources and subsequently to human diets. In general, the first trophic level (aquatic plants) possesses the highest accumulation potential for cadmium. Marine foods for human consumption are harvested from many trophic levels. So the ingestion of seafood represents an important route by which many people become exposure to cadmium.

The highest cadmium concentrations are generally found in offal, shellfish, seeds, whole grain products, and certain mushrooms. Cereals, roots, vegetables, and in certain populations seafood, Shellfish, such as mussels, scallops and oysters, may be a major source of dietary cadmium and contain 100 to 1000 µg/kg. Meat, fish and fruit contain 1 to 50 µg/kg, grains contain 10 to 150 µg/kg, and greater concentrations are in liver and kidney (Klaasen, 2001) (Table 4, 5 and 6).

Table 4. Concentration of cadmium in biological samples of poultry from Slovakia (Skalická *et al*., 2002)

Biological samples (N)	Cadmium concentration (mg/kg, origin matter)	
	Range	Mean ± SD
Leg mucle (72)	0.010-0.025	0.019 ± 0.005
Breast muscle (72)	0.017-0.025	0.021 ± 0.003
Heart (72)	0.042-0.080	0.061* ± 0.016
Liver (72)	0.075-0.121	0.099* ± 0.017

*statistical significance at $p \leq 0.05$

Table 5. Cadmium levels (ppm, wet weight) in commercial fish from New Jersey market (Adapted from Burger and Gochfeld , 2005)

Species	N	Cd levels	
		Mean ± SEM	GM
Bluefish	51	0.006 ± 0.002	0.003
Chilean sea bass	7	0.004 ± 0.001	0.002
Cod	7	0.005 ± 0.003	0.00009
Croaker	14	0.001 ± 0.0004	0.001
Shrimp (small)	12	0.00013 ± 0.0004	0.03
Shrimp (large)	12	0.004 ± 0.002	0.001
Yellow fin tuna	50	0.03 ± 0.005	0.02

The data are shown in mean ± SEM.

N = Number

GM = Geometric mean

Table 6. Concentration of cadmium in food plants grown on uncontaminated and contaminated soils (Kabata-Pendias and Pendias, 1992).

Plant	Cd concentration (mg/kg dry weight)	
	Uncontaminated soils	Contaminated soils
Leafy vegetables	0.05-0.66	5.2-45
Root/tuber vegetables	0.03-0.24	1.7-3.7
Grain/cereal	0.01-0.21	0.72-4.2

While cadmium is found in most food types, this remains at a low concentration, unless contamination has occurred (Table 6) (Reilly, 1991). Plants readily take up cadmium from soil contaminated by fallout from the air, cadmium containing water used for irrigation and cadmium-containing fertilizers. Another source of concern about potential sources of cadmium toxicity is the use of

commercial sludge to fertilize agricultural fields, which may contain up to 1500 mg of cadmium per kilogram of dry material (Klaasen, 2001).

1.7 Toxicokinetics of cadmium

It has been well established that excess cadmium exposure produces adverse health effects on human health. For virtually all chemicals, adverse health effects are noted at sufficiently high total exposures. The relevant questions with regard to cadmium exposure are the total exposure levels and the principal factors which determine the levels of cadmium exposure and the absorption rate of the ingested/inhaled cadmium by the individual, in other words, the pathways by which cadmium enters the food chain, the principal pathway of cadmium exposure for most human.

1.7.1 Absorption

Inhalation exposure of cadmium primarily occurs in the workplace. Cadmium and its salts have low volatility and exist in air primarily as fine suspended particular matter, either as fumes or dust (ASTDR, 1999). After inhalation, the exposure of cadmium compounds may vary greatly and depends upon the particle sizes and their solubility. Some fractions of the particulate matter would be deposited in the respiratory tract and the remaining fractions exhaled. Based on the physiology of the human respiratory tree, Norberg *et al.* (1985) has developed a model to predict the kinetics of inhaled cadmium in humans. Modelling results indicated that only about 5% of the large particulate (greater than 10 μm) remains deposited in the upper respiratory tract. Much of the particulate that is not exhaled is transported up the respiratory tree and eventually swallowed. In contrast, about 50% the finer particulate of about 0.1 μm tends to get deposited into the lung tissues. While the major site of absorption is the alveoli, between 50-100% of cadmium deposited in the alveoli will ultimately be absorbed into the blood (Nordberg *et al*., 1985). Therefore, for the purpose of this analysis, about 25% of cadmium fine particulate is considered absorbed. The respiratory cadmium intake can be diverted to the gastrointestinal tract due to the clearance of cadmium deposited on the mucosa of nasopharynx, trachea or bronchi.

Most ingested cadmium from food and water passes through the gastrointestinal tract without being absorbed, only about 5-8% is absorbed (Klaasen, 2001). The intestinal uptake process consists of two separable steps; transport over the luminal membrane into epithelial cytoplasm and transport over the basolateral membrane into serosal fluid. Cadmium ion was absorbed mainly in duodenum and early jejunum (Andersen *et al.*, 1994). The available information indicated that various dietary factors and physiological status can vary in the amount of cadmium absorption. Dietary factors affecting cadmium absorption include metal-metal (e.g. iron, calcium, chromium, magnesium, and zinc) and metal-protein interactions (glutathione and sulfhydryl containing enzyme) in the body and in food or water (ASTDR, 1999). Low dietary calcium stimulates synthesis of calcium-binding protein, which enhances cadmium absorption (Klaasen, 2001). Other physiological status such as age, body stores of ion, calcium, and zinc, pregnancy history also influence cadmium absorption. Human studies showed that reduced body iron storage, which commonly found in women of child-bearing age, is associated with elevated absorption of cadmium (Klaasen, 2001). Probably there is a common absorption mechanism for cadmium and iron, involving DMT1, a duodenal iron transporter (Nicolas, *et al.*, 2003), which is upregulated at low iron stores. It has been shown that the body burden of cadmium increases during pregnancy, mainly due to the decreasing iron status.

Dermal absorption of cadmium is generally very low (about 0.5%), and there is a significant risk from skin exposure unless contact for long periods of time or at very high levels (ASTDR, 1999).

1.7.2 Distribution

Cadmium is not known to undergo any direct metabolic conversion. But cadmium ion can bind to anionic group such as sulphydry group in proteins, in particular albumin and metallothionein (ASTDR, 1999). After uptake from the lung or the gastrointestinal tract, cadmium is transported in blood plasma initially bound to albumin and preferentially taken up by the liver. In the liver, cadmium induces the synthesis of metallothionein and a few days after exposure metallothionein-bound cadmium appears in the blood circulation. Because of its low molecular weight,

cadmium-metallothionein is efficiently filtered through the glomeruli and thereafter reabsorbed by the tubules and accumulated in the kidney cortex.

Relatively large amounts of cadmium can be retained in the body in this manner and in this form, cadmium is relatively non-toxic. The metallothionein may be degraded releasing cadmium which may then induces fresh synthesis of metallothionein in the kidney. Cadmium induced renal toxicity is believed to be due to the action of cadmium not bound to metallothionein.

Distribution of cadmium is relatively independent of the route of exposure. Once absorbed, cadmium is rapidly cleared from blood into various tissues and has a high volume of distribution. Deposition occurs principally in the liver and kidneys, with the latter generally containing about one third of the total body burden. Smaller amounts are distributed to the bone, testes, spleen, various endocrine organs, brain, and muscle tissue (Lee and White, 1980).

1.7.3 Elimination

Most cadmium that is ingested or inhaled and transported to the gut via mucociliary clearance is excreted in the feces. Almost all fecal cadmium represented the portion that was not absorbed from the gastrointestinal tract. Most absorbed cadmium is excreted very slowly, with urinary and fecal excretion being approximately equal. It was estimated that about 0.007% of body burden is eliminated daily in feces and about 0.009% in urine (ASTDR, 1999). Cadmium is also eliminated through hair and breast milk, but these routes are of limited importance as compared to the total excretion. The placenta is only a partial barrier to fetal exposure to cadmium.

Several studies have shown that in the general population, urinary cadmium excretion increases with age, and this increase coincides with the increased body burden. Smokers have higher urinary excretion than non-smokers. The amount of cadmium excreted represents only a small fraction of the total body burden unless renal damage is present.

The most dangerous characteristic of cadmium is that its very slow excretion makes it accumulate throughout lifetime. Cadmium accumulates in the liver and kidneys and has a long biological half-life of 17-30 years in man (Goyer, 1995). The summary of kinetics of cadmium is shown in Figure 3.

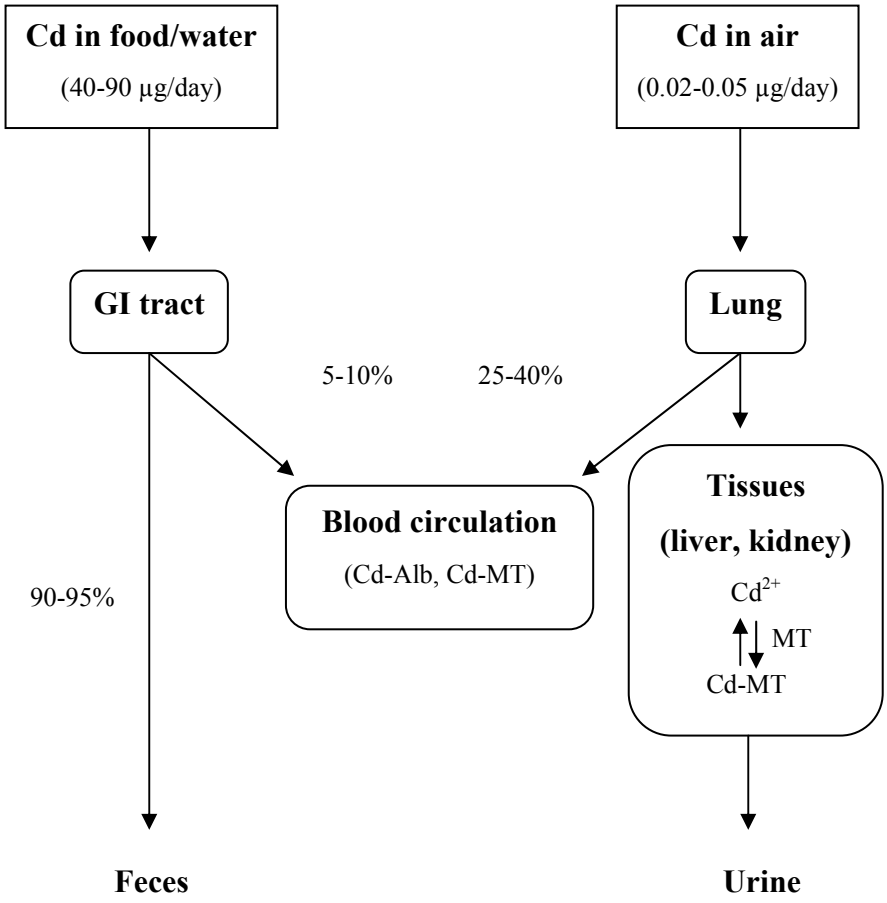


Figure 3. Kinetics of cadmium in human (Cd-Alb: Cd-Albumin bound, Cd-MT: Cd-Metallothionein bound)

1.8 Toxicity of cadmium

Cadmium is listed by the US Environmental Protection Agency as one of 126 priority pollutants. The classical example of the importance of cadmium as an environmental contaminant is the outbreak at the Jinzu River in Japan of a severe disease (Itai–Itai disease) characterized by severe pain, bone fractures, proteinuria and severe osteomalacia, which appeared mainly among women. It seems that the disease was caused by the ingestion of rice and water contaminated with cadmium originating in a mine slag, in combination with nutritional factors (Chang, *et al*., 1996).

In general, the toxicity of cadmium does not depend on its chemical form. Cadmium oxide is of most interest for inhalation exposure because it is the main form of airborne cadmium. However, other forms of cadmium appear to exhibit similar toxicological properties as cadmium oxide on inhalation. For oral exposure, the more

soluble salts of cadmium, such as cadmium chloride and carbonate are more important. The toxicity of cadmium is complex because of the large number of biological systems that can be affected and many different toxic effects that may be elicited. The important target organs of cadmium toxicity are kidneys, bone tisuues and respiratory system (ASTDR, 1999).

Kidneys: (Renal effects): The kidney is considered to be the critical target organ for the general population as well as for the occupationally exposed population. Within the kidney, the cortex is the site where the first adverse effect occurs.

Long-term exposure to cadmium causes renal tubular dysfunction with proteinuria, glucosuria, and aminoaciduria, as well as histopathological changes, in both experimental animals and humans. These are usually the first effects to occur after ingestion or inhalation exposure. In working environment with high cadmium exposure level, workers have also developed hypercalciuria, phosphaturia, and polyuria and some have suffered from renal colics due to recurrent stone formation. As the renal dysfunction progresses in severity, the glomeruli may also be affected and, in a few cases, the cadmium-induced damage may lead to renal failure.

Bone tissues: Case studies indicated that calcium deficiency, osteoporosis, or osteomalacia can develop in some workers after long-term occupational exposure to high levels of cadmium. Bone lesions (accompanied by renal damage) have also been reported in aged and malnourished women living in cadmium-polluted areas in Japan. Effects on bone generally arise only after kidney damage has occurred and are likely to be secondary to resulting changes in calcium phosphorus and vitamin D metabolism. The daily intake via food and exposure in air at which the bone effects occurred are probably in the same range as those produced kidney effects.

Respiratory system: Long-term occupational exposure to high levels of cadmium has been reported to cause emphysema and dyspnea in humans. The dose needed to produce these effects is higher than the dose needed to produce renal effects. Chronic inflammation of nose, pharynx, and larynx have been reported in some studies. Anosmia is a frequent symptom in cadmium workers after prolonged exposure.

1.8.1 Effects on Humans

1.8.1.1 Acute Toxicity

Acute, high level exposure to cadmium by inhalation or oral routes can be fatal. The cause of death is pulmonary edema following inhalation exposure, and massive fluid imbalance and widespread gastrointestinal, liver and other organ damage after oral exposure. The effects are mainly due to destruction of cell membranes at the point of entry.

High levels of cadmium oxide fumes or dust are extremely irritating to the respiratory tissues. A single high exposure can lead to long term impairment of the lung functions. Typical symptoms include tracheobronchitis, pneumonitis and pulmonary edema.

Oral exposure to acute, high level of cadmium causes severe irritation to the gastrointestinal epithelium. Common symptoms include nausea, vomiting, abdominal pain, cramps and diarrhea. Gastrointestinal toxicity is not observed after low level of oral exposure or after inhalation exposure.

1.8.1.2 Chronic Toxicity

Although cadmium can be acutely toxic, it is mainly a cumulative toxicant, and long term exposure is of most concern to human. Kidney is the main target of cadmium toxicity after intermediate to chronic exposure to this toxicant by either inhalation or oral route. The early sign of kidney damage from cadmium is the presence of a number of low molecular weight proteins (e.g. β_2-microglobulin and α_1-microglobulin) due to decreased reabsorption of these proteins, signifying dysfunction of the proximal tubule of the kidney (Chang *et al*., 1996). With longer exposures or at higher doses, there is a reduction in filtration of high molecular weight proteins and even necrosis. Renal function can deteriorate further, even after cessation of cadmium exposure. The impact of early kidney damage on the overall health of affected individuals is not clear. However, the damage affects the vitamin D metabolism in the kidney, this in turn leads to the disruption in calcium absorption and excretion, calcium imbalance and reduction in bone density (ASTDR, 1999).

Tubular dysfunction occurs only after cadmium reaches a minimum threshold level, generally referred to as the “critical concentration”, in the renal cortex.

The critical concentration of cadmium in the renal cortex associated with increased incidence of kidney dysfunction in an adult human population on prolonged exposure has been estimated to be 200 mg/g wet weight by several investigators (Friberg, 1974; Kjellstrom *et al.*1984; Roels *et al.* 1983).

Long term inhalation exposure to low levels of cadmium causing lung inflammation results in impairment in lung functions and emphysema. Some tolerance to cadmium induced lung irritation can develop in some workers.

Both oral and inhalation exposure can cause anemia in human, likely by reducing gastrointestinal uptake of iron from the diet. Cadmium-induced anemia is unlikely among populations that have adequate iron intakes to compensate for reduced iron absorption (ASTDR, 1999).

1.8.1.3 Genotoxicity

Cadmium has been shown to induce chromosomal aberration in human lymphocytes *in vitro* or from exposed workers in some studies but not in others. In those studies where significant responses were observed, the chromosomal aberrations tended to occur in the more heavily exposed group. Similarly, cadmium was shown to be genotoxic in some of the experimental tests, both *in vivo* and *in vitro*. Overall, cadmium appears to have the capability of altering genetic materials, particularly chromosomes in mammalian cells. Furthermore, cadmium and its compounds inhibit repair of DNA damaged by other agents, thereby enhancing their genotoxicity (ASTDR, 1999).

1.8.1.4 Carcinogenicity

Cadmium has been classified as a category 1 carcinogen (human carcinogen) by the International Agency for Research on Cancer (IARC, 1993), because cadmium has been associated with cancers of the lung, the prostate, the pancreas, and the kidney (Waalkes and Rehm, 1994, Schwartz and Reis, 2000, Pesch *et al*., 2000; Hu *et al*., 2002). The classification of cadmium as a human carcinogen is supported by strong evidence from animal experiments. In rodents, cadmium-induced tumors in various organs; adenocarcinomas of the lung in rats after inhalation (Glaser *et al.*, 1990; Takenaka *et al*., 1983), tumors of the prostate and the pancreas in rats by

subcutaneous injection, tumors of the testes by oral exposure of rats, and local tumors at various sites of injection, typically sarcomas, in rats and mice (Waalkes, 2000). Cadmium predominantly is a non-genotoxic carcinogen. It is essentially non-mutagenic in bacterial tests and only weakly mutagenic in mammalian cells in vitro (IARC, 1993). However, cadmium compounds have been proven to be comutagenic in mammalian cell tests when combined with genotoxic agents, and this property has been explained by the inhibition of DNA repair processes by this metal (Hartwig and Schwerdtle, 2002).

