[illegible] OF CADMIUM IN [illegible] (CHEOCHROMIS NILOTICUS)

PREM KUMAR

FACULTY OF MARINE STUDIES AND MARINE SCIENCE
UNIVERSITI MALAYSIA TERENGGANU

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Perpustakaan Sultanah Nur Zahirah (UMT)
Universiti Malaysia Terengganu

PERPUSTAKAAN SULTANAH NUR ZAHIRAH UMT

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In-vitro genotoxic effects of cadmium in tilapia fingerlings (Oreochromis niloticus) / Prem Kumar.

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Lihat sebalah

HAK MILIK
PERPUSTAKAAN SULTANAH NUR ZAHIRAH UMT

IN-VITRO GENOTOXIC EFFECT OF CADMIUM IN TILAPIA FINGERLINGS (*OREOCHROMIS NILOTICUS*)

By

Prem Kumar

Research Report submitted in partial fulfillment of
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Bachelor of Science (Marine Science)

Department of Marine Science

Faculty of Maritime M and Marine Science

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JABATAN SAINS MARIN
FAKULTI PENGAJIAN MARITIM DAN SAINS MARIN
UNIVERSITI MALAYSIA TERENGGANU

PENGAKUAN DAN PENGESAHAN LAPORAN PROJEK PENYELIDIKAN I DAN II

Adalah ini diakui dan disahkan bahawa laporan penyelidikan bertajuk:

In-Vitro Genotoxic Effect of Cadmium in Tilapia Fingerlings (*Oreochromis nioticus*) oleh **Prem Kumar**, no.matrik:**UK10616** telah diperiksa dan semua pembetulan yang disarankan telah dilakukan. Laporan ini dikemukakan kepada Jabatan Sains Marin sebagai memenuhi sebahagian daripada keperluan memperolehi **Ijazah Sarjana Muda Sains (Sains Samudera)** Fakulti Pengurusan Maritim dan Sains Marin, Universiti Malaysia Terengganu.

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LIST OF ABBREVATIONS/SYMBOLS

ICP-MS	-	Inductively Coupled Plasma – Mass Spectrometry
Cd	-	Cadmium
Cd^{2+}	-	Cadmium in ionic form
ppb	-	parts per billion equivalent to ug L^{-1}
ppm	-	parts per million equivalent to mg L^{-1}
HNO_3	-	Nitric Acid
H_2SO^4	-	Sulfuric Acid
HCI	-	Hydrochloric acid
H_2O_2	-	Hydrogen Peroxide
Mg L^{-1}	-	milligram per liter
Ug L^{-1}	-	microgram per liter
L	-	Liter
PCR	-	Polymerase Chain Reaction
RAPD	-	Random Amplified Polymorphism DNA
mg	-	milligram
cm	-	centimeter

ABSTRACT

The ultimate aim of this study is to determine the genotoxic effect which takes place in aquatic organisms following exposure of heavy metal. *O. niloticus* fingerlings (2.5 cm ± 0.5) was exposed for a period of 21 days to various sub-lethal Cadmium concentrations (0.4683 ppm, 0.9366 ppm, 1.8552 ppm and 2.8098 ppm), designed from 96-h LC_{50} value (4.688 ppm) which was obtained from 96 hours acute toxicity test. The exposed fingerlings were harvested at each 7days for determination of Cadmium concentration in different body parts as well as determination of Cadmium induced genotoxic effect on fingerlings. Detection through ICP-MS indicated that significant mean difference for cadmium concentrations were found in gills, muscle and viscera only for exposure concentrations 1.8552 ppm and 2.8098 ppm when compared to control at all time intervals. However significant differences were found in whole body of every fingerling treated in all the exposure concentrations at all time intervals. Fluctuating pattern of Cadmium concentration which was found in all parts studied with increasing concentrations at various time intervals could be attributed to varying bioavailability as well other factors of temperature, size and physiological response towards heavy metal between individuals. The ICP-MS detection also indicated that Cadmium accumulated the most in muscle tissues, followed by viscera, gills and last, whole body for all time intervals. RAPD fingerprinting of *O.nilotiucs* fingerlings revealed appearance/disappearance of stable bands (400bp in OPA 9 and 900bp, 700bp in OPB 8) and changes in band intensity among samples treated with various concentrations at various time intervals, indicating damage had occurred at genomic levels. The Genomic