1.8.2 Effects on aquatic organisms

River water receives discharges from a variety of sources including industrial plants sewage drainage, agricultural drain water, atmospheric pollution, and other natural sources which lead to heavy metal pollution. Living in aquatic ecosystems, fish are particularly subjected to aquatic pollution, because most of them spend the whole of their life-cycle in water and their assimilation takes place through sensitive epithelia in intimate contact with their surroundings (Laale, 1981). The other reason is also because they, as representatives of relatively high trophic levels in the aquatic food chain. Therefore they are endangered by diet-borne pollutants, which are transferred along the food chain and, owing to bioaccumulation effects.

Fish accumulate heavy metals in their tissues to higher concentration levels than in their environment by the absorption along the gill surface and in the gut tract wall. The important food sources of fish, phytoplankton or invertebrates, are able to uptake cadmium into themselves. In some species of fish, such as rainbow trout, eel, and lake white fish, cadmium accumulates mainly through ingestion of contaminated food (Kraal *et al.*, 1995). Cadmium accumulates in fish through the food chain and may accumulate over years, while elimination is very slow. Heavy metals mainly accumulate in metabolically active tissue (Kock and Hofer, 1998). In fish, cadmium accumulates predominantly in the kidney, liver, gills, and digestive tract (Miliou *et al*., 1998). Metal distribution between the different tissues varies depending on the source of uptake, diet and/or waterborne exposure.

Cadmium is not a biologically essential element in fish, but may be a potential toxicant. Even at low concentration, it disturbs central function of fish by

affecting various basic biochemical and physiological processes. Furthermore, it leads to reproductive failure, behavioral changes, bone distortion and calcium deficiencies (Miliou *et al*., 1998). Cadmium-induced toxicity in fish is a function of water quality (e.g. salinity, water hardness, pH, alkalinity, and temperature) and parasitism (Sorensen, 1991).

The acute toxicity of cadmium to freshwater fish is highly variable. Salmonids seem to be particularly sensitive with lethal concentration (LC_{50}) of 0.001 to 0.03 mg/l (Sprague, 1987). Marine species similarly show considerable variability in their response to cadmium. For example, the LC_{50} for the mummichog *Fundulus heteroclitus* is 60 mg/l, while those for the Atlantic silverside *Menidia menidia* and the flounder *Pseudopleuronectes americanus* are 6.4 and 0.6 mg/l, respectively (Sprague, 1987).

Numerous subacute effects of cadmium in freshwater teleosts have been reported. Plasma electrolytes (sodium, potassium, calcium, and chloride) (Fu *et al*., 1989) and their growth (Shukla and Pandey, 1988) decreased after exposure to cadmium.

The gill damage by exposure to heavy metals is seen as the fusion of secondary gill lamellae, proliferation of chloride and mucus cells, and hyperplasia of gill epithelial cell (Forlin *et al*., 1986). The structure and function of the gills are likely to be severely altered after exposure to waterborne cadmium (Gony and Gostan, 1997). The main toxic action of cadmium is the impairment of ionic regulation rather than respiratory or nervous functions. The mechanism of impairment of Ca^{2+} uptake by cadmium is attributed mainly to the competitive inhibitory action of cadmium on basolateral Ca^{2+}-ATPase of bronchial chloride cells (Cinier *et al*., 1997). The symptoms of metal exposure also vary between the waterborne and dietary routes. Waterborne heavy metals enter the organism mainly via gills, Therefore, gills constitute the first target organ for metals. In contrast, a significant increase in cell death and proliferation was found in the intestine of fish from dietary cadmium (Berntssen *et al*., 2001).

Hematological changes following cadmium exposure include anemia and erythrocyte abnormalities, such as chromatin condensation, increase interchromatin spaces in erythrocyte nuclei, nuclear puffs, hypochromia, cytoplasmic erythrocyte

stippling, cell membrane deterioration, schistocytosis, lymphocytosis, thrombocytosis, neutropenia, and eosinophilia. Cadmium and other nonessential metals have been reported to cause anemia both in mammals and on fish. The mechanism involved in these animals is reportedly to be due to a reduction in absorption of iron from the gut for the synthesis of hemoglobin (Sorensen, 1991).

2. The walking catfish (*Clarias batrachus*)

2.1 Catfish

Catfish is an extremely large group of varied fish in the order Siluriformes (sometimes also called the Nematognathi) (Smith, 1965), which is classified into subclass Teleostoei; the group of ray finned fish as most dominant of fish in the world today (Greenwood *et al.*, 1966); they are comprise the bulk (96%) of all living fishes, estimated to be around 20,000 species (Srivastava, 1996).

There are approximately 2,211 species belonging to 400 genera of 31 families of catfish group (Nelson, 1984). About 1,300 species inhabit the water of the New World, while the remainders are scattered over the other continents of the world with the highest concentrations in the tropical regions of Africa and Asia (Burgess, 1989). Most of catfish in order Siluriformes are found in freshwater, except two families, which can be found in saltwater; Plotosidae and Ariidae.

Catfish are so-named due to the barbels (or whiskers) that extend from the upper jaw and, in some species, from the lower jaw, resembling the whiskers of a cat. Catfish are distinguished by the presence of barbells, the lack of true scales, the presence of bony plates, the strong spines at the front of the dorsal and pectoral fins, and an adipose fin, which is a flab of fatty tissue cover with skin that locate on the back between the dorsal fin and the caudal fin. The head of most catfish is well ossified, with a cephalic shield in which the dermal bones may be rugose.

Many catfish are inactive during the day, coming out to feed at night, thus they spend much of their time in hiding places. Most catfish have small eyes and therefore they detect food primarily with their sense of taste, using taste buds that are found over the entire external surface and barbels as well as inside mouth, pharynx and gill arches (Wellborn, 1988).

2.2 The Walking Catfish (*Clarias batrachus*)

The walking catfish, *Clarias batrachus* (Linnaeus 1758) is classified into subclass Teleostoei; order Nematognathi (or Siluriformes); family Clariidae (Smith, 1965). Members of the family Clariidae are collectively known as "air breathing" catfish, although they are not the only catfish family capable of breathing atmospheric air (Robins 2005).

The walking catfish is generally found in freshwater, but also occur in slightly brackishwater. Its range extends from India and Myanmar to Malaysia, Thailand, Indochina and the Philippines (Robins,2005; Chinabut *et al.*, 1991). Within these areas, it is valued as a food fish and is the focus of both subsistence fishers and commercial farming operation. In Thailand, it was introduced in the mid of 1951 and became a popular species of aquatic animal commercially cultured, especially in central region of Thailand (Piumsombun, 2000).

Figure 4. Catfish, *Clarias batrachus*

2.2.1 Taxonomy

Kingdom Animalia
Phylum Chordata
Class Actinopterygii
Order Siluriformes
Family Clariidae
Genus Clarias
Species *Clarias batrachus*

The walking catfish was formally described in 1758 as *Silurus batrachus* in Carolus Linnaues' 10th edition of Systema Nature. Johannes established the genus *Clarias* in 1777 (Robins, 2005) . *Clarias* is derived from Latin, meaning "shining".

Synonyms of *Clarias batrachus* include *Macropteronotus fuscus* by Lacepede in 1803, *Macropteronotus jagur* by Hamilton in 1822, *Clarias assamensia* by Day in 1877 and *Clarias punctatus* by Valenciennes in 1840 (Robins, 2005). *Clarias Magur*, *Clarias marpus*, *Macropteronotus batrachus* and *Macropteronotus magur* are also the other scientific names appearing in the literature of this species (Gulf State Marine Fisheries Commission, 2005).

2.2.2 Common name

Walking catfish is the most common English name for this species. The Thai name "Pla duk dan" translates to "dull colored wriggling-fish". Other English common names include clarias catfish, freshwater catfish and Thailand catfish (Robins, 2005).

2.2.3 Geographic distribution

The walking catfish is a widely distributed species, considered native to Pakistan, eastern India, Sri Lanka (Ceylon), Bangladesh, Myanmar, Thailand, Malaysia, Singapore, Indonesia and the Philippines (Gulf Coast Research Laboratory, 2005) and valued in aquaculture by some Southeast Asian people. In the United States, this species was imported from Asia and have escaped into the river in southern Florida, and replaced native fish in some local water (Robins, 2005).

2.2.4 Habitat

The walking catfish can be found in a wide variety of habitats including lakes and rivers but are best known for it ability to thrive where many fish cannot. It prefers warm, stagnant, muddy and often hypoxic waters such as ponds, canals, swamps, lakes, ditches, rice paddies and marshes (Chinabut *et al.*, 1991). It usually lives close to the bank, in shallow water, in hollow logs and other hiding place, and comes to the surface for aerial respiration at intervals of between 1 and 16 minutes (Hora, 1935). It is also known to inhabit slightly brackish water. The walking catfish

is a tropical species with a moderate tolerance to colder water, with the optimal temperature is between 20-25^{o}C (68-77^{o}F) (Gulf Coast Research Laboratory, 2005).

2.2.5 Biology

The walking catfish has an elongated body (typically 22.5-30.0 cm, with a maximum of 60 cm reported) (Courtenay and Miley, 1975) in a manner of a slender tadpole shape (Robins, 2005), with a rather broad and short strongly sloping head. The distinguishing features of this species are its long dorsal and anal fin that each is terminated in a lobe near but separated from the rounded caudal fin (Gulf Coast Research Laboratory, 2005). The walking catfish is unique in the development of spinous rays in the pectoral fins, the pectoral spine is stout, sharp, smooth or sometimes a little roughened, and covered with skin except near its tip (Chinabut *et al.* 1991). These vicious spines are capable in inflicting painful wounds. Its spines are very strong, especially the leading spine, also help its odd lifestyle of moving over land (Gulf Coast Research Laboratory, 2005).

The mouth is broad with small teeth, those of the maxillaries being smaller than those on the mandible. There are four pairs of barbels, one pair of nasal, one pair of maxillary and two pairs of mandibular barbels (Gulf Coast Research Laboratory, 2005), with the maxillary pair extending to the middle of the pectoral fins (Chinabut *et al*., 1991).

There are a few varieties of walking catfish. The young are pale brown in color and the color gradually changes to a dark gray or bluish black on top and a white around the belly and throat as they grow (Chinabut *et al.*, 1991). The albino, unusual to most fish species, is also occasionally observed in nature. The albino is white all over with red eyes, another much prettier one is piebald/pink and has normal eyes (Gulf Coast Research Laboratory, 2005). The albino variety of this species has been quite popular in the aquarium fish industry (Axelrod *et al*., 1971).

The “walking catfish” received its common name on its ability to walk over land from pond to pond when it original habitat dries up or after a heavy rainfall by the help of a large accessory breathing organ (Axelrod *et al*., 1971, Courtenay *et al*., 1974). It possesses a much reduced air-blade and are stiffened to prevent their collapse when out of water. In a special part of the gill chamber are spongy tree-like organs

growing from the upper ends of the gill arches. These and the skin surrounding them are well supplied with blood vessels and operate efficiently in water lacking in oxygen or when the fish is out of water. It keeps its gills closed when out of water and as long as its body is kept moist, it can stay on land for a considerable length of time (Gulf Coast Research Laboratory, 2005).

In the native range of the walking catfish, spawning is coincide with the onset of the rainy season during which the species may construct nest in the flooded environment (Robins, 2005). Adhesive eggs are laid in a nest or in submerged vegetation, and the male guards the eggs.

The walking catfish is a bottom feeder with nocturnal habits; it searches the bottom with its barbels vigorously sifting through detritus and soft substrates. It lies quietly under weeds or in the lee of stones throughout the day, only coming up occasionally to the surface for atmospheric oxygen. It feeds largely on aquatic invertebrates such as insect larvae and other bottom fauna (Chinabut *et al*., 1991), as available, but is omnivorous. Under pond conditions, it readily becomes carnivorous and feeds on the young of other fish, such as *Fundulus*, *Gambusia* and *Lepomis* (Courtenay *et al*., 1974), trash fish or pellets.

Within area of its native range, the Southeast Asia countries, the walking catfish is one of the important commercial species of freshwater teleosts. Owing to its ability to survive extended period out of water, it can be sold and traded live with ease, ensuring a fairly fresh food product. Outside of its native range, the walking catfish is a demonstrated pest, with the potential to do severe ecological harm. Numerous countries, including the United States, have "blacklisted" the walking catfish and classified all members of the family Clariidae as injurious wildlife, illegal to possess without a federal permit (Robins, 2005).

2.3 Chromosome of walking catfish, *Clarias batrachus*

The chromosome characteristics of walking catfish, *Clarias batrachus*, were studies by several researchers as summarized in Table 7.

Table 7. Chromosome characteristics of walking catfish, *Clarias batrachus*

Chromosome number	Karyotype	N.F. (arm number)	References
52	52a	52	Nayyar, 1966
52	ND	58	Srivastava and Das, 1968
50	16m + 8sm + 14st + 12a	74	Rishi, 1976
50 (male)	16m + 10sm + 4st + 20a	76	Pandey and Lakra, 1997
50 (female)	16m + 11sm + 4st + 19a	77	Pandey and Lakra, 1997

The chromosome numbers and standard karyotypic details for walking catfish, *Clarias batrachus* were ascentained by Pandey and Lakra (1997) as shown in Table A and Figure 5 and 6. The fish showed a diploid chromosome number, 2n = 50, exhibited 25 homomorphic pairs in male and 24 homomorphic and one heteromorphic pair in female karyotype. The karyotype was found to possess 50 chromosomes comprising 8 metacentric, 5 submetacentric, 2 subtelocentric and 10 acrocentric pairs in male specimens. The female fish also revealed the same karyotype except for one additional large submetacentric chromosome and one fewer samall acrocentric chromosome.

Figure 5. Mitotic metaphase chromosome of male *Clarias batrachus* (2n = 50) (Pandey and Lakra, 1997)

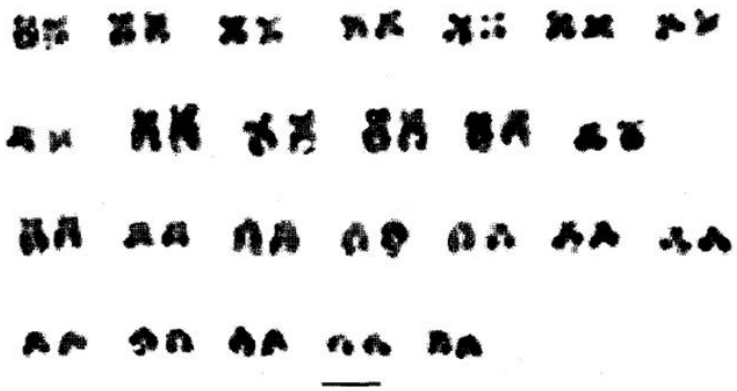


Figure 6. Karyotype of male *Clarias batrachus* (2n = 50), scale indicated 5 μm (Pandey and Lakra, 1997)

2.4 Erythrocytes of walking catfish, *Clarias batrachus*

The blood is the important circulating, transportation medium of the animal body. The nomenclature applied to fish blood cells has been borrowed from mammalian haematology. The cells of walking catfish circulating blood are: erythrocytes, lymphocytes, thrombocytes, monocytes and polymorpholeucocytes (neutrophil granulocytes).

The mature erythrocyte is oval to round with a deeply stained nucleus and abundant cytoplasm (Figure 7). The overall size of the walking catfish red blood cell is 10×11 micrometres to 12×13 micrometres, and the diameter of the nucleus is 4-5 micrometres. Chinabut *et al.* (1991) have defined the erythrocyte count as 3.18×10^6 cells/mm^3 which is higher than the 2.25×10^6 cells/mm^3 from the report of Belsare (1975) who also studied the haematology of *C. batrachus* (Chinabut *et al.*,1991). The immature erythrocytes are called reticulocytes, and these are mainly found in the anterior kidney (pronephros), spleen and only rarely in the circulating blood. Its size is about the same as that of the mature erythrocyte but the nucleus is larger. The cytoplasm of reticulocytes stains a light blue or grey colour by the Wright and Giemsa method.

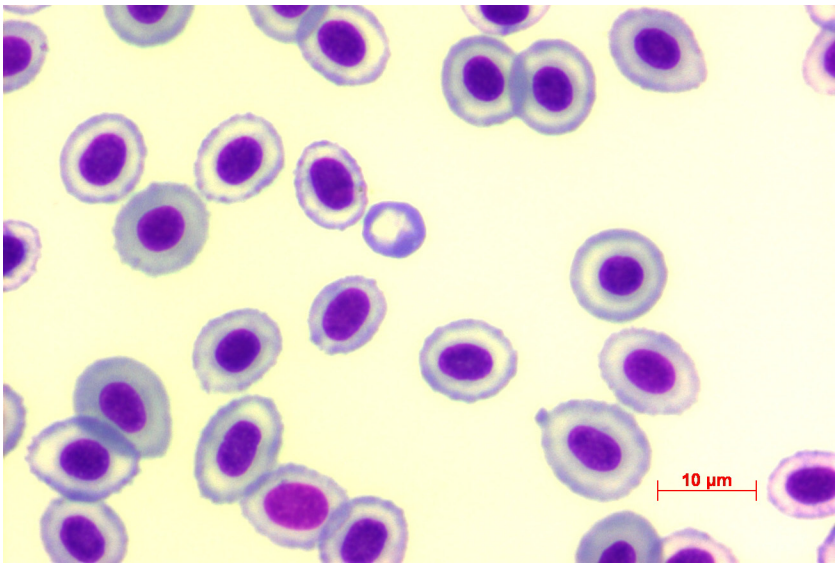


Figure 7. Erythrocytes of catfish, *Clarias batrachus*, from pheripheral blood (May-Grünwald-Giemsa staining)

3. Micronucleus assay

3.1 Introduction

Genotoxicity is a general term referring to alterations to the gross structure or content of chromosomes (as clastogenicity) or base-pair sequences of DNA (as mutagenicity) by exposure to toxic agents. Research on the genotoxic effects of different pollutants in aquatic environment to fish and other aquatic organisms has been used to determine the toxic potential for chemical carcinogenesis (Powers, 1989).

For the detection of genotoxic effect of aquatic pollutant, a cytogenetic investigation with fish was carried out by several authors. Cytogenetic assays were used to determine chemical induced changes in structure or number of chromosomes, as seen through light microscope. Many techniques are available for cytogenetic testing both in vivo and in vitro, but they are divided into three different types:

(a) Test to detect chromosomal aberrations.

(b) Test to detect exchanges of chromosomal material between sister chromatids.

(c) Test to detect chromosome fragments, or “micronuclei” resulting in chromosome damage.

Cytogenetic observations in fish used as an indicator of genotoxic agents under actual field and laboratory conditions are primarily based on chromosomal aberration analysis, although such tests exhibit some disadvantages such as low mitotic activity, the difficulty of finding a sufficient number of metaphase for scoring chromosome aberrations and the limitation of a suitable fish karyotype (Kligerman *et al*., 1975; Hooftman and Raat, 1982). The fish karyotype consists of a large number of small and irregular chromosomes (AI-Sabti and Metcalfe, 1995). While species of mudminnow *(Umbra* sp.) have a suitable karyotype for metaphase analysis of genotoxicity (Kligerman, 1982), but these species are of little use for studies because they are relatively rare and of no commercial value. An alternative cytogenetic endpoint for genotoxicity is the sister chromatid exchange (SCE); however, only a few studies have used this endpoint in fish (Al-Sabti, 1991). Two other endpoints for cytogenetic damage are micronucleus assay (MN) and anaphase aberrations.

The micronucleus assay is a mutagenicity test system used for detecting the effect of mutagenic agents on chromosomes by the identification of acentric fragments and/or lagging chromosomes, as small membrane-bound DNA fragments (micronuclei) that separate from the main nucleus (Heddle *et al.,* 1978, Schmid, 1975). These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division. The assay thus has the potential to detect the activity of both clastogenic and aneugenic chemicals (Kirsch-Volders, 1997, Parry and Sorrs, 1993).

Micronucleus assays have been used extensively to test for the genotoxic activity of chemicals (Heddle *et al.,* 1983). The application of this assay in genotoxic biomonitoring of clastogenic effects of pollutants has proven the most practical tool in environmental mutagenesis. This endpoint is widely used in aquatic organisms such as mussels (*Mytilus galloprovincialis*), bivalve molluscs (*Crassotrea gigas* and *Chamelea galina*), rana tadpoles (*Rana perezi*), rainbow trout (*Oncorhynchus mykim*), salmoniform fish (*Umbra pygmea*), Prussian carp (*Carassius auratus gibelio*) and tilapias (*Oreochromis mossambica*) (Manna *et al*., 1985; Metcalfe, 1988; Rodriguez-Ariza *et al*., 1992; Al-Sabti, 1994; Al-Sabti and Metclafe, 1995; Vernier, *et al*., 1997).

The micronucleus assay has a number of advantages over the metaphase analysis to measure chromosome aberrations (OECD, 2004). Because micronuclei in interphase cells can be assessed much more objectively than chromosomal aberrations in metaphase cells, there is not as rigorous a requirement for detailed training for testing personnel to achieve competence in this assay. Also, there is no requirement to count the chromosomes in a metaphase preparation, to evaluate subtle chromatid and chromosome damage, but only to determine whether or not a cell contains a micronucleus. As a result, the preparations can be scored much more quickly. This makes it practical to score thousands instead of hundreds of cells per treatment, and this imparts greater statistical power to the assay. Finally, as micronuclei may contain whole (lagging) chromosomes, there is the potential to detect aneuploidy-inducing agents that are currently very difficult to study in conventional chromosomal aberration tests.

3.2 Micronucleus formation

During cell division, the genetic material replicates and then divides equally into two daughter cells. If the process is disrupted, or the chromosomes are broken or damaged by chemicals or radiation, then the distribution of genetic material between the two daughter nuclei during cell division may be affected and resulted in formation of “micronucleus” (NTP).

Micronuclei are formed by condensation of acentric chromosomal fragments or by whole chromosomes that are not incorporated in the main nuclei following mitosis. Micronuclei are formed in the cytoplasm as described by Heddle (1973) and Schmid (1975) through the following events (Figure 8). In anaphase, acentric chromatid and chromosomal fragments lag behind when the centric elements move towards the spindle poles. After telophase the undamaged chromosomes, as well as the acentric fragments, give rise to regular daughter nuclei. The lagging elements are included in the daughter cells, too, but a considerable portion is transformed into one or several secondary nuclei that are much smaller than the principal nucleus (1/5 to 1/20) and are therefore called micronuclei. Heddle (1973) and Schmidt (1975) suggested that the micronuclei in fish could be smaller in size than mammalian cells, which have large

chromosome. Fish chromosomes on the other hand are much smaller, the micronuclei could be about 1/10 to 1/30 smaller than the principal nucleus.

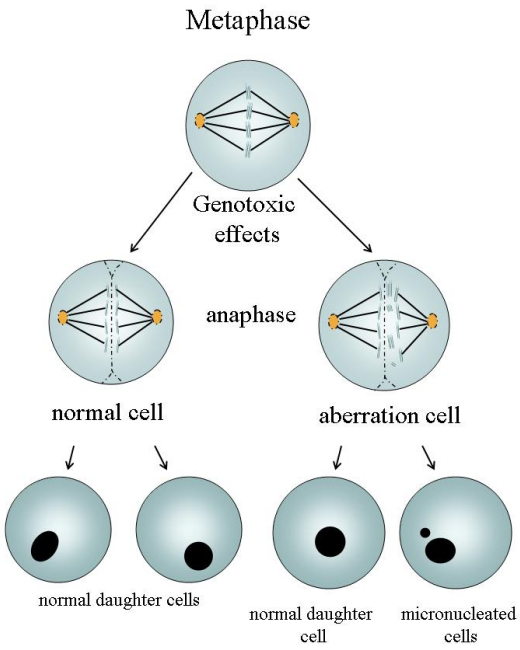


Figure 8. Schematic illustration of the mechanism of micronucleus formation in mononucleated cells (Al-Sabti, 1995)

3.3 Method of micronucleus assay

The micronucleus assay is performed in a variety of ways, depending upon the questions the investigator is attempting to answer such as the test organism, the cell type that is assayed, and the mode of action of the chemical. The NTP conducts micronucleus tests in rats or mice, and the tissues assessed are often the bone marrow and peripheral blood (NTP, 2006). Erythrocytes (red blood cells) are the cells that are scored in the bone marrow or the blood for the presence of micronuclei. The assay is an *in vitro* method, where either cell lines or primary cultures of human lymphocytes may be used.

The micronucleus assay using any types of cells requires that a substantial fraction of the cell population treated with a genotoxic agent must undergo mitosis, so that the centric fragments induced during the first cell cycle are visible as micronuclei

in the cytoplasm during the second or subsequent cell cycles (Tates *et al.,* 1980).

For the *in vivo* micronucleus assay, whole animals were exposed to the different concentrations of test substance for periods of time. After exposure to test substance, peripheral blood erythrocytes, gill and liver cells will be collected from the animals. Then the cell suspension was softly smeared on the whole surface of slides. The dried slides were fixed in methanol and stained with Giemsa solution. The stained slides were analyzed under light microscope at a magnification of 1000. The *in vitro* micronucleus assay is described by NTP. Cell cultures were exposed to the test substances both with and without an exogenous source of metabolic activation. After exposure to the test substance, cell cultures were grown for a period sufficient to allow chromosome damage to lead to the formation of micronuclei in interphase cells (NTP, 2006). Harvested and stained interphase cells were then analysed microscopically for the presence of micronuclei.

Micronuclei scoring: Micronucleus assays with fish have indicated that it is necessary to score at least 1,000 erythrocytes from each fish specimen to evaluate clastogenicity. However, Metcalfe (1988) suggested that scoring of more than 4,000 erythrocytes per fish is necessary to detect genotoxic activity with weak clastogens. Slides should be coded and scored without prior knowledge of the treatment group (i.e. 'blind'). The coded slides can be scanned under low magnification and selected for scoring at × 1,000 on the basis of good staining and slide quality. Overlapping and damaged cells should be disregarded during scoring, and cells that contain abnormal nuclei should also be disregarded. Often, debris and precipitates from the staining procedure that are superimposed on the cells being scored can be mistaken for micronuclei. This material can be easily identified by adjusting the focus up and down on the microscope. Extranuclear material that is not in the same focal plane as the main nucleus of the cell cannot be a micronucleus.

The established scoring criteria for the identification of micronuclei in fish erythrocyte (Matsumoto and Colus, 2000, Cavas *et al.*, 2005) were described as follow:

(a) Only the cells that clearly isolated from the surrounding cell will be scored,

(b) The cells must show an oval appearance and intact cytoplasm, with oval nuclei and intact nuclear membrane,

(c) Micronuclei size must be smaller than or equal to one-third of the main nuclei size,

(d) Micronuclei must be clearly separated from the main nuclei, and

(e) Micronuclei must be on the same plane of focus and have the same color.

3.4 Micronucleus assay in fish

Environmental pollution forces the studies in the direct and indirect effects of the disposal of industrial and other wastes on the aquatic environment. Many waterborne pollutants have cytogenetic properties which in fish cause enhanced frequency of chromosomal aberration (Al-sabti *et al.*, 1984, Al-sabti 1985, Al-sabti 1986). Interest in the actions of chemical mutagens in inducing chromosomal damage stems not only from the possibility that the presence of a chemical mutagens in the environment could result in an increased incidence of cancer, but also from the fact that exposure to these agents may result in an increased incidence of transmitted genetic disease (Evans 1983).

The study in toxic effects of pollutants in aquatic environment is important for man himself because of exposure throughout his lifetime to low levels of toxicants present in both the water and aquatic food. Fish are proven to be very important indicator organisms for the assessment of the mutagenic and/or carcinogenic potential of water samples since they are aquatic vertebrate organisms that can metabolize, concentrate and store waterborne pollutants.

The micronucleus assay in fish also has the potential for detecting clastogenic substances in aqueous media. Since teleost erythrocytes are nucleated, micronuclei have been scored in fish erythrocytes as a measure of clastogenic activity. Various studies have shown that the peripheral erythrocytes of fish have a high incidence of micronuclei after exposure to different pollutants under field and laboratory conditions (Hooftman and de Raat, 1982; AI-Sabti, 1986a,b; Metcalfe, 1988; AI-Sabti and Hardig, 1990; A1- Sabti, 1991; Schultz *et al*., 1993; A1-Sabti *et al*., 1994; A1-Sabti, 1994).

The micronucleus assay is a rapid mean of qualitative assessment of frequencies of chromosomal damage, and indirectly gives insight into the risk to human health from environmental contaminants and many other various induced and natural

pollutants in water ecosystem. It may serve as a primary screening test of genotoxic substances or agents. Observations of micronuclei can be made very rapidly. In most fish species the erythrocytes are sizeable and have a large nucleus, therefore micronuclei in fish erythrocytes are quite distinct, easily scored, and persist in the cytoplasm enable to use them in the investigation. Their recognition is technically much easier and the technique is fifteen times more rapid than the direct scoring of chromosomes during the metaphase (Al-Sabti, 1991).

In general the basic principles of fish micronuclei methods are the same as in most other animal groups (i.e.: obtaining and preparing the micronuclei from the dividing cells of different tissue either directly or by cell culture). Al-Sabti and Metcalfe (1995) summarized the micronucleus assay as follow:

3.4.1 Test protocols with fish erythrocytes

3.4.1.1 Laboratory studies

Many micronucleus assay methods have been used to detect MN in the erythrocytes of fish exposed to clastogens in the laboratory. It is reasonable to expect that many of the conditions governing induction of micronuclei in fish erythrocytes will vary with test species, exposure protocol, water temperature, etc. Since many of these variables have not been studied adequately, most in vivo tests have involved several exposures and sampling protocols in an attempt to detect a maximum MN response in fish. An analysis of many studies indicated that fish are generally exposed to clastogens by repeated intraperitoneal (i.p.) injections or continuous exposure in water for a period of days to weeks (Al-Sabti and Metcalfe, 1995).

Typically, several test doses and a control treatment are used in order to determine whether there is a dose response, and 3-12 fish are used in each treatment. In the laboratory tests, fish were killed between 1 and 58 days after cessation of exposure to a clastogen, but maximal MN induction typically occurred within 1-5 days post-exposure (Al-Sabti and Metcalfe, 1995). However, because of the potential effects of variables such as test species and temperature on the MN response, fish should be killed at various times post-exposure until standard test protocols are established.

It is instructive to examine a few typical in vivo tests protocols for induction of MN in the erythrocytes of fish. A1-Sabti (1986) used an erythrocyte MN assay with three cyprinid species exposed to chemicals in the laboratory where prior to the experiment, fish were acclimatized for 14 days in water at a temperature suitable for each species. Genotoxic chemicals were injected i.p. into the fish, or were dissolved in water. Peripheral blood samples for smears were obtained 48 h after exposure from the caudal vein or heart of the fish with a syringe. Blood was smeared on clean microscope slides and the smears fixed in pure ethanol for 20 min. The fixed smears were then left to air dry at room temperature, and finally stained with 10% Giemsa in Sorenson buffer for 10 min. Since Giemsa stains the nuclear content darker than the cytoplasmic content, the micronuclei were readily visible next to the normal nuclei of erythrocyte cells when observed under a light microscope. Staining with Schiff's, followed by counter-staining with aqueous green (Hooftman and de Raat, 1982; Metcalfe, 1988) also allows easy scoring of micronuclei.

3.4.1.2 In situ studies

Several in situ studies of MN in fish erythrocytes have been conducted. Typically, fish are captured from one or more polluted sites as well as a relatively pristine 'reference' site. Alternatively, fish from a reference site are caged in polluted locations for several days or weeks before they are retrieved and killed. Studies conducted in rivers where fish can be caged above and below a point-source of pollution (A1-Sabti et al., 1994) are particularly useful for establishing relationships between exposure to contaminants and a clastogenic response.

3.4.2 Test protocols with other fish tissues

Other fish tissues besides circulating erythrocytes have been examined for frequencies of MN. Cells from the gills and kidneys of Tilapia *(Oreochromis mossambicus)* exposed to X-rays or chemicals (aqueous exposure) were isolated and smeared on slides for analysis of MN frequency (Ueda et al., 1992). The cells isolated from gill tissue appeared to be more sensitive to the clastogenic effects of these treatments than kidney tissue and circulating erythrocytes. Williams and Metcalfe developed an in vivo hepatic micronucleus assay with trout (Williams and Metcalfe,

1992). The fish liver micronucleus assay gave a positive response with two direct-acting clastogens (EMS and MMC), and with diethylnitrosamine (DEN), which requires metabolic transformation in the liver to an active intermediate compound. However, this assay involves exposure of fish to allyl formate in order to induce regenerative proliferation of hepatocytes.

CHAPTER IV
MATERIALS AND METHODS

Study I: The survey of cadmium levels in commercial catfish (*Clarias batrachus*) in Bangkok metropolitan

Experiment 1: Specimen collection and sample preparation

1.1 Materials

1.1.1 Apparatus

1. Water purification system (Milli-Q, RiOs): Millipore
2. Blender and homogenizer: TRI-R, STIR-R and Waring Blendor, Deluxe
3. Balance (Top load): Mettler Toledo
4. Freeze-dryer: Christ model, Alpha 1-4

1.2 Methods

Catfish (*Clarias batrachus*) were purchased from 6 markets; including Wongweanyai market, Bangkoknoi market, Sainert market, Yingcharoen market, Khlongtoei market, and Ratchawat market, which covered six districts of Bangkok metropolitan (Appendix A). Six catfish were collected from each market; they were packed in icebox and transported to the laboratory on the same day.

For sample preparation, fish were measured for weight and length, then the whole fish were cleaned with tap water and followed by distilled water to remove foreign particles, excessive mucus coating and other materials that could absorb metals (Kargin, 1998). Afterward, the fish were abdominal dissected and separated into parts including liver, gills, muscle and skin. The organs were then rinsed with Milli-Q water and blended or minced into small pieces by blender, homogenizer or scissors. The tissue homogenate was weighted, kept at -84^{o}C in glass flasks for at least an hour and

then freeze-dried by freeze-dryer at -50^{o}C for 48 hours at less than 200 millitorr vacuum. The dried samples were crushed to powdered, weighted, and kept in plastic bags until analysis. The diagrams are shown in Figure 9 and 10.

Experiment 2: Determination of cadmium residues in various tissues of catfish

2.1 Materials

2.1.1 Apparatus

1. Digestive microwave oven: CEM Model, MarsX
2. Graphite-furnace atomic absorption spectrophotometer (GFAAS): Varian SpectrAA 220Z, spectrometer equipped with GTA 110Z Furnace atomizer control unit, PSD and HP 1200 series printer
3. Glassware

All glassware and apparatus used in analytical work were soaked in 20% HNO_3 overnight, rinsed with distilled water twice, and rinsed with Milli-Q water. Then they were dried in hot air oven, and kept in the closed clean container until used.