DNA template stability analysis also showed that significant decrease in genomic stability had occurred in all samples tested with OPB 1, OPA9, OPA 16 and OPB 8. Dendogram analysis meanwhile showed that genetic diversity occurred to some extent between all samples tested with primers mentioned. Comet assay had also revealed that significant Strand Breakage (assessed through analysis of mean comet tail length) occurred in all samples treated with various Cadmium concentrations. However, DNA repairing was also found occurring following exposure to the lowest sub-lethal concentration (0.4688 pm), with increasing time intervals. The present study concludes that Cadmium accumulates in *O.niloticus* at some significant level in various parts and induces genotocic effect as well in aquatic organisms.

KESAN GENOTOKSIK CADMIUM TERHADAP ANAK IKAN TILAPIA (*Oreochromis niloticus*)

ABSTRAK

Tujuan utama kajian ini adalah untuk menentukan kesan genotoksik terhadap organisma akuatik akibat pendedahan kepada logam berat. Anak ikan *O.niloticus* berukuran (2.5 cm ± 0.5) didedahkan selama 21 hari kepada beberapa kepekatan sub-lethal yang berbeza (0.4683 ppm, 0.9366 ppm, 1.8552 ppm and 2.8098 ppm) yang direka berdasarkan dari nilai 96-h LC_{50} yang diperolehi dari 96 hours ujian toksik akut. Anak-anak ikan yang didedahkan kepada Cadmium akan diambil seminggu sekali untuk mengaji kandungann Cadmium di dalam pelbagai bahagian ikan serta mengaji kesan genotoksik yang diakibatkan oleh logam berat Cadmium terhadap ikan tilapia. Melalui ICP-MS, adalah didapati bahawa terdapat perbezaan yang signifikan dalam kandungan cadmim di dalam insang, otot dan bahagian dalam perut hanya ditemui pada kepekatan dedahan 1.8552 ppm and 2.8098 ppm bila dibandingkan dengan kawalan. Walaubagaimanapun, kepekatan cadmium dalam badan menunjukkan perbezaan ang signifikan untuk semua kepekatan dedahan pada setiap masa. Bioavailability dikatakan sebagai punca utama kepada trend kandungan cadmium yang naik/turun di dalam semua bahagaian yang dikaji, selain factor lain seperti suhu, saiz dan respon fisiologi yang berbeza antara individual. Melalui ICP-MS juga diketahui bahawa cadmium berkumpul paling banyak di dalam tisu, diikuti bahagian dalaman perut, insang dan akhirny badan. Profil RAPD O.niloticus yang didedahkan kepada cadmium mendedahkan bahawa

kemunculan/kehilangan jalur stabil (400bp untuk OPA 9 dan 900bp, 700bp untuk OPB 8) dan perbezaan dalam kecerahan jalur berlaku. Ini menunjukkan bahawa terdapat kesan genotoksi berlaku pada peringkat genomic. Stabiliti templat DNA juga menunjukkan penurunan yang signifikan untuk semua sample yang diuji dengan primer OPB 1, OPA 9, OPA 16 dan OPB 8. Analisa dendogram juga menunjukkan bahawa diversity genetic berlaku antara pada takat tertentu dalam populasi yang didedahkan kepada cadmium. Comet assay turut mendedahkan berlakunya pemecahan jalur DNA yang signifikan (dinilai berdasatkan min pnjang ekor comet) berlaku di dalam semua sample yand diuji dengan cadmium. Walaubagaimanapun, pembaikpulihan DNA ditemui berlaku pada kepekatan pendedahan terkecil (0.4688 ppm), dengan pertambahan masa. Kajian ini menyimpulkan bahawa cadmium berkumpul di dalam O.niloticus pada taket yang signifikan di dalam pelbagai bahagian dan menyebabkan kerosakan genotoxic terhadap organisma akuatik.

CHAPTER 1

INTRODUCTION

Health, vitality and vigor of the environment around us directly or indirectly govern the quality of human life. Through the multifarious interactions of abiotic factors such as air, water, food and biotic factor such as plants and animals, life is continued. Decline in health of any of thee components can have an unpleasant effects on the continuity of life (Jha, 2004).