2.1.2 Reagents

All chemicals and reagents used were of analytical grade or equivalent. Nitric acid (65% HNO_3, suprapur), cadmium standard solution (1,000 mg/l cadmium, cadmium nitrite in nitric acid 0.5 mol/l) and palladium were purchased from Merck (Germany).

2.2 Methods

2.2.1 Digestion method

Approximately 0.5 g of dried sample was digested with 10 ml of concentrated nitric acid (65% HNO_3) in 50 ml PE Teflon pressurize vessels by digestion microwave oven as followings parameter:

Vessel type:	HP500
Temperature:	200^{o}C
Pressure:	200 atm
Holding time:	10 minutes

After cooling, the clear digested sample was filtered by a Whatman No.1 filter paper and adjusted volume to 50 ml with Milli-Q water in 50 ml volumetric flasks and kept in PE bottles at 4^{o}C to avoid loss of metal ions due to adsorption (Batley and Gardner, 1977). Blank digestions were treated similarly.

2.2.2 Determination of cadmium content by Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS)

The cadmium concentration in each organ was measured by Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS). The operation of GFASS is shown in Table 8. Cadmium standard curve was prepared at the range of 0-2 ppb (0, 0.4, 0.8, 1.2, 1.6 and 2.0 ppb). For cadmium level determination, 250 µl of digested sample was diluted with 750 µl of Milli-Q water to lower the concentration of HNO_3 to below 4%, in order to prevent the corrosive action to graphite tube. Ten µl of diluted sample was added with 5 µl of 1000-ppm palladium as modifier, before injected into GFAAS. The concentration of detected cadmium was determined from the prepared cadmium standard curve.

Table 8. Graphite Furnace operation parameter (Rothery, 1988)

Step	Final temperature (^{o}C)	Ramp Time (Sec)	Gas flow (L/min)	Gas type	Read Command
1	85	5	3.0	Normal	No
2	95	40	3.0	Normal	No
3	120	10	3.0	Normal	No
4	250	5	3.0	Normal	No
5	250	1	3.0	Normal	No
6	250	2	0	Normal	No
7	1800	0.8	0	Normal	Yes
8	1800	2	0	Normal	Yes
9	1800	2	3.0	Normal	No

Wavelength:	228.8	nm
Lamp current:	4	mA
Spectral bandwidth:	0.5	nm
Background:	BC on	
Measurement:	Peak area	

2.3 Data presentation

The data of cadmium concentration was calculated statistically and expressed as mean values ± standard error of mean (SEM) for each market.

The experimental protocol

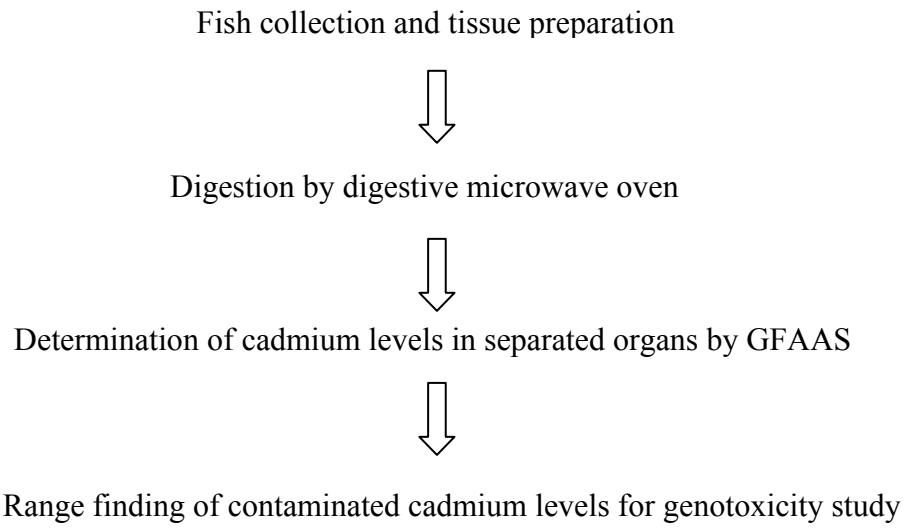
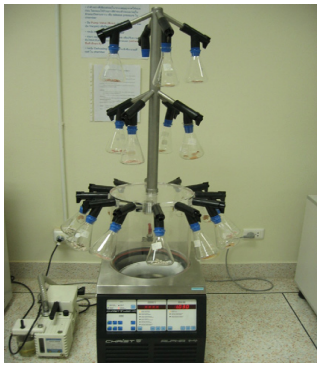


Figure 9. Diagram shows the protocol for analysis of cadmium levels in catfish

Sample preparation

Muscle, skin, gills and

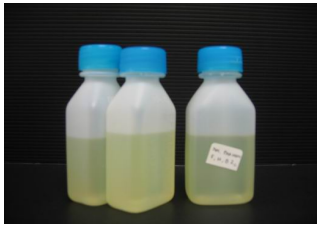


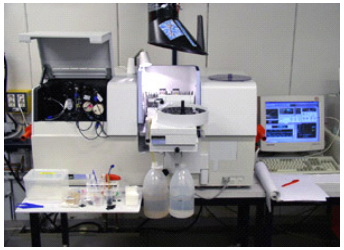
Drying

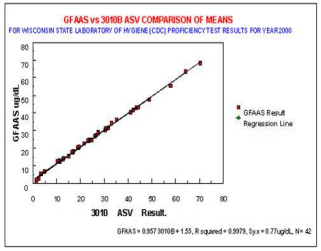
At -50 ^{0}C for
48 hours
By freeze dryer

Digestion

Dried wt.0.5 g + 10 ml HNO_3
Temperature : 200 ^{o}C
Pressure : 200 atm
Holding time : 10 min







Graphite Furnance Atomic Absorption

Spectrophotometer (GFAAS)

Figure 10. The picture shows the protocol of sample preparation and cadmium

Study II: Evaluation of catfish (*Clarias batrachus*) as a model system for genotoxicity study of water pollution

Experiment 3: The cadmium uptake by catfish

3.1 Materials

3.1.1 Test organism

The freshwater catfish, namely *Clarias batrachus* (Clariidae), was selected as test species. *C. batrachus*, locally known as "Pla duk dan" is an indigenous species since it is commonly found in local freshwater of Thailand. It is widely cultivated and available throughout the year. *C. batrachus* has not been extensively evaluated as a test species.

Two-month-old freshwater catfish were obtained from farming ponds in Samut Sakhon province. Test fish were selected from a population of a single stock, transported to a controlled laboratory environment and acclimatized for 2 weeks to laboratory conditions in glass aquaria measuring 40 cm in width x 20 cm in length and 25 cm in height, containing standard water (pH 7.2±2) (Appendix C). The room temperature and photoperiod during the experiment were 25±1 ^{o}C and 12 h light and 12 h dark, respectively. The aquaria were well aerated. The growth, tolerance and behavior of fish were kept under observation. Throughout the study, fish were fed commercial catfish food pellet once daily in the morning. Healthy individuals, apparently free of overt diseases, were randomly allocated to various treatment groups.

3.1.2 Apparatus

1. Glass aquaria with a capacity of 20 liters and 40 cm x 20 cm x 25 cm in dimension, equipped with aeration.
2. Water purification system (Milli-Q, RiOs): Millipore
3. Blender, homogenizer: TRI-R, STIR-R and Waring Blendor, Deluxe
4. Balance (Top load): Mettler Toledo
5. Freeze-dryer: Christ model, Alpha 1-4
6. Digestive microwave oven: CEM Model, MarsX

7. Graphite-furnace atomic absorption spectrophotometer (GFAAS): Varian SpectrAA 220Z, spectrometer equipped with GTA 110Z Furnace atomizer control unit, PSD and HP 1200 series printer

8. Glassware

All glassware and apparatus used in analytical work were soaked in 20% HNO_3 overnight, rinsed with distilled water for two times, and rinsed with Milli-Q water. Then they were dried in hot air oven, and kept in the closed clean container until used.

3.1.3 Reagents

Cadmium uptake in catfish

All chemicals used for study cadmium uptake were of analytical grade or equivalent used without further purification. Cadmium chloride anhydrous ($CdCl_2$) and calcium chloride dihydrate ($CaCl_2.2H_20$) were purchased from Fluka Chemie AG (Switzerland). Sodium bicarbonate ($NaHCO_3$), magnesium sulphate ($MgSO_4.7H_2O$) and potassium chloride (KCl, extra pure) were, respectively, purchased from May & Baker LTD (England), Farmitalia Carlo Erba and Merck (Germany).

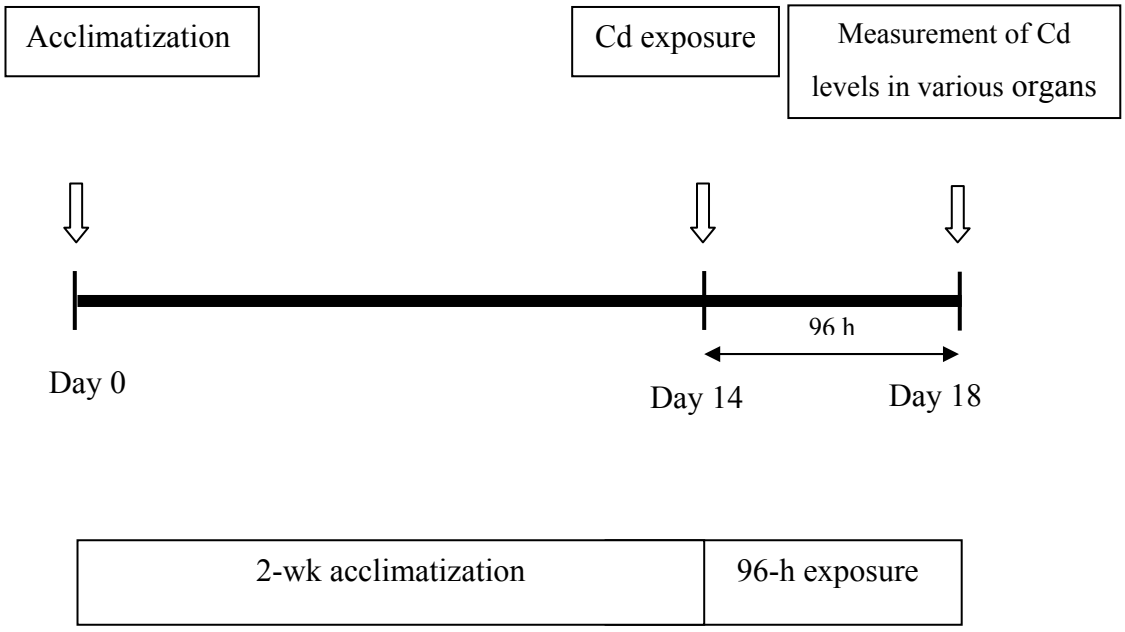
Measurement of cadmium uptake

All chemicals and reagents used were of analytical grade or equivalent. Nitric acid (65% HNO_3, suprapur), cadmium standard solution (1000 mg/l cadmium, cadmium nitrite in nitric acid 0.5 mol/l) and palladium were purchased from Merck (Germany).

3.2 Methods

3.2.1 Experimental design

The cadmium chloride salt ($CdCl_2$) and standard water were used in treatment as test and negative control group, respectively. Since cadmium chloride is completely soluble in water; stock solutions were then prepared in sterile distilled water into various concentrations of cadmium chloride (0.005, 0.5, 1, 5, 10 and 20 mg/l) for the study of cadmium uptake in cafish. This concentration range was based on the work of Al-Sabti and Metcalfe (Al-Sabti and Metcalfe, 1995). A treatment period of 96 hours was used for the short-term exposure study (Figure 11).



Negative control: Standard water, 96-h exposure

Test groups: $CdCl_2$ (0.005, 0.05, 0.5, 1, 5, 10 and 20 ppm), 96-h exposure

Figure 11. Schematic representation of the experimental design on cadmium uptake by catfish (*Clarias batrachus*)

3.2.2 Cadmium uptake in catfish from short-term cadmium exposure

After the 2-week acclimatization period, fish were transferred from the stock aquaria to the experimental aquaria. Assays were conducted under the same conditions described for the acclimatization period in the previous section and the static method was employed.

Seven aquaria were filled with 10 l of standard water and cadmium salt was added to each aquarium to make the final concentrations of 0.005, 0.05, 0.5, 1, 5, 10 or 20 ppm of $CdCl_2$. The eighth aquarium was used as a control. Four catfish were placed in each aquarium, loading densities about 2.5 l/fish. Each concentration was done in three replicates. Exposure was continued up to 96 hours (4 days) and the levels of cadmium in various organs of fish; muscle (including skin), gills and liver, were determined.

3.2.3 Determination of cadmium uptake by catfish

At the end of exposure period, cadmium uptake in various organs of catfish was determined. The fish were anesthetized by immersion in ice-cold water for 1-2 minutes and killed by severing the spinal column behind the opercular. The whole fish were cleaned first with distilled water and dried using drying paper. After that, the fish were abdominal dissected and separated into parts including muscle covered with skin, gills and liver. The organs were first wet weight and then rinsed with Milli-Q water and minced into small pieces by scissors. Each organ was pooled within the same treatment group, kept at -84^{o}C in glass flasks for at less an hour and then freeze-dried by freeze-dryer at -50^{o}C for 48 hours to less than 200 millitorr vacuum. The dried samples were crushed to powdered, weighted, and kept in plastic bags until analysis.

3.2.4 Digestion method

Approximately 0.5 g of dried sample was digested with 10 ml of concentrated nitric acid (65% HNO_3) in 50 ml PE Teflon pressurize vessels by digestion microwave oven as described in Experiment 2.

After cooling, the clear digested sample was filtered by a Whatman No.1 filter paper and adjusted volume to 50 ml with Milli-Q water in 50 ml volumetric flasks and kept in PE bottles at 4^{o}C to avoid loss of metal ions due to adsorption (Batley and Gardner, 1977). Blank digestions were treated similarly.

3.2.5 Determination of cadmium residues in catfish organs by Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS)

The cadmium concentration in each organ of catfish was determined by Graphite furnace atomic absorption spectrophotometer (GFAAS) as described in Experiment 2. Digested sample was diluted with Milli-Q water to lower the concentration of HNO_3 and to lower concentration of cadmium level in digested sample to the detectable ranges of the instrument. Ten µl of diluted sample was added with 5 µl of 1000-ppm palladium as modifier, before injected into GFAAS. The concentration of detected cadmium was determined from the prepared cadmium standard curve.

3.3 Data presentation

The data of cadmium concentration was calculated statistically and expressed as mean values ± standard error of mean (SEM) for each exposure group.

Catfish were acclimatized for 2 weeks to laboratory conditions in glass aquaria.

⇩

4 fish/group were exposed to various concentrations of cadmium
(0.005, 0.05, 0.5, 1, 5, 10 and 20 ppm of $CdCl_2$)

⇩

Exposure was continued up to 96 hours.

⇩

Cadmium levels in various organs of fish were determined by GFAAS.
(muscle (including skin), gills and liver)

Figure 12. The diagram of study of cadmium uptake in catfish

Experiment 4: The relationship between cadmium exposure levels and the cadmium residues in catfish tissues.

The detected levels of cadmium in catfish from Experiment 3 were analyzed for their relationship with the levels of cadmium exposure by ANOVA analysis.

Study III: Evaluation for genotoxicity of cadmium in catfish (*Clarias batrachus*)

Experiment 5: Effect of short-term cadmium exposure on micronucleus frequencies in catfish

5.1 Materials

5.1.1 Test organism

The freshwater catfish, *Clarias batrachus*, was used as test species, as described in Experiment 3. Healthy individuals, apparently free of overt diseases, were randomly allocated to various treatment groups.

5.1.2 Apparatus

1. Glass aquaria with a capacity of 20 liters and 40 cm x 20 cm x 25 cm in dimension, equipped with aeration.
2. Needle, syringe and scissors
3. Glass slides and cover slip
4. Light microscopy

5.1.3 Reagents

Micronucleus induction in catfish

All chemicals used for the induction of micronuclei were of analytical grade or equivalent used without further purification. Cadmium chloride anhydrous ($CdCl_2$) and Calcium chloride dehydrate ($CaCl_2.2H_2O$) were purchased from Fluka Chemie AG (Switzerland). Sodium bicarbonate ($NaHCO_3$), magnesium sulphate ($MgSO_4.7H_2O$) and potassium chloride (KCl, extra pure) were purchased from May & Baker LTD (England), Farmitalia Carlo Erba and Merck (Germany), respectively. cyclophosphamide was purchased from Baxter (Germany).

Slide preparation

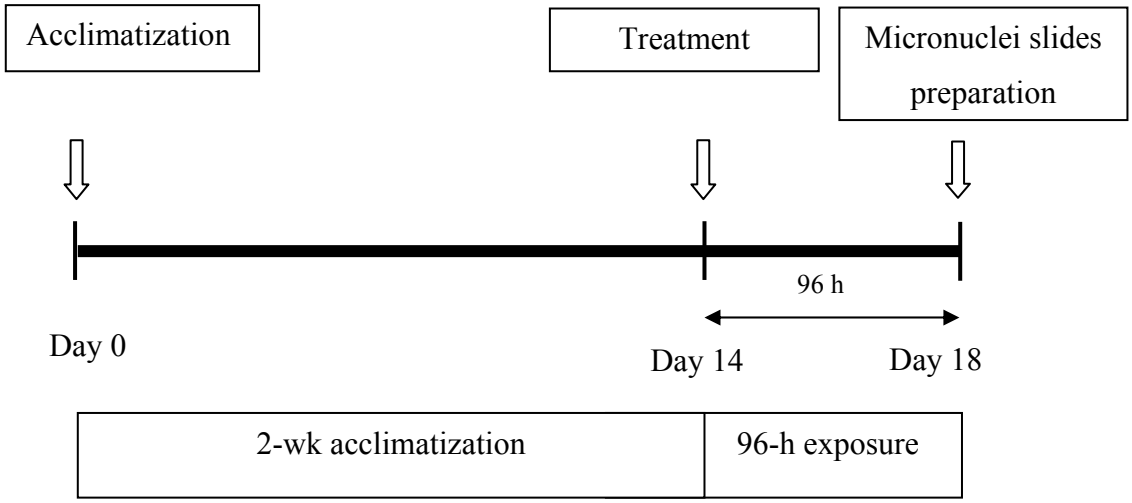
All chemicals used for cytogenetic preparation were of analytical grade or equivalent. Ethanol (C_2H_5OH), methanol (CH_3OH), May-Grünwald's eosin-methylene

blue solution (modified for microscopy, contains methanol), Geimsa's azur-eosin-methylene blue (for microscopy), xylene (C_6H_4 $(CH_3)_3$) and immersion oil (for microscopy, contains benzyl benzoate $C_{14}H_{12}O_2$) were obtained from Merck (USA). Glycerine ($CH_2OHCHOHCH_2OH$) and Permount® (histological mounting medium) were purchased from Carlo Erba and Fisher Scientific, respectively.

5.2 Methods

5.2.1 Experimental design

Nine aquaria, one of which was designed as negative control, were used to conduct the experiment. Seven aquaria were filled with 10 l of standard water and cadmium chloride salts was added to each aquarium to make the final concentration of 0.005, 0.05, 0.5, 1, 5, 10 or 20 mg/l $CdCl_2$. The ninth aquarium was used as a positive control, 4 fish were pretreated with 40 mg/kg BW cyclophosphamide, i.p. before placed into the aquarium contained 10 l standard water. All fish were kept in aquaria for 96 hours. A treatment period of 96 hours was used for the acute genotoxic study (Figure 13).



Negative control: Standard water, 96-h exposure

Positive control: Cyclophosphamide 40 mg/kg BW (i.p.), single dose

Test groups: $CdCl_2$ (0.005, 0.05, 0.5, 1, 5, 10 and 20 ppm), 96-h exposure

Figure 13. Schematic representation of the experimental design on effects of cadmium levels on catfish (*Clarias batrachus*)

5.2.2 Slide preparation

At the end of experiment, fish were removed from aquaria and anesthetized by immersion in ice-cold water for 1-2 minutes. The peripheral blood smears were obtained by the caudal incision on clean grease free microscopic slides. Three fine blood smears were prepared for each fish. The smears were fixed in absolute ethanol for 15 minutes after drying at room temperature for 12 hours. Slides were stained with May- Grünwald Giemsa staining by the procedure of Sanchez-Galan et al. (1998). Slides were sequentially stained with May-Grünwald for 2 minutes, May-Grünwald/distilled water 1:1 for 3 minutes, and Giemsa/distilled water 1:6 for 10 minutes. Then slides were rinsed through running tap water and allowed to dry at room temperature. Finally the slides were cleared in xylene and permanently mounted by Permount. The diagram of cytogenetic preparation is shown in Figure 14.

Slides were selected for scoring the micronuclei frequencies on the basis of staining quality, then coded, randomized and scored blindly. For each dosage 5,000-8,000 erythrocytes (a minimum of 2,000 cells per slide) were scored under microscope by 1000x magnification, to determine the frequencies of micronuclei (Ateeq, 2002). For the nuclear abnormalities, the important variations included binucleated and enucleated erythrocytes, microcytes, and erythrocyte with nuclear bud, nuclear bleb and nuclear lobe were observed for 3,000 erythrocytes per each exposure.

Fish were anesthetized by immersion in ice-cold water for 1-2 min.

⇩

The caudal incision and peripheral blood were smeared on microscopic slides.

⇩

Slides were dried at room temperature and fixed in absolute ethanol for 15 min.

⇩

Slides staining
- May-Grünwald for 2 min
- May-Grünwald/distilled water 1:1 for 3 min
- Giemsa/distilled water 1:6 for 10 min

Rinsed through running tap water and air-dried

⇩

Slides were cleared in xylene and permanently mounted by Permount.

Figure 14. The diagram of slide preparation for micronuclei study

5.3 Scoring criteria for micronuclei and nuclear abnormalities

The established scoring criteria for the identification of micronuclei (Matsumoto and Colus, 2000, Cavas *et al.*, 2005) were strictly followed to ensure authentic scoring.

(a) Only the cells that clearly isolated from the surrounding cells were scored,
(b) The cells must showed an oval appearance and intact cytoplasm, with oval nuclei and intact nuclear membrane,
(c) Micronuclei size must be smaller than or equal to one- third of the main nuclei size,
(d) Micronuclei must be clearly separated from the main nuclei, and
(e) Micronuclei must be on the same plane of focus and have the same color.

Following Carrassco *et al*. (1990), the nuclear abnormalities observed were classified. Briefly, blebbed nuclei present a relatively small evagination of the nuclear

envelope, which seemed to contain euchromatin. Lobed nuclei are those presenting evaginations larger than the blebe nuclei. Finally, a notch nucleus presents an appreciable depth into a nucleus that does not contain nuclear material. Enucleated erythrocytes represent erythrocytes with the absence of nucleus, while binucleated erythrocytes represent erythrocytes having two nuclei with approximately equal sizes.

5.4 Statistical analysis

The statistic used to compare the effects of chemicals on micronucleus frequencies over different concentrations. Micronucleus frequencies differences between treatments and their controls were submitted to univariate analysis of variance to test for the effects of chemicals and their correlation. The result from micronuclei analysis was given as mean $\pm$ standard error of mean (mean $\pm$ SEM). Analysis of variance (ANOVA) and LSD are used for testing the difference of the mean values of more than two groups. Significant differences between groups are defined as those with p -values less than 0.05.

Experiment 6: The relationship between cadmium uptake and micronucleus frequencies in catfish

Catfish from Experiment 3 and 5 were sacrificed after blood collection by severing the spinal column behind the opercular, and then length and weight were recorded. Various organs; muscle (including skin), gills and liver, were collected for detection of cadmium residues. The results of cadmium uptake in catfish tissues will be analyzed for the correlation with the micronucleus frequencies induced in catfish peripheral erythrocytes.

CHAPTER V
RESULTS

Study I: The survey of cadmium levels in commercial catfish (*Clarias batrachus*) in Bangkok metropolitan

Experiment 1: Specimen collection and sample preparation

Some of the aquatic environment world-wide has been contaminated by heavy metals and as a result of this, animals living in contaminated water showed high metal concentrations (Bryan and Langston, 1992; Kargin, 1998; Kalay *et al*., 1999; Rashed, 2001; Yilmaz, 2003; Ashraf, 2005). Over a few decades, there has been growing interest to determine heavy metal levels in the aquatic environments and attention was drawn to find out contamination level of public food supplies particularly fish (Kalay *et al*., 1999). Levels of contaminants in fish are of particular interest because of the potential risk to human who consume them. While attention has focused on self-caught fish, most of the fresh water fish consumed by the Thai people comes from farming sources.

Catfish was chosen for the study because this fish is a common and relatively sedentary species, and is available all the year round in most fish farms. Catfish is one of the most important commercial species of freshwater teleosts in Thailand and other Southeast Asian countries. In this experiment, catfish were obtained from six markets around Bangkok area.

Fish sampling was done during the dry season (March and April) of 2004. On average, sample sizes were 34-39 cm in length (35.93 ± 0.25 cm) while the weight of the samples were within the ranges of 330-390 g, with mean value of 354.92 ± 2.34 g (Table 9). The highest mean values were found in fish collected from Ratchawat market, 362.04 ± 5.81 g and 36.93 ± 0.45 cm, while the lowest mean values were found in fish collected from Bangkoknoi (344.65 ± 3.72 g) and Wongweanyai (35.00 ± 0.62 cm) for weight and length, respectively.

Table 9. Biological characteristics of the commercial catfish samples

Markets	Characteristics		N
	Weight (g)	Length (cm)	
Wongweanyai	352.63 ± 6.14	35.00 ± 0.62	6
Bangkoknoi	344.65 ± 3.72	35.42 ± 0.51	6
Sainert	352.28 ± 5.99	35.42 ± 0.68	6
Yingcharoen	361.26 ± 7.14	36.83 ± 0.67	6
Khlongtoei	356.66 ± 4.05	36.00 ± 0.45	6
Ratchawat	362.04 ± 5.81	36.93 ± 0.45	6
Mean	354.92 ± 2.34	35.93 ± 0.25	36

The values are means ± SEM.

N = number of fish

Catfish were not significantly different within the same market for their weights and lengths (all the ANOVAs were not significant); therefore they can be considered homogenous. There were consistent differences in weight and length among the six markets (Table 9).

Experiment 2: Determination of cadmium residues in various tissues of catfish

Cadmium residues were determined in muscle, skin, gills and liver. Detected cadmium levels in muscle, skin, gills and liver of catfish from six markets in Bangkok were given in Table 10 and the distribution of cadmium in various organs of fish was shown in Figure 15. The data showed relatively large standard error of mean (SEM), nevertheless, it is possible to describe the profile of tissue accumulation.

Average cadmium levels in muscle, skin, gills and liver were 0-36.31, 0-45.27, 0-67.47 and 10.14-185.04 ppb (μg/kg wet weight), respectively. The results showed significantly differences in cadmium accumulation according to organs, the highest level was found in liver, followed by gills, skin and muscle (liver > gill ~ skin ~ muscle). Total cadmium contents in whole body of catfish from six markets, are showed in Table 11 and Figure 16. There was a significant difference among the sampling sites in cadmium levels in whole body. The highest level of cadmium (31.12 ± 7.31 ppb) was found in fish collected from Khlongtoei market, while the lowest level of cadmium (5.53 ± 1.44 ppb) was found in fish collected from Wongweanyai market.

Table 10. Cadmium contents in organs of catfish collected from 6 districted in Bangkok metropolitan

Sample sites (Markets)	Cd levels (ppb wet weight)				Sample sites (Markets)	Cd levels (ppb wet weight)			
	Muscle	Skin	Gills	Liver		Muscle	Skin	Gills	Liver
Wongweanyai					**Yingcharoen**				
Fish 1	11.31	6.23	11.07	32.58	Fish 19	11.61	28.02	25.78	29.40
Fish 2	2.76	10.61	3.12	20.28	Fish 20	36.31	19.28	17.99	78.43
Fish 3	0.68	8.00	1.26	67.50	Fish 21	32.52	8.15	11.27	29.00
Fish 4	6.41	18.46	5.03	23.68	Fish 22	2.40	2.80	0.00	18.45
Fish 5	0.00	10.54	3.11	50.43	Fish 23	0.00	4.34	4.50	16.14
Fish 6	1.04	2.95	2.96	57.52	Fish 24	0.00	8.73	3.70	46.50
Bangkoknoi					**Khlongtoei**				
Fish 7	2.39	1.37	2.13	11.22	Fish 25	71.42	29.13	25.42	71.54
Fish 8	0.37	5.51	1.35	10.14	Fish 26	19.11	25.06	22.01	71.96
Fish 9	2.37	20.30	0.00	8.28	Fish 27	17.75	35.00	38.76	133.93
Fish 10	16.28	16.36	4.16	18.48	Fish 28	13.58	10.59	18.11	121.41
Fish 11	5.17	28.97	4.01	38.42	Fish 29	27.88	45.27	15.86	141.97
Fish 12	17.87	35.36	10.99	18.00	Fish 30	34.47	0.00	27.94	163.12
Sainert					**Ratchawat**				
Fish 13	20.91	15.17	8.91	18.04	Fish 31	6.75	11.66	11.68	107.27
Fish 14	4.81	14.41	11.69	47.47	Fish 32	16.31	29.47	63.09	108.43
Fish 15	12.84	16.60	3.66	43.99	Fish 33	0.00	16.54	0.00	130.15
Fish 16	6.79	12.59	18.44	26.26	Fish 34	6.26	13.62	4.71	46.85
Fish 17	19.21	12.10	5.01	15.31	Fish 35	5.62	3.78	67.47	185.04
Fish 18	16.81	27.76	16.72	37.95	Fish 36	12.72	13.29	2.87	30.88

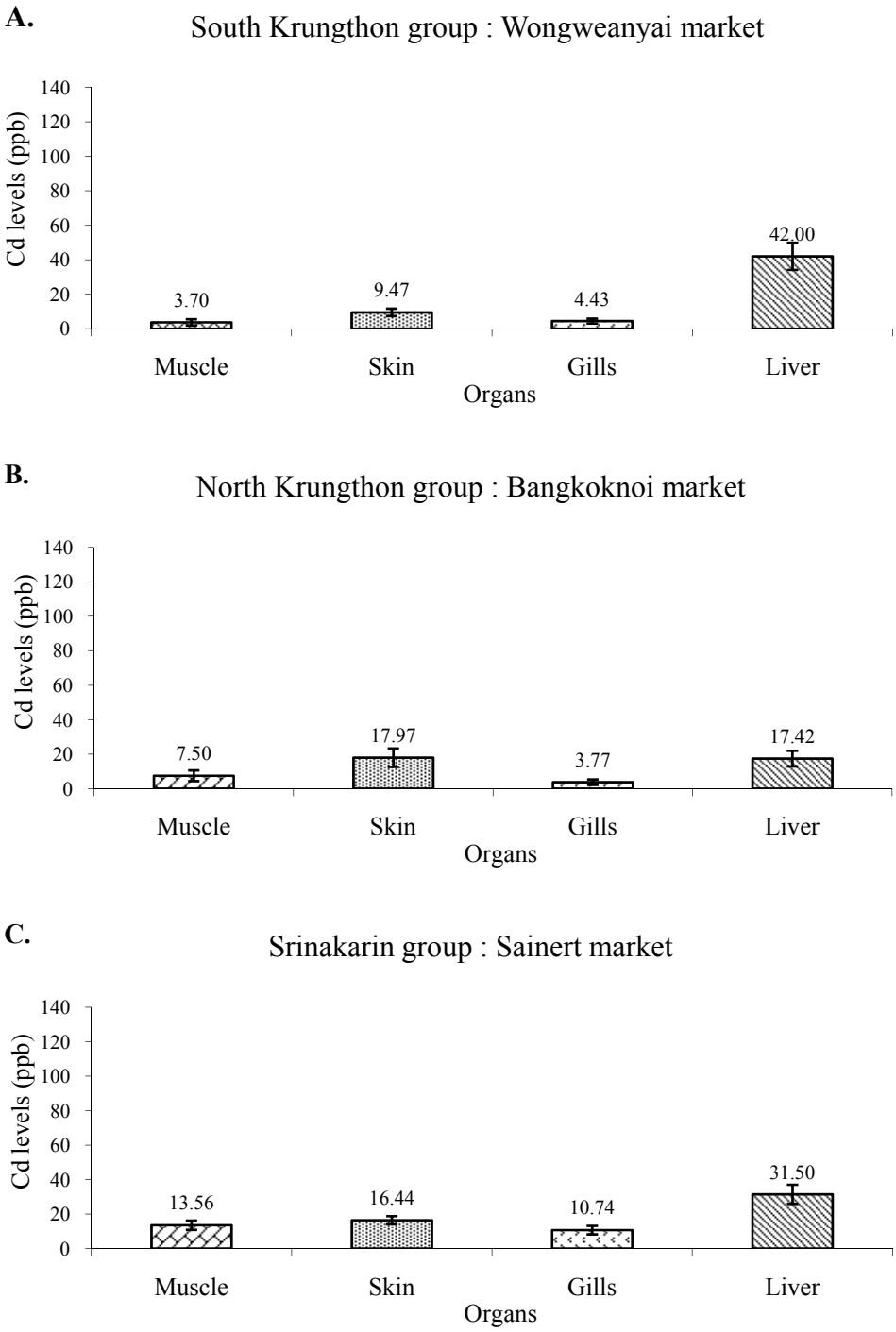


Figure 15. Distribution of cadmium in organs of catfish collected from six districts area of Bangkok metropolitan (Means ± SEM)

A) South Krungthon group, Wongweanyai market

B) North Krungthon group, Bangkoknoi market

C) Srinakarin group, Sainert market

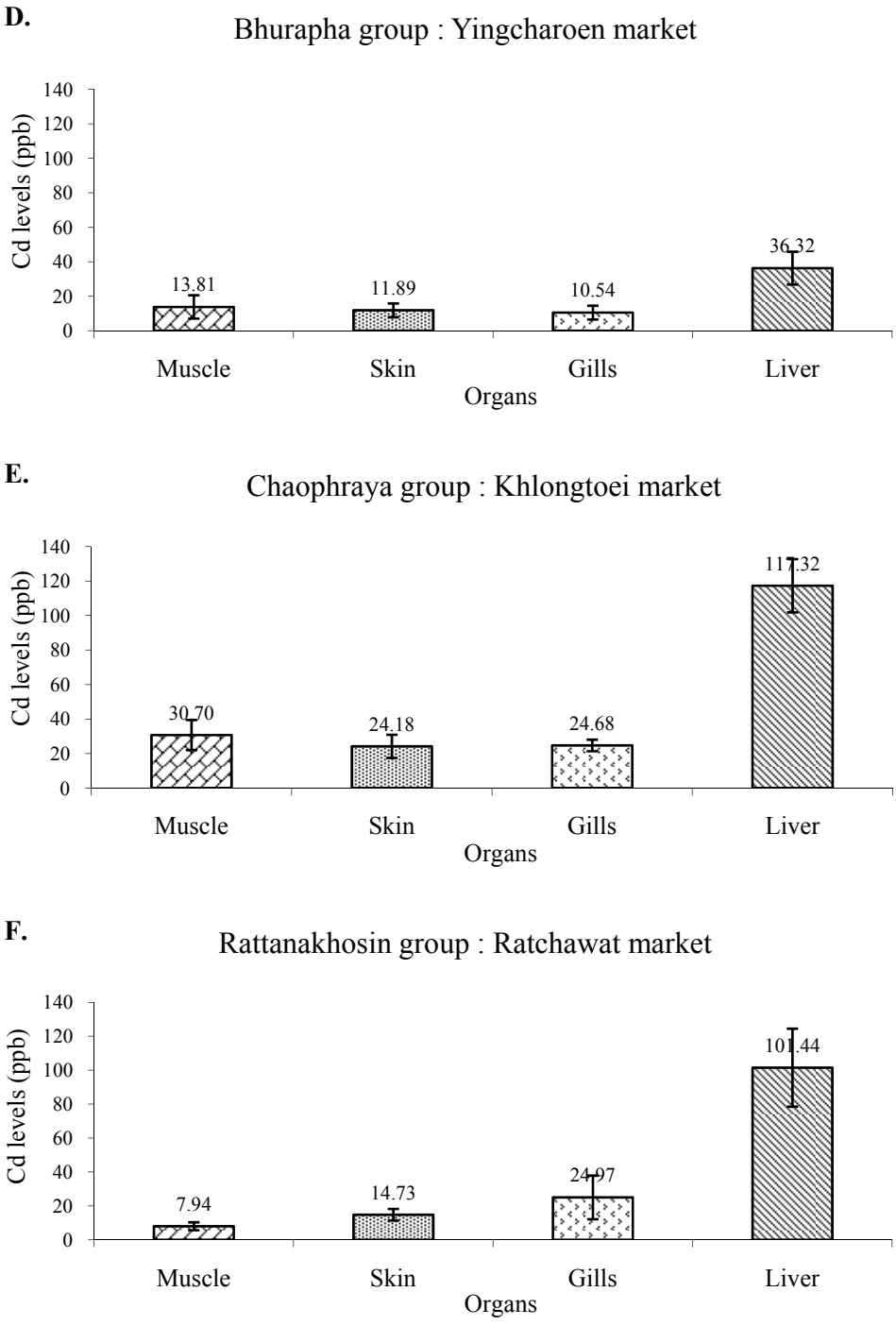


Figure 15. Distribution of cadmium in organs of catfish collected from six districts area of Bangkok metropolitan (cont.)