The aquatic environment, which covers two-thirds of the planet, is populated by the majority of extant species in different ecological niches; moreover many of them are vital sources of human food. Therefore, ecosystem function, human health and civilization are very dependant of aquatic environment. However, human populace flourish together with industrial development continues to raise the production, consumption and dumping of anthropogenic wastes. Sadly, all these potentially genotoxic and carcinogenic contaminants ends up in aquatic environment. Besides the hazard to human health via food chain, exposure of aquatic organism to genotoxic contaminants also may lead to heritable mutations and loss in total genetic diversity (either intra or inter species), with significant implications for long-term survival of natural populations (Jha, 2004).

Many metal ions are trace elements by nature but in higher concentrations, they become toxic. Unlike many other pollutants, heavy metals are difficult to be removed from the environment and can not be biologically or chemically degraded. Today, heavy metals constitute a global environmental hazard (Yoshida *et al.*, 2005).

Redistribution of metals from the earth's crust to the environment had been accelerated by the rapid development and industrial activities. Natural waters are increasingly gaining heavy metals from geological and anthropogenic sources. These heavy metals can cause serious genotoxic effects in organisms. Genotoxicants are substances capable of modifying the genetic material of living organism (Forbes, 1999). Contamination of aquatic ecosystems with heavy metals has seriously increased worldwide attention, and a lot of studies on heavy metals in aquatic environment have been published. Under certain environmental conditions, heavy metals may accumulate to toxic concentrations and cause ecological damage (Jha, 2004).

In aquatic environment, fishes are usually the top predator and can assimilate a large concentration of some metals from the water. Furthermore, fishes are used as an indicative factor in estimating trace metals pollution in aquatic system and risk of human consumption (Broek *et al.*, 2002). Heavy metals are taken up through different organs of fish because of the affinity between them. In this process, heavy metals concentrate at different levels and rate in different organs of the fish. Eventually, enrichment takes place at each trophic level and finally when man consumes these contaminated fishes as his protein source, he suffers dire consequences. Hence it is important to determine the

concentrations of heavy metals in commercial fishes in order to evaluate the possible risk of fish consumption (Yilmaz, 2005).

Bioaccumulation of metals in organisms in contaminated water is an important aspect of environmental awareness, because it may affect all members of the food chain, including fish (Atli *et al.*, 2006). Aquatic ecosystems are influenced by heavy metals and addition from anthropogenic sources. Biota can access the chemical forms of these metals through significant accumulation in food chain. The distribution of metals of major environment concern is in human brain, heart, liver, kidneys, lungs, and skin. From genetic point of view, complex formation of metals with DNA constitutes the most serious reaction. Induction of cross-links between both the strands of DNA has been reported following exposure to cobalt, copper, manganese, platinum and zinc. The underlying reactions are important in the context of their possible mutagenic, carcinogenic or teratogenic effects (Mohamad and Vohora, 1995).

OBJECTIVES

This research was conducted to investigate the changes in genome and cell of tilapia fingerlings due to Cadmium exposure. Objectives of this research:

- To determine the 96 hour median lethal concentration ($96hLC_{50}$) of Cadmium on tilapia fingerlings and detection of Copper concentration through Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).
- To study the affects in the genome of exposed tilapia fingerlings by using Randomly Amplified Polymerase Chain Reaction (RAPD-PCR).
- To investigate the degree of nucleus damaged caused by Cadmium in exposed tilapia fingerlings through Single Cell Gel Electrophoresis (Comet Assay).

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CHAPTER 2

LITERATURE REVIEW

2.1 Nile Tilapia (*Oreochromis niloticus*)

Fish species are often the top consumers in aquatic ecosystems and thus metal concentrations in fish can act as an environmental indicator of the state of the environment. Fish are known to bioaccumulate metals and therefore their use as biomonitors also has the advantage of allowing the comparison of metal concentrations among sites, where water samples are near or below the detection limits of the atomic absorption technique (Broek *et al.*, 2002).