D) Bhurapha group, Yingcharoen market

E) Chaophraya group, Khlongtoei market

F) Rattanakhosin group, Ratchawat market

Table 11. Total cadmium contents in whole body of catfish collected from 6 markets in Bangkok metropolitan

Sampling sites (Markets)	Farm locations (Province)	Cd levels (ppb wet weight)						
		Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Fish 6	Means ±
Wongweanyai	Suphan Buri	10.85	4.88	2.97	8.93	3.16	2.37	5.53 ± 1.44
Bangkoknoi	Suphan Buri	2.42	1.33	5.03	16.00	8.89	20.01	8.95 ± 3.10
Sainert	Ang Thong	19.76	6.86	13.48	8.04	17.85	18.32	14.05 ± 2.26
Yingcharoen	Ang Thong	14.06	35.16	29.75	2.63	0.71	1.78	14.02 ± 6.19
Khlongtoei	Sukhothai	64.86	20.78	21.59	14.98	31.28	33.21	31.12 ± 7.31
Ratchawat	Suphan Buri	9.82	21.44	4.94	7.95	23.90	12.63	13.45 ± 3.11

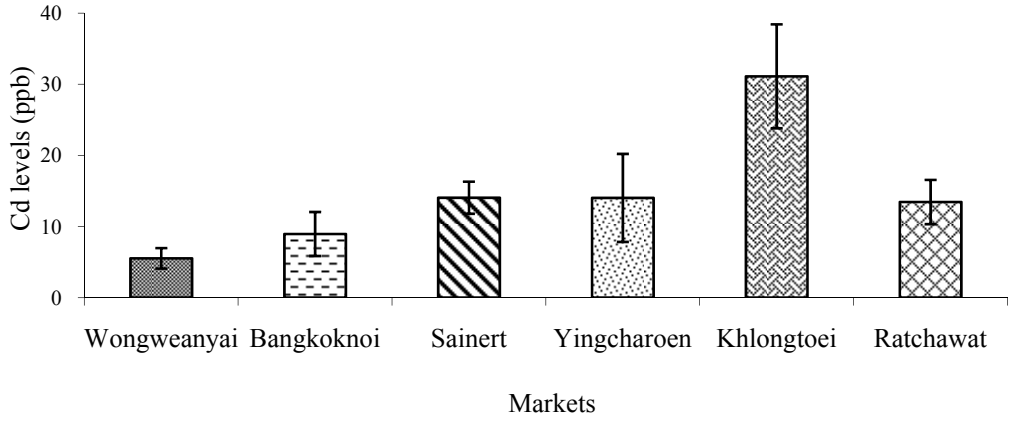


Figure 16. Total cadmium contents in whole body of catfish collected from six districts area of Bangkok metropolitan (Values are means ± SEM)

Study II: Evaluation of catfish (*Clarias batrachus*) as a model system for genotoxicity study of water pollution

Experiment 3: The cadmium uptake by catfish

For the assessment of the toxic effects of different pollutants in the aqueous environment, fish are proven to be very important indicator organisms, due both to their top position in the food chain and their requirement for great volumes of water in respiration, making their exposure to pollutants very intensive (Al-Sabti, 1991). Fish accumulate pollutants from polluted environment resulting in accumulation in their tissues.

Catfish were exposed to various concentrations of cadmium chloride ($CdCl_2$), 0.005, 0.05, 0.5, 1, 5, 10 or 20 ppm, in short-term period (96 hours) and further evaluated for cadmium uptake in various organs by GFAAS. The results of cadmium uptake in catfish tissues, as a function of exposure concentration, are presented for muscle (including skin), gills and liver in Table 12. These graphs clearly showed that the cadmium uptake had different accumulation according to organs, cadmium levels were highest in liver, followed by gills and muscle including muscle respectively (liver > gills > muscle including muscle). Cadmium uptake in catfish also showed a dose-dependent manner with exposure concentrations. In gills and liver, the concentration of Cd^{2+} significantly increased as the concentration of cadmium in the water increased. Conversely, cadmium levels in muscle (including skin) showed less difference than gills and liver.

Table 12. Cadmium uptake in various organs of catfish from exposure to various concentrations of $CdCl_2$

Exposure conc. of $CdCl_2$ (ppm)	Cd uptake levels (mg/kg wet weight)		
	Muscle + Skin	Gills	Liver
0	0.015	0.063	0.093
0.005	0.018*	0.089*	0.155*
0.05	0.018*	0.152*	0.373*
0.5	0.22*	1.34*	1.72*
1	0.26*	1.48*	3.29*
5	0.29*	1.86*	9.07*
10	0.35*	2.58*	14.57*
20	0.54*	5.2*	18.46*

* Significant different from the negative control group at p-value < 0.05

Experiment 4: The relationship between cadmium exposure levels and the cadmium residues in catfish tissues

The results of cadmium uptake in catfish tissues, as a function of exposure concentration, are presented for muscle (including skin), gills and liver in Figure 17. Cadmium uptake in catfish showed a dose-dependent manner with exposure concentrations. These dose- dependent levels of cadmium uptake by skin and muscle may be used as a biomarker of cadmium exposure.

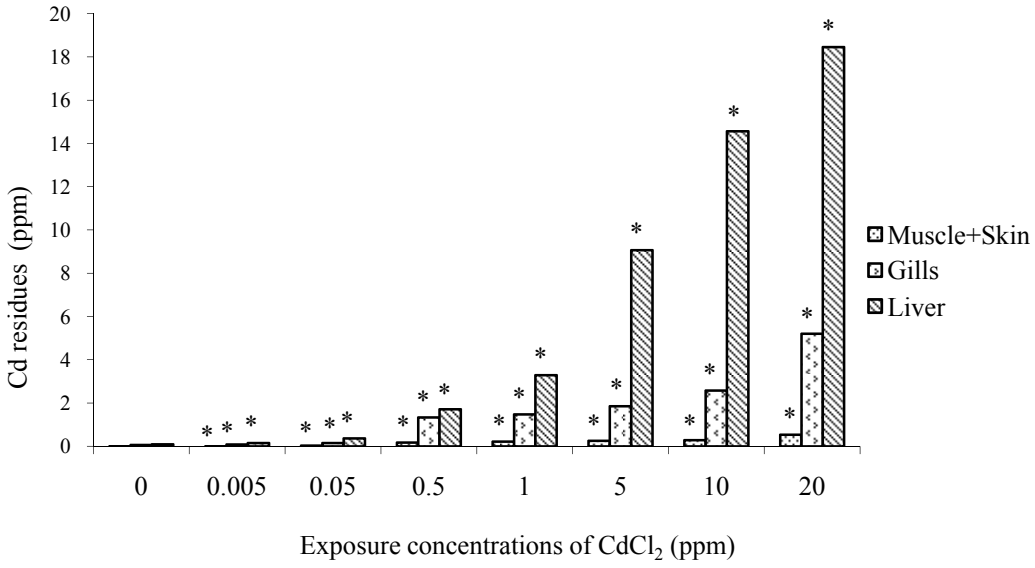


Figure 17. Cadmium uptake by various organs of catfish after 96 h exposed to $CdCl_2$

*Significant different from the negative control group ($CdCl_2$ 0 ppm) at *p*-value < 0.05

Study III: Evaluation for genotoxicity of cadmium in catfish (*Clarias batrachus*)

Experiment 5: Effect of short-term cadmium exposure on micronucleus frequencies in catfish

Fish are proven to be very important indicator organisms for the assessment of the mutagenic and/or carcinogenic potential of water samples since they are aquatic vertebrate organisms that can metabolize, concentrate and store waterborne pollutants.

The micronucleus assay in fish has the potential to detect clastogenic substances in aqueous media. Since teleost erythrocytes are nucleated, micronuclei have been scored in fish erythrocytes as a measure of clastogenic activity. Various studies have shown that the peripheral erythrocytes of fish have a high incidence of micronuclei after exposure to different pollutants under field and laboratory conditions.

In the evaluation for genotoxic effect of short-term cadmium exposure on micronucleus frequencies in catfish, fish were exposed to various concentrations of cadmium chloride ($CdCl_2$), 0.005, 0.05, 0.5, 1, 5, 10 or 20 ppm for 96 hours (4 days). After that, blood slides were done for micronucleus examination under microscopy. Figure 18 shows the pictures of peripheral erythrocytes with and without micronucleus.

The data of micronuclei counts obtained in the experimental groups, as well as the summary of the statistical analysis are shown in Table 13. Micronuclei were significantly induced in catfish by exposing to cyclophosphamide (40 mg/kg BW, i.p.) as compared to unexposed catfish. However, micronuclei were not significantly induced in catfish from exposing to low cadmium concentrations ($CdCl_2$ 0.005-5 ppm), whereas they were significantly induced when exposed to high concentrations ($CdCl_2$ 10 and 20 ppm). Dose-dependent effect of micronuclei frequencies as a function of exposure concentration was observed (Figure 19), in fact, micronucleus counts increased with the increase cadmium concentration in water.

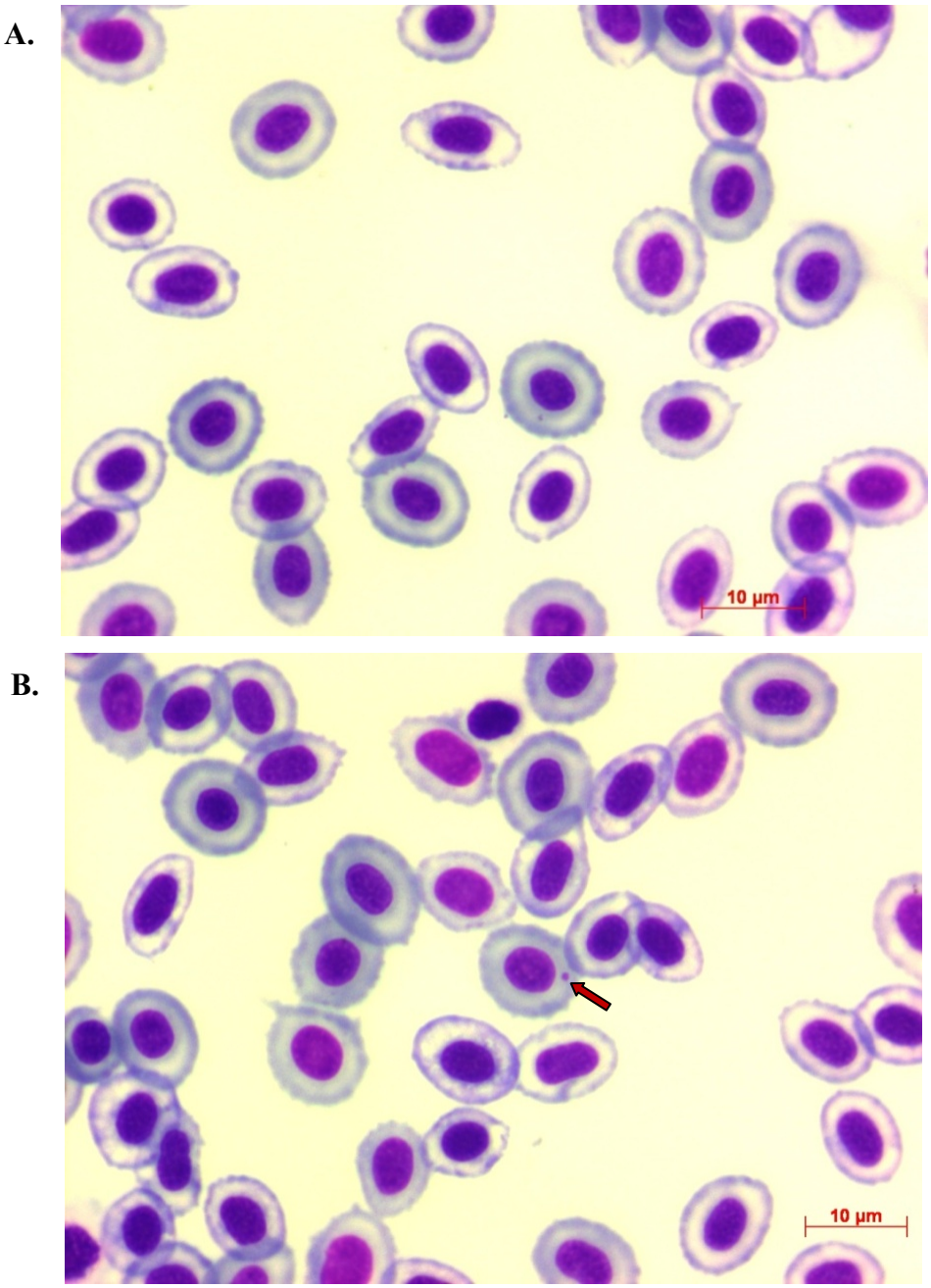


Figure 18. Peripheral erythrocytes of *C. batrachus,* May-Grünwald-Giemsa staining (1,000x magnification)

A) The normal peripheral erythrocyte from negative control group

B) Micronucleus (red arrow) in peripheral erythrocytes after exposure to positive control, cyclophosphosphamide (40 mg/kg BW, i.p.)

Table 13. The micronuclei frequencies (‰) in catfish peripheral erythrocytes after exposed to various concentrations of cadmium.

Treatment	Total cells scored	Number of MN		Total number of MN	MN frequencies (‰) (mean ± SEM)
		1MN	2MN		
Negative control					
Standard water	60314	5	0	5	0.083 ± 0.031
Positive control					
Cyclophosphamide 40 mg/kg BW (i.p.)	20101	31	2	33	1.643* ± 0.129
Test groups					
$CdCl_2$ 0.005 ppm	48231	5	0	5	0.104 ± 0.037
0.05 ppm	48356	6	0	6	0.124 ± 0.037
0.5 ppm	80573	12	0	12	0.149 ± 0.033
1 ppm	56417	9	0	9	0.160 ± 0.042
5 ppm	56317	10	0	10	0.178 ± 0.041
10 ppm	36205	7	1	8	0.221* ± 0.065
20 ppm	36197	7	2	9	0.249* ± 0.059

*Significant different from negative control group (p-value < 0.05)

MN frequencies (‰) = MN/1000

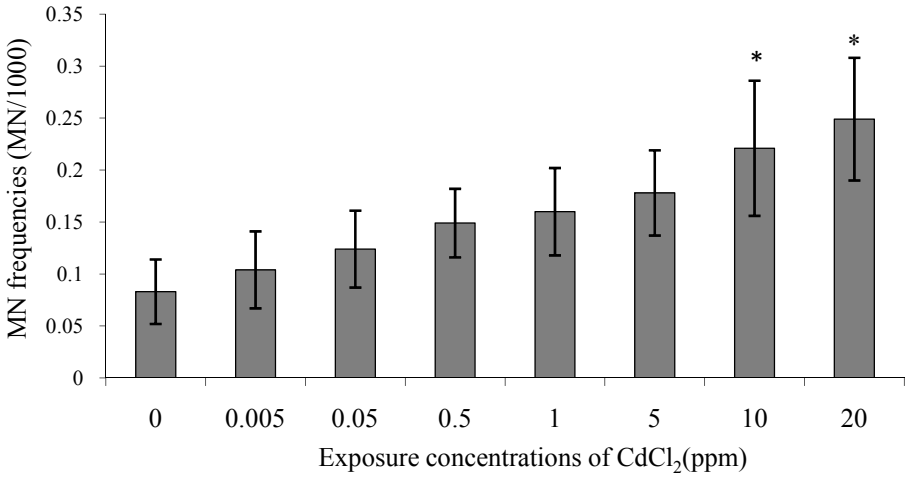


Figure 19. Micronucleus frequencies in peripheral erythrocytes of catfish exposed to various concentrations of cadmium

*Significant different from negative control group (p-value < 0.05)

In the observation of nuclear abnormalities, the important variations include binucleated and enucleated erythrocytes, microcytes, and erythrocytes with nuclear bud, nuclear bleb and nuclear lobe. These abnormalities were observed in catfish peripheral erythrocytes after exposed to cadmium. The results showed their increases due to the increase in concentration of cadmium (Table 14). Table 14 shows the micronucleus frequencies and the incidence of nuclear abnormalities from exposing to various concentration of cadmium. Relationship between these two parameters and cadmium concentrations was observed (Table 15). The nuclear abnormalities and micronucleus increased with the increase in cadmium concentration.