The type of fish which will be used in this study is Nile/Red tilapia (*Oreochromis niloticus*). Tilapia, second only to carps as the most widely farmed freshwater fish in the world. Tilapia is the generic name of a group of cichlids endemic to Africa. The group consists of three aquaculturally important genera; Oreochromis, Sarotherodon and Tilapia. Several characteristics distinguish these three genera, but possibly the most critical relates to reproductive behavior (Popma and Masser, 1999). All the tilapia in the broad sense have in common a mainly herbivores diet in distinction to the majority of fishes which feed predominantly on small invertebrates or on young or small sized fishes. They are therefore only one step from the primary producers (plant

life) and as they grow to a good size, they are valuable food source for man, the omnivore (Pullin, 1984).

Tilapia is the dominant fish species found in contaminated rivers and estuarine regions (Lam *et al.*, 1998). Tilapia is a tropical species native in Africa; it cannot survive when water temperature is below 13°C, but it tolerates poor water quality, resistant to viral, bacterial and parasitic disease, and hence is a pollutant resistant species ideal for biomonitoring of water pollution (Andrew *et al.*, 2004)

Tilapias can withstand not on low levels of dissolved oxygen but also very high levels of carbon dioxide with maximum tolerance levels of 72.6 ppm. Other gasses (NH_3, H_2S) which originate from the decomposition of organic matter are also well tolerated (Pullin, 1984). According to Pullin (1984), the available observations in nature and in ponds, cages and tanks suggests that tilapias are very resistant to low levels of dissolved oxygen and the lowest short term dissolved oxygen limit recorded around 10 ppm.

2.2 Heavy metal

The term heavy metal has variously been used to denote; metal with atomic number 23 onwards except for Rb, Sr, Y, Cs, Ba and Fr, metals with density greater than 5 gcm^{-3} and metals which are toxic to man and other life forms when found in the environment (Yilmaz *et al.*, 2005). The eight most common heavy metal pollutants listed by the Environment Protection Agency (EPA) are: Cd, Cr, Cu, Hg, Ni, Pb and Zn

(Mohammad and Vohora, 1995). Living organism require trace amounts of some essential heavy metal including cobalt, copper, iron, manganese, molybdenum, vanadium, strontium and zinc. Excessive levels of essential metals however can be detrimental to the organisms (Atli *et al.*, 2006).

2.3 Cadmium

Cadmium is a soft, silvery gray metal that is malleable and ductile, similar to zinc. When heated, it burns in air with a bright light to form the oxide CdO. In nature, essentially all cadmium exists as seven stable isotopes and one radioactive isotope. Cadmium is found in rare ores such as sphalerite and greenockite, and it is formed as a byproduct during production of zinc, copper, and lead. The majority of cadmium that enters the environment is from mining, smelting, oil and coal combustion, and waste incineration. Most cadmium in the United States (about 75%) is used in nickel-cadmium batteries. It has also been used as an anticorrosive coating for steel and cast iron, and it is a component of certain specialty alloys. Cadmium is used in semiconductors (such as cadmium selenide and telluride), in dyes and pigments, as a stabilizer in plastics such as polyvinyl chloride, and as a neutron absorber in nuclear reactor control rods and shields (Hodgson, 2004).

2.4 Gills, muscle tissue and viscera of fish

There are two main ways heavy metals can enter the aquatic food chain, either directly through the digestive tract due to consumption of contaminated water or food, or non-dietary routes across permeable membranes such as gills. Fish gills are directly and continuously exposed to water borne toxicants (Broek *et al.*, 2002). Fish gills acts as a major site of uptake and depuration for these toxicants and also is highly sensitive to waterborne toxicants relative to mammalian systems (Bingsheng, 2005).

As well as their respiratory function, the gills are responsible for regulating the exchange of salts and water and play a major role in the excretion of nitrogenous waste products. Even a slight structural damage can thus render a fish very vulnerable to osmoregulatory as well as respiratory difficulties. Their vulnerability is thus considerable because their external location and necessarily intimate contact with the water means that they are liable to damage by any irritant materials whether dissolved or suspended in the water. Because of its relative simplicity in structure only the limited number of reactions can be manifested by the diseased gills. External irritants are the most frequent causes of significant gill pathological change (Roberts, 1997).

When fish are exposed to potential toxicants the chemicals may both enter the body over and cause damage to the gills membranes. The toxicants may then affect the physiological functions of the fish in a variety ways, but most commonly disturbance to ion and osmoregulation are observed (Jobling, 1998). These disturbances arise primarily

due to damage to the gills which may in turn affect ion and respiratory gas exchange, acid-base balance and the excretion of waste products. These chemicals also may be deposited and stored in various tissue of the body, such that tissue concentrations continue to rise with prolonged exposure to that chemical (Jobling, 1998). Heavy metal levels in two different fish species, concentrations of heavy metals detected in the muscle tissue, gills and liver samples showed different capacities for accumulation. The highest metal concentrations were found in the liver and gill while muscle tended to accumulate less metal (Yilmaz *et al*, 2005).

2.5 Median Lethal Concentration

Toxicity tests are carried out in order to determine the potency or toxicity of chemicals to a group of test organisms. Usually in aquatic toxicity tests utilizing acute concentrations of chemical, short term tests of up to 96 hours. Acute toxicity can be defined as toxicity elicited immediately following short-term exposure to a chemical. Effects encountered with acute toxicity commonly consist of mortality or morbidity (Morrall *et al*., 2003).

From a quantitative standpoint these effects are measured as the 96hLC_{50} (96 hour median lethal concentration). The LC_{50} value represents the concentration of the material to which the organisms were exposed that causes mortality (LC_{50}) in 50 % of an exposed population. Since ecotoxicology focuses upon the adverse effects of chemicals in the

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environmental, acute toxicity in this discipline is more commonly described by the LC_{50} (Hodgson, 2004).

Clearly, the LC_{50} value is not indicative of an acceptable level of the chemical in the environment. Allowing an environmental concentration of chemical that is predicted to kill 50% of the exposed organisms is hardly an example of good environmental stewardship. Rather the LC_{50} is used as an indicator of relative acute toxicity. The LC_{50} is used to this end rather than a more relevant descriptor of an environmentally suitable environmental concentration because the LC_{50} value has the greatest level of confidence associated with it due to its central location on the concentration response line (Ros-Chumillas *et al.*, 2005).

2.6 DNA

DNA is the genetic material of any form of life with a universal code and a natural rate of spontaneous mutation, which is possibly increased by exposure to chemicals. DNA, or deoxyribonucleic acid, determines our physical characteristics- what we look like, our ailments etc. many also believe that DNA is partly responsible for our behavior. DNA is passed on from one generation to generation. DNA must carry information from parent cell to daughter cell. It must contain information for replicating itself. It must be chemically stable, relatively unchanging. However, it must be capable of mutational change. (Atienzar *et al.*, 1998)

2.7 Comet Assay

The alkaline comet assay is increasingly used in industrial genotoxicity testing in vitro and is also becoming an important tool for evaluating the genotoxic potential of compounds *in vivo* (Gabianelli, 2006).

With this test, cells with increased DNA damage display increased migration of genetic materials in the direction of an electrophoresis current. The extent of DNA damage is quantified by measuring the displacement of genetic material between the cell nucleus and the resulting tail. Compared with other genotoxicity assays, the advantages of the comet assay include the relatively short time needed to complete an experiment, its sensitivity in detecting low levels of DNA damage in individual cells, and its use as a high throughput screening assay. Different physical agents (solar radiation and X-rays) and chemical compounds can induce DNA damage. In aquatic organisms, such damage is associated with reduced growth, abnormal development and reduced survival of embryos, larvae and adults. Among the DNA lesions produced by chemical and physical agents are strand breaks, modified bases, DNA–DNA cross links and DNA–protein cross links (Gabianelli, 2006).

2.8 Randomly Amplified Polymerase Chain reaction (RAPD-PCR)

A DNA marker was issued to detect the genetic diversity and changes. The Randomly Amplified Polymorphic DNA (RAPD) technique was developed in 1990 by Williams. It has been used in recent yeas to study populations' subdivision, genetic diversity and genetic distance, species identification and construction of genetic linkage maps in various fish species (Forbes, 1998). The randomly amplified polymorphic DNA (RAPD) assay and related techniques like the arbitrarily primed polymerase chain reaction (AP-PCR) have been shown to detect genotoxin-induced DNA damage and mutations. The changes occurring in RAPD profiles following genotoxic treatments include variation in band intensity as well as gain or loss of bands (Atienzar *et al*, 2001). The advantages of using this method are the same primers can be used for all plants, the process can be automated and it was very simple (no radioactivity, no genomic libraries, and no DNA hybridizations). It can be used to produce genetic markers off certain phenotypic characteristics and so may provide markers of loci that are responsive to contaminant selection. Thus, RAPDs might be useful in monitoring for the effects of selection and in the identification of markers of contaminant resistance (Forbes, 1998).