Table 14. The nuclear abnormalities in catfish peripheral erythrocyte after exposed to various concentrations of cadmium

Treatment	Total cells scored	Types of nuclear abnormalities						Total of nuclear abnormalities	Nuclear abnormalities (‰ ± SEM)
		Binucleated	Nuclear bleb	Nuclear lobe	Nuclear bud	Enucleated	Microcyte		
Negative control									
Standard water	3017	0	0	2	0	0	1	3	0.994
Positive control									
Cyclophosphamide 40 mg/kg BW (i.p)	3004	3	17	15	4	4	12	55	18.31*
Test groups									
$CdCl_2$ 0.005 ppm	3008	0	1	2	0	0	2	5	1.66*
0.05 ppm	3012	0	2	3	1	0	4	10	3.32*
0.5 ppm	3005	1	2	4	1	1	5	14	4.66*
1 ppm	3003	0	3	5	0	0	4	12	4.00*
5 ppm	3021	0	4	5	1	1	5	16	5.30*
10 ppm	3001	0	6	8	1	1	6	22	7.33*
20 ppm	3019	1	9	9	2	2	6	29	9.61*

* Significant different from negative control group (p-value < 0.05)

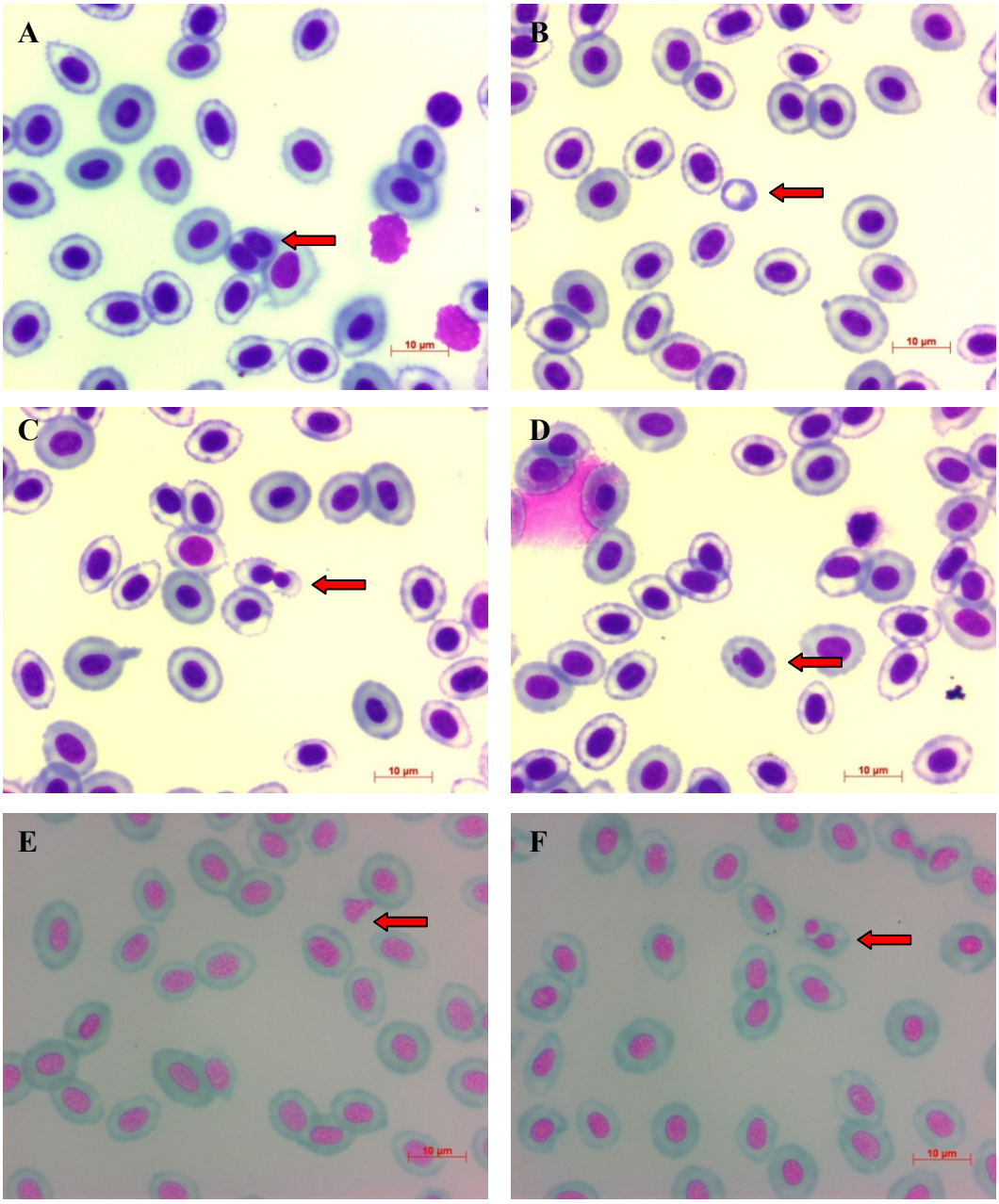


Figure 20. Micronucleus and nuclear abnormalitites in peripheral erythrocytes of *C. batrachus*, after exposure to positive control (cyclophosphosphamide 40 mg/kg BW, i.p.)

(A-D) May-Grünwald-Giemsa staining and (E-F) Schiff's reagents staining (Fuelgen reaction); (A) Binucleate, (B) Enucleated, (C) Nuclear lobe, (D) Nucleur bud, (E) Nuclear bleb and (F) Binucleate (red arrow).

Table 15. The micronuclei frequencies and nuclear abnormalities in catfish peripheral erythrocytes after exposed to various concentrations of cadmium

Treatment	MN Frequencies	Nuclear abnormalities
Negative control		
Standard water	0.083	0.994
Positive control		
Cyclophosphamide 40 mg/kg BW (i.p.)	1.643*	18.31*
Test groups		
$CdCl_2$ 0.005 ppm	0.104	1.66*
0.05 ppm	0.124	3.32*
0.5 ppm	0.149	4.66*
1 ppm	0.160	4.00*
5 ppm	0.178	5.30*
10 ppm	0.221*	7.33*
20 ppm	0.249*	9.61*

The values are means ‰ ± SEM.

* Significant different from negative control group (p-value < 0.05)

Experiment 6: The relationship between cadmium uptake and micronucleus frequencies in catfish

The relationship between cadmium uptake and micronucleus frequencies in catfish exposed to various concentrations of cadmium are shown in Figure 21A. Dose-dependent effect of micronuclei frequencies and cadmium uptake as a function of exposure concentrations was observed at high concentration of cadmium (10 and 20 ppm $CdCl_2$). However, seven tested concentrations of $CdCl_2$ induced a significant increase of the nuclear abnormalities. These nuclear abnormalities increased with the increase exposed concentration of cadmium (Figure 21B) and cadmium uptake. Micronucleus induction assay in catfish may suitable for screening the genotoxic potential of cadmium and other water polluted heavy metals but this model may not a good biomarker of cadmium pollutant effect since the catfish is less sensitive for cadmium induced micronucleus frequencies.

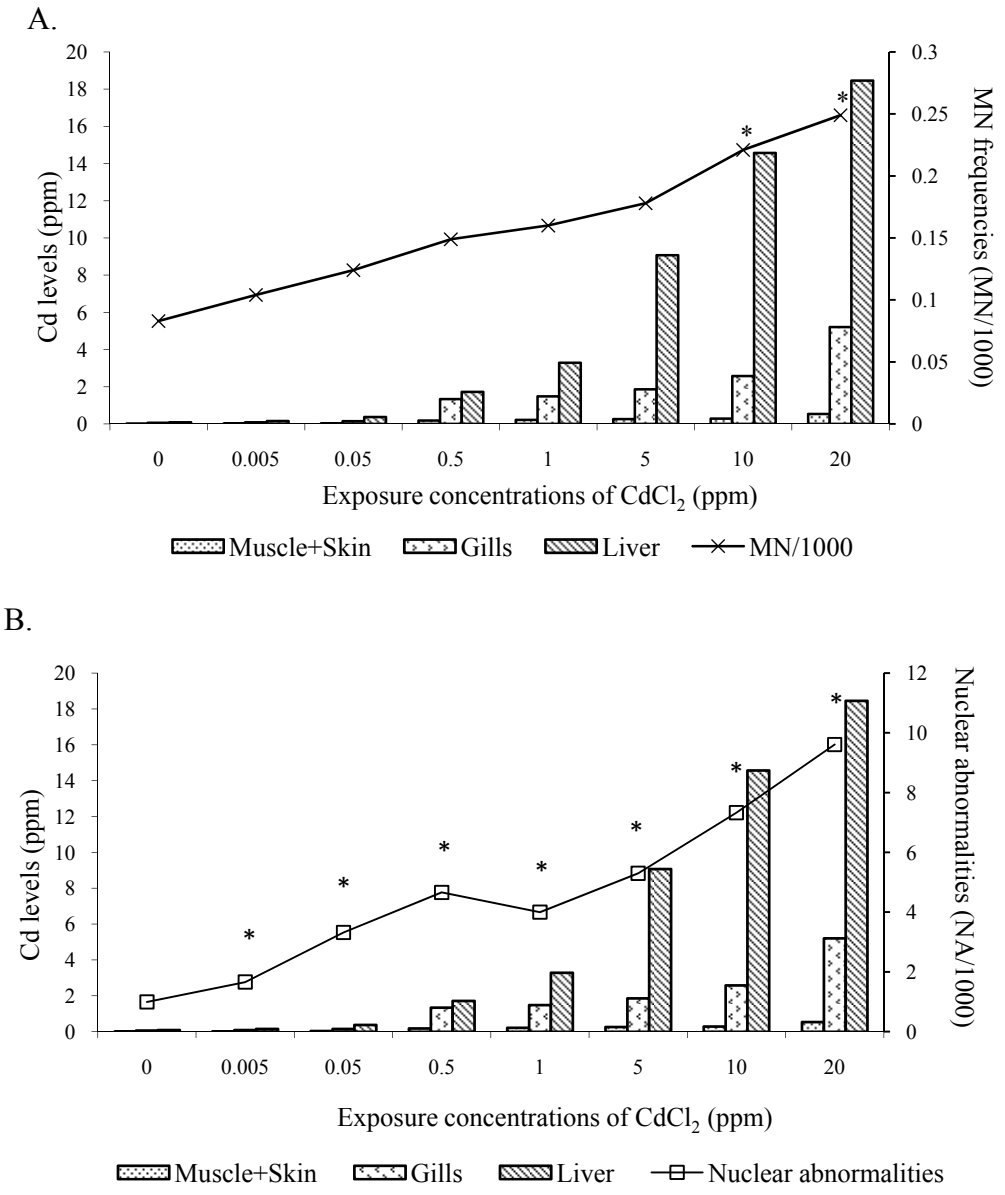


Figure 21. The relationship between micronucleus frequencies and nuclear abnormalities in peripheral erythrocytes and cadmium uptake levels in catfish exposed to various concentrations of cadmium

A) Cadmium uptake and micronucleus frequencies

B) Cadmium uptake and nuclear abnormalities

* Significant different from negative control group (p-value < 0.05)

CHAPTER VI
DISCUSSION

Growing interest to determine heavy metal contamination in the environment, particularly fish, provides insight into possible health risk of consumers. Tissue concentration of heavy metals is one way to determine human exposure to the environmental pollutants (Kalay *et al.*, 1999). Cadmium is a metal without biological function in vertebrates, and can be contaminated in water sources that receive residues from industrial processes associated with the refining of other metal (Pratap *et al.*, 1989). This contamination and its ability to accumulate in living organisms is a significant threat to environment and public health, since it accumulate through bioaccumulation up the tropic level and into the human through consumption of aquatic food (Kalay, *et al*., 1999). It strongly affects the stability of ecosystems and produced adverse effects in human and other living organisms. This study thus aimed to determine the Cd contamination in catfish, an important commercial food fish, and its genotoxic potential.

This study has three principal experiments: the survey of cadmium contamination in tissues of commercial catfish (*Clarias batrachus*) in Bangkok metropolitan area, the evaluation of catfish as a model system for genotoxicity study of water pollution by determination of cadmium uptake after cadmium exposure, and the evaluation for genotoxic potential of cadmium in catfish (*Clarias batrachus*) by micronucleus assay.

In the first experiment, commercial catfish were purchased from six markets in Bangkok, and the average cadmium contaminations in catfish tissues (muscle, skin, gills, and liver) were determined. The results showed a variation in cadmium contents among sampling sites, samples from Wongweanyai and Bangkoknoi market showed low levels of cadmium in catfish tissues and whole body, while samples from Khlongtoei market showed the high accumulation. Data from the survey revealed that the catfish sampling from Wongweanyai and Bangkoknoi markets were farmed in Suphan Buri province, while catfish from Khlongtoei market were farmed in

Sukhothai province. The difference in farm locations of fish may reason for the difference in cadmium contents in tissues, due to the variation in contamination. The results also showed the difference in cadmium accumulation according to organs, the highest level was found in liver, while accumulation in gills, skin, and muscle were relatively small and similar (liver > gills ~ skin ~ muscle). The cadmium concentrations in muscle found in this study are lower than the national guidelines for human consumption. The cadmium levels in muscle samples were in the range of 0 to 36.31 ppb indicating that the fish muscles in this study were in the safe baseline levels for human consumption. The highest level of cadmium was found in liver of a fish from Ratchawat market at 185.04 ppb, while the whole body cadmium level were in the range of 1.33 to 64.86 ppb. These levels are still below the national guidelines for human consumption (1.5 ppm) set by the Fish Inspection and Quality Control Division, Thailand. Therefore, it was concluded that the fish is safe for consumption.

The detected level of Cd contamination obtained from surveying the commercial catfish were further tested for genotoxic potential in laboratory condition *in vivo*. Catfish were cultured in aquaria and exposed to various concentrations of cadmium to determine the sensitivity and suitability in *in vivo* genotoxicity testing by measuring cadmium uptake and by induction of micronuclei after short-term exposure. The results from cadmium exposure (0.005, 0.05, 0.5, 1, 5, 10 or 20 ppm of $CdCl_2$, 96 hours exposure) clearly showed that the cadmium uptake had significant different accumulation according to organs. The cadmium levels were highest in liver, followed by gills and muscle (including skin) respectively (liver > gills > muscle including skin). The results show that cadmium concentrations are high in liver and gills while it is low in muscle (including skin). These results are consistent with previous studies which are carried out on freshwater fish (Kargin, 1998). Cadmium uptake in catfish also showed a dose-dependent manner with exposure concentrations. In gills and liver, the concentration of cadmium significantly increased at the concentration of cadmium in the water increased. Conversely, cadmium levels in muscle (including skin) showed less difference.

Pollutants rarely distribute uniformly within the body tissues of fish, but are accumulated by particular target organs. In summary, the liver was found to be the site

for cadmium accumulation in catfish, followed by gills and muscle (including skin). Muscle contamination was low and occurred only at high cadmium exposure.

Metal distribution between the different tissues varies depending on the source of uptake, diet and/or waterborne exposure. Food seems to be a significant route of uptake by intestine, whereas waterborne cadmium plays a dominant role in accumulation by gills. Whichever the exposure route may be, this study found that cadmium accumulates significantly in liver, whereas muscle, skin, and gills accumulate relatively small and similarly. Consequently, most prior studies focused on cadmium accumulation in liver, gills, and kidney, but not muscle. Nevertheless, cadmium accumulation in fish muscle may be very important from the point of view of human health. Since catfish are cultured for human consumption (Piumsombun, 2002), the kinetics of cadmium accumulation in their muscle tissue are obviously of great interest.

Gills and liver are chosen as target organs for assessing metal accumulation. The concentrations of metals in gills reflect the concentrations of metals in waters where the fish species live, whereas the concentrations in liver represent storage of metals (Yilmaz, 2003). The gills is tissue which active and passive exchanges occur between the animal and aquatic environment, while heavy metals mainly accumulate in metabolically active tissues; therefore liver is highly active in the uptake and storage of heavy metals (Kargin, 1998). Induction of metallothioneins (MTs) in liver is the main form of storage and detoxication of metals in fish (Hamilton and Mehrle, 1986). Generally, freshwater fish muscle is not considered as a metal accumulating tissue but some studies have demonstrated elevated concentration of heavy metal in muscle tissue fish from exposure of high cadmium concentrations. A few researches reported on the residues of metals in fish skin consumed by human. This study indicated that concentrations of cadmium were higher on almost of the skin of commercial catfish than in muscles. This may be the result of cadmium formed complex with the skin mucus that can not remove completely from the muscle before cadmium analysis. Since the uptake of cadmium into the liver and muscle (including skin) by catfish is correlated well with the cadmium concentrations in water, these uptake levels are good indicators for water polluted degree by cadmium.