Randomly amplified polymorphic DNAs (RAPD) have been so far used to analyze genetic similarity and diversity in genetics and breeding research of animal/plant/microbes. The polymerase chain reaction-randomly amplified polymorphic DNAs (RAPD-PCR) has been particularly used for genetic and molecular studies as it is a simple and rapid method for determining genetic diversity and similarity in various

organisms. It also has the advantage that no prior knowledge of the genome under research is necessary. RAPD technique is one of the most frequently used molecular methods for taxonomic and systematic analyses of various organisms (Jong-Man and Gye-Woong, 2001).

2.8.1 Primers

Primers are short, artificial DNA strands-not more than 50 (usually 18-25 bp) nucleotides that exactly match the beginning and end of the DNA fragment to be amplified. They anneal (adhere) to the DNA template at these starting and ending point, where the DNA- Polymerase binds and begins the synthesis of new DNA strand (Thiel *et al.*, 2002).

Primers generally contain 50 – 60 % guanosine and cytidine (G + C). Each primer of the pair should have a similar temperature of melting T_m, which is defined as the temperature at which 50% of the hydrogen bonds are distrusted. Upon denaturation of the template DNA, the primers anneal to their complementary sequences. The annealing temperature is slightly below the T_m of the primers. Generally, T_m is provided by the primer supply company. Although one could choose primers by simply analyzing the DNA sequence, computer programs can more efficiently choose primer pair that are likely to give a good yield of product. Commercial laboratories synthesize PCR primers with the desired nucleotide sequences (Thiel *et al*, 2002).

CHAPTER 3

Materials and Methods

3.1 Background of Study Area

The study took place in INOS and FST Oceanography laboratories.

3.2 Experimental Design for Toxicity Tests

A total of 18 plastic aquariums, each measuring 42 cm (length) X 28 cm (width) X 28 cm (height) and capable of withholding more than 20 liters of waters were used in this toxicity test. Also, one large fiber tank was used to contain dechorinated water from which water was distributed evenly to the other aquarias. All aquariums and tanks were aerated all the time both for the purpose of maintaining the dissolved oxygen content of the water and to dechlorinate it.

3.2.1 Equipments

Equipments used were contaminant and toxic free. All glass wares as well as plastic wares were immersed in 10 % nitric acid (HNO_3) prior to use for approximately 48 hours and then rinsed with distilled water before drying them in a hot air oven at a constant temperature of 60 ^{0}C. Physical and physiochemical parameter measuring equipment were constantly calibrated prior to use.

3.2.2 Water Source

Tap water was used mainly for the toxicity test while deionozed water was used for the cleaning of the apparatuses. Tap water was dechlorinated through active aeration. Tap water in big container was aerated for 36 to 48 hours (Hall *et al.*, 1998). Physical and physiochemical parameters of water were measured using Hydrolab Quanta from time to time to maintain the predetermined water parameters as following: pH 7.5, dissolved oxygen 7.5-7.8 mg L^{-1}, alkalinity 100 mg L^{-1}, hardness 109 mg L^{-1} (as $CaCO_3$) (Zirong and Shijun, 2006).

3.2.3 Fish Source

Disease free tilapia fingerlings ranging from 2.5 cm to 3.0 cm in length were purchased from an unpolluted hatchery farm in Ajil and acclimatized to laboratory conditions at optimal water temperature of 24-26 ^{o}C for three weeks under a natural photoperiod prior to experimentation. During the experimental period the fish were fed ad-libitum with commercial dry pellets. Single parental fingerlings were chosen to decrease genetic variations among the same population.