However, the efficiency of metal uptake from contaminated water and food may differ on relation to ecological needs, metabolism, and the contamination gradients of water, food, and sediment, as well as other factors such as salinity, temperature, and interacting agents (Canli and Furness, 1995). Mathis and Cummings (1973) studied the metal accumulation of 10 freshwater fish from the Illinois River and they found that omnivorous fish had higher metal accumulation than carnivorous fish. In this study, this research was conducted side-by-side with the study by Sarunya (thesis 2006). In the study by Sarunya (2006), snake-head fish, which is a carnivorous fish, was found to have high cadmium contamination in all organ tissues. On the contrary, this study found that catfish, which benthic omnivorous fish, have lower cadmium contamination. This finding contradicts with the study by Mathis and Cummings (1973). The difference in findings may be due to the contamination in the culture area, and feeding behavior. It is also possible that the difference in accumulation of heavy may be due to in species variation. In addition, snake-head fish have scale, whereas catfish doesn't. The presence of scale may support the absorption of heavy metals.

The third study, the evaluation for genotoxicity of cadmium in catfish (*Calarias batrachus*) by micronucleus assay, the assay in fish had proved to provide an estimate of the frequencies of chromosomal damage. It also indirectly give insight into the risk to human health from environmental contaminants and many other various induced and natural pollutants in water ecosystem. It may serve as a primary screening test of genotoxic substances or agents.

The micronucleus test has been performed to evaluate the environmental mutagenicity in ecosystem biomarkers. Consequently, in order to evaluate the quality of aquatic environments, this test has been carried out in several organisms, especially, fish. It represents an important tool for the environmental diagnosis of areas under human influence, by detecting mutagenic agents responsible for genetic instability increases.

Cytogenetic methods, such as chromosome aberration tests and sister chromatid exchanges, are probably the most sensitive and efficient means of detecting the effects of genotoxic substances (Matsumo and Colus, 2000). However, fish are not normally suitable for certain cytogenetic techniques, because they have a large number of small chromosomes (Belpaeme *et al*., 1996). The micronucleus test has been used

successfully as a mutagenic assay in fish model since it is simple, reliable, sensitive, and is not strongly dependent on any karyotypic characteristics (Heddle *et al.*, 1983). The method using fish erythrocytes are not time consuming and can be done without causing suffering to the animals (Minissi *et al.*, 1996). For those reasons, the micronucleus test using fish erythrocytes is a promising assay for investigations in environmental mutagenesis (Al-Sabti and Metcalfe, 1995).

The study of micronucleus induction was performed in catfish model; fish were exposed to various concentrations of cadmium (0.005, 0.05, 0.5, 1, 5, 10 or 20 ppm of $CdCl_{2)}$ as well as cyclophosphamide as positive control, and observed for micronuclei after short-term exposure (96 hours). The result from positive control is that micronuclei were significantly induced in catfish by cyclophosphamide exposure (40 mg/kg BW, i.p.) compared to unexposed catfish. These suggested methods in general yielded significant differences from the control group when compared to the applied carcinogenic agents. The second evident result is that micronuclei were not significantly induced in catfish from the low cadmium exposure ranging from 0.005-5 ppm), whereas they were significantly induced when exposed to higher concentrations ($CdCl_2$ 10 and 20 ppm). The other interesting aspect is that both of micronuclei frequencies and nuclear abnormalities showed dose-dependent effects as a function of exposure concentrations. However, nuclear abnormalities were induced even if micronuclei were not; therefore we suggest including these anomalies in fish genotoxicity analyses based on micronuclei counts. From these data, catfish and its erythrocytes may not suitable as a model for cadmium genotoxic screening by micronucleus assay since catfish showed lower micronucleus frequencies at low concentration of cadmium, when compared to negative control and positive control. Only high concentrations of cadmium showed the induction in micronucleus frequencies. Further increasing the concentrations of cadmium may other forms of toxicities as mortalities.

The frequencies of spontaneous and induced micronucleus were low (Matsumo and Colus, 2000). According to Rizzoni *et al*., (1987), a dose- and time- dependent response has been observed in many studies using fish, although the micronucleus frequencies are lower than mammals. Formation of micronuclei occurs spontaneously in fish cells, but the frequency of spontaneous micronuclei appears to be lower in fish

than in rodent test organisms (Williams and Metcalfe, 1992). The erythrocyte micronucleus assay with fish has some drawbacks, however, including a lack of sensitivity (Metcalfe, 1988) and the potential for mistaking nuclear damage from viral erythrocyte necrosis as a clastogenic response (Carrasco *et al*., 1990).

Organisms with micronucleated erythrocytes (MNE) values close to zero have a very efficient system to withdraw them from the circulation, so when exposed to clastogents, the MNE levels in peripheral blood are not increased (Corazza *et al*., 1990; Schlegel *et al*., 1986). On the other hand, organisms with a high MNE number, like the mouse, have been used to test genotoxic agents by counting micronuclei in polychromatic erythrocytes (PCE) and reticulocytes (Kishi *et al*., 1992). The less efficient clearance by the reticulo-endothelial system in the organisms with the greater spontaneous MNE numbers means variations in the MNE observed in peripheral blood, as result of their accumulation.

In the observation of nuclear abnormalities, the important variations includes binucleated and enucleated erythrocytes, microcytes, and erythrocyte with nuclear bud, nuclear bleb and nuclear lobe were observed in catfish peripheral erythrocytes after exposed to cadmium. The results showed their increases due to increase in concentration of cadmium.

The formation of morphological alterations in the nuclear envelope described by Carrasco *et al*. (1990), as blebbed (nuclei that present a relatively small evagination from the envelope, which seems to contain euchromatin), lobed (nuclei presenting evaginations larger than those from blebbed nuclei) and notched (nuclei present a remarkable notch containing nuclear material) have also been reported in fish erythrocytes, as a consequence of exposure to environmental and chemical contaminants of genotoxic, mutagenic or carcinogenic action, although the mechanisms responsible for such abnormalities have not been described yet.

Changes in water quality (e.g. salinity, hardness, pH, alkalinity, temperature and organic fraction) and species differences impact cadmium toxicity (Eisler, 1971, Pickering & Gast, 1972, Pascoe & Cram, 1977, Pascoe & Mattey, 1977, McCarty et al., 1978). It is well known that water hardness has a direct ameliorative effect against cadmium toxicity, with Ca^{2+} having a greater protective effect than Mg^{2+} (Wood, 2001). Mortality due to waterborne cadmium is reduced with the increase of

waterborne Ca^{2+}, as Ca^{2+} and Cd^{2+} compete for the same transport pathway (Playle *et al.*, 1993), this explains why the presence of Ca^{2+} protected against cadmium toxicity in fish (Baldisserotto *et al*., 2001). Several studies showed that the change in water hardness resulted in changes of cadmium LD_{50}. Sorensen (1991) summarized the approximate water hardness and its effect on 96-h LC_{50} in cadmium aqueous exposed fish. Various species were tested, and goldfish exposed to cadmium in water hardness of 2.1 ppm $CaCO_3$ has LC_{50} of 2.1 ppm, while water hardness increased to 140 cause the increase in LC_{50} as 46.8 ppm (McCarty et al., 1978). Thus, the increase in water hardness decreased cadmium toxicity in goldfish. In addition, in this study, at the same cadmium concentration, the frequency of micronucleus was found to be lower than previous reports (Rodriguez-Cea, *et al.*, 2003). This difference in finding may be due to the differences in treatment condition, namely the water hardness. In this study, Ca^{2+} concentration in standard water was 80.26 mg/l (ppm) from $CaCl_2.2H_20$. This water hardness may reduce cadmium toxicity.

CHAPTER VII
CONCLUSION

In conclusion, the results showed:

1. Consumption of commercial catfish available in Bangkok Metropolitan may not cause acute cadmium toxic on consumers.

2. The detected contaminated cadmium levels in commercial catfish were not toxic to the fish and cause no genotoxicity as tested by micronucleus induction assay.

3. Under short-term exposure condition to cadmium, the studies demonstrated the induction genotoxic damage at high cadmium concentrations as revealed by the micronucleus assay on catfish erythrocytes.

4. Catfish has shown a dose-dependent of cadmium uptake and at high cadmium concentration catfish shown a dose-dependent of micronucleus induction frequencies. Catfish uptake levels may suitable as a biomarker of cadmium exposure.

5. For screening of genotoxic potential of cadmium in catfish model, catfish erythrocytes were not suitable for micronucleus induction assay, since the high concentrations of cadmium are required. These high doses may cause other forms of cadmium toxicities. The micronucleus induction in catfish by cadmium may not a sensitive biomarker of cadmium genotoxic effect.

6. Since other nuclear abnormalities were induced by cadmium exposure in catfish even if micronuclei were not, these anomalies should be included in fish genotoxicity analyses based on micronuclei counts.

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APPENDIX

APPENDIX A

SAMPLING AREA

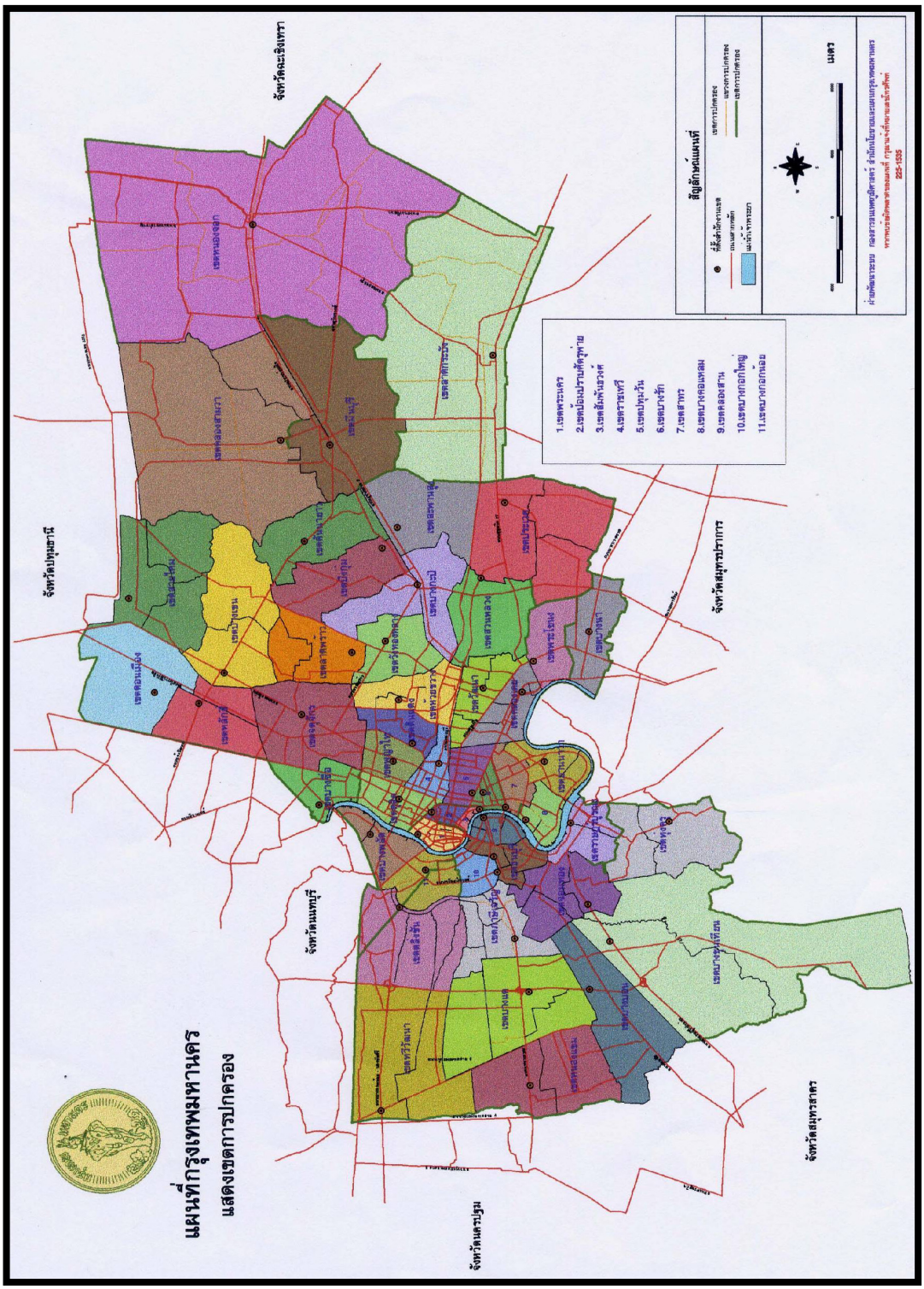

Figure 24: The map represented 40 districts of Bangkok metropolitan

SIX DISTRICTS OF BANGKOK METROPOLITAN

1. South Krungthon group

1. Bang Khun Thian
2. Bang Bon
3. Chom Thong
4. Rat Burana
5. Thung Khru
6. Thon Buri
7. Khlong San*
8. Bang Khae

*Sampling site: Wongweanyai market
Farming site: Suphan Buri province

2. North Krungthon group

1. Bang Phlat
2. Taling Chan
3. Bangkok Noi*
4. Bangkok Yai
5. Phasi Charoen
6. Nong Khaem
7. Thawi Watthana

*Sampling site: Bangkoknoi market
Farming site: Suphan Buri province

3. Srinakharin group

1. Saphan Sung
2. Min Buri
3. Khlong Sam Wa
4. Nong Chok
5. Lat Krabang
6. Prawet
7. Suan Luang
8. Khan Na Yao*

*Sampling site: Sainert Market
Farming site: Ang Thong province

4. Bhurapa group

1. Don Mueang
2. Lak Si
3. Sai Mai
4. Bang Khen*
5. Chatuchak
6. Lat Phrao
7. Bueng Kum
8. Bang Kapi
9. Wang Thonglang

*Sampling site: Yingcharoen market
Farming site: Ang Thong province

SIX DISTRICTS OF BANGKOK METROPOLITAN (cont.)

5. Chaophraya group

1. Din Daeng
2. Huai Khwang
3. Watthana
4. Khlong Toei*
5. Bang Na
6. Phra Khanong
7. Sathon
8. Bang Kho Laem
9. Yan Nawa

*Sampling site: Klongtoei Market
Farming site: Sukhothai peovince

6. Rattanakhosin group

1. Bang Sue
2. Dusit*
3. Phaya Thai
4. Ratchathewi
5. Pathum Wan
6. Phra Nakhon
7. Pom Prab Sattru Phai
8. Samphanthawong
9. Bang Rak

*Sampling site: Ratchawat market
Farming site: Suphan Buri province

APPENDIX B
CADMIUM CONTENT DETERMINATION

Cadmium contents in the organs of catfish were determined by using GFAAS from the cadmium standard curve.

Table 16. Calibration data for cadmium

Standard ID	Concentration (µg/l)	Absorption (Peak area)	SD	% RSD
Std 1	0	0.0055	0.0002	3.6
Std 2	0.4	0.0219	0.0002	0.9
Std 3	0.8	0.0364	0.0009	2.4
Std 4	1.2	0.0533	0.0005	0.9
Std 5	1.6	0.0691	0.0009	1.3
Std 6	2.0	0.0845	0.0007	0.8

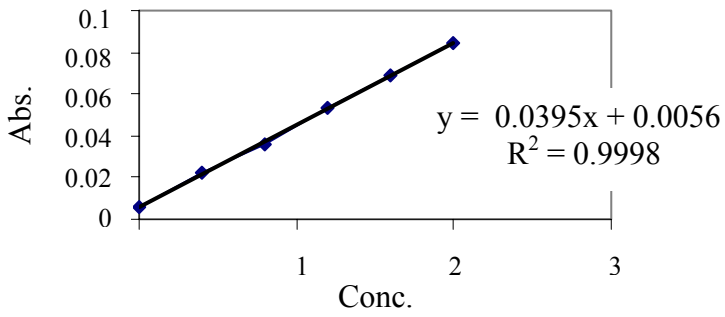

Figure 25. Standard curve of cadmium

APPENDIX C
REAGENTS PREPARATION

1. Standard water

Standard water was freshly prepared as described in International Standard ISO 7346/2-1984*. This standard water contains 25 ml of each of the following solutions in 1 liter of deionized water; $CaCl_2.2H_2O$ 11.76 g/l, $NaHCO_3$ 2.59 g/l, $MgSO_4.7H_2O$ 4.93 g/l and KCl 0.23 g/l to the final pH of 7.8 ± 0.2.

* International Organization for Standardization. (1984). *International Standard ISO 7346/2: Water quality-determination of the aquatic lethal toxicity of substances to a freshwater fish, part 2: Semi-static method.* Switzerland.

2. Cyclophosphamide stock solution (CP)

The stock of cyclophosphamide was freshly prepared by dissolving 10 mg cyclophosphamide into 1 ml distilled water at a final concentration of 10 mg/ml.

3. Stock of Giemsa staining

Giemsa powder	1.0 g
Glycerine	66.0 ml
Methyl alcohol	66.0 ml

Giemsa power was mixed well with glycerine. The mixture was placed in the hot plate and set temperature at 60^{o}C for 2 h. Finally, methyl alcohol 66 ml was added in the mixture while stirring gently.

4. Giemsa solution

Giemsa solution (1:6) was prepared by mix Giemsa's stock solution 1 part into distilled water 6 parts. Then mixed solution was filtered through a medium-fast paper. Giemsa solution should be prepared freshly before use.

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