3.2.4 Stock Solution of Cadmium

Stock solution is a concentrated aqueous solution that can be stored. Measured volumes of a stock solution were added to dilution water in order to prepare the required strengths of solutions (Hall *et al.*, 1998). 1000 ppm of Cadmium stock solution was prepared by dissolving 1.5986 g of Cadmium Chloride in 1000 mL of de-ionized water.

3.2.5 Control

The control was used to determine the absence of measurable toxicity due to basic test condition (e.g., quality of the control/dilution water, health or handling of test organisms) (Hall *et al.*, 1998).

3.3 Experimental Design for Acute Toxicity Test

3.3.1 Range finding test

Preliminary range finding test was conducted in order to establish an appropriate range of Cd^{2+} concentration for acute toxicity test. Broader concentrations of Cadmium ranging from 1 ppm to 10 ppm were used in this test and the duration was set at 48 hours. An appropriate geometric dilution series was used, in which each successive concentration was about 10 % of the previous one (e.g., 100, 10, 1.0, 0.1) (Hall *et al.*, 1998). Those concentrations causing mortality in between 10-90 % were selected to construct concentrations for acute toxicity test. A total number of ten fingerlings were used for each concentration.

3.3.2 Acute toxicity test

Based on the range finding test, five different concentrations within the range of concentrations causing 10-90 % mortality were chosen. This test was done in three replicates for duration of 96hours. This test was conducted in similar manner under the same environment conditions as definitive tests (temperature 20 °C, pH 7.0 ±, salinity 0 ppt, DO > 6.0 mg L^{-1}) using the same number of fingerlings per concentration. 24 hours

before the experiment and throughout the duration of the test, the fingerlings were not fed. Mortalities of fingerlings were recorded every 2 hours after the test begun and every 12 hours after the first 24 hours.

The criterion for death was the failure of fingerlings to respond to gentle stimuli. Fingerlings were observed during the holding time and were excluded if they are moribund or showing signs of stress such as loss of buoyancy control and orientation, lethargy or erratic swimming behavior. Dead fingerlings were removed as soon as detected.

3.3.3 Statistical Analysis

96-h LC_{50} value represents the median lethal concentration where 50 % of fishes died in the 96 hours duration. Based on the numbers of mortalities from each concentration, the 96-h LC_{50} value was calculated by using FORTON Toxicology Analysis software Version: 1 and using probit analysis method.

3.4 Experimental Design for Sub-Lethal Toxicity Test

Assuming that the 96-h LC_{50} stands as 100 %, four nominal concentrations, each representing 10 %, 20 %, 40 % and 60 % of the 96-h LC_{50} were constructed for sub-lethal toxicity test.

The sub-lethal toxicity test was run for a period of 21 days. The test was run using four nominal concentration build based upon the 96-h LC_{50} value and one control. The test was run in 3 replicates. 20 fingerlings were used in each aquarium and these

fingerlings were fed throughout the test period. 48 hours before the exposure, the fingerlings were transferred into the aquariums and let to acclimatize. Constant monitoring was done throughout the test period for any mortality and changes. To maintain a steady physical and physiochemical parameter of water, water was checked every 3 days using Hydrolab Quanta. Every 7^{th}, 14^{th} and 21^{st} day of the test, 6 fingerlings were harvested from each aquaria for the purpose of heavy metal detection using ICPMS method and DNA damage detection using RAPD-PCR and COMET assay method.

3.4.1 Continuous Flow-Through System

The sub-lethal toxicity test was done using continuous flow-through recycled system. Using this system, the fingerlings were exposed to a low concentration of Cadmium for a long period of time. The system continuously introduced toxicants and fresh water into the aquariums. 200 mL of fresh water and 6 ml of Cadmium solution flowed into the aquariums every 1 minute. Water discharged from each aquarium was equal to the amount of new mixture of fresh water and heavy metal introduced (Rogers *et al.*, 2003). Cadmium solutions of different concentrations were injected with a peristaltic pump into a mixing chamber where they were mixed with dechlorinated tap water before released into the aquariums. Test concentrations in the chambers were measured at day 0 and approximately weekly thereafter (Morrall *et al.*, 2003). Water samples were obtained daily, filtered using 0.45 μm filter paper. Filtered water samples were taken to quantify the concentration of Cu^{2+} concentration in the treatment tanks. Figure 3.1 shows a brief overview of how this system works.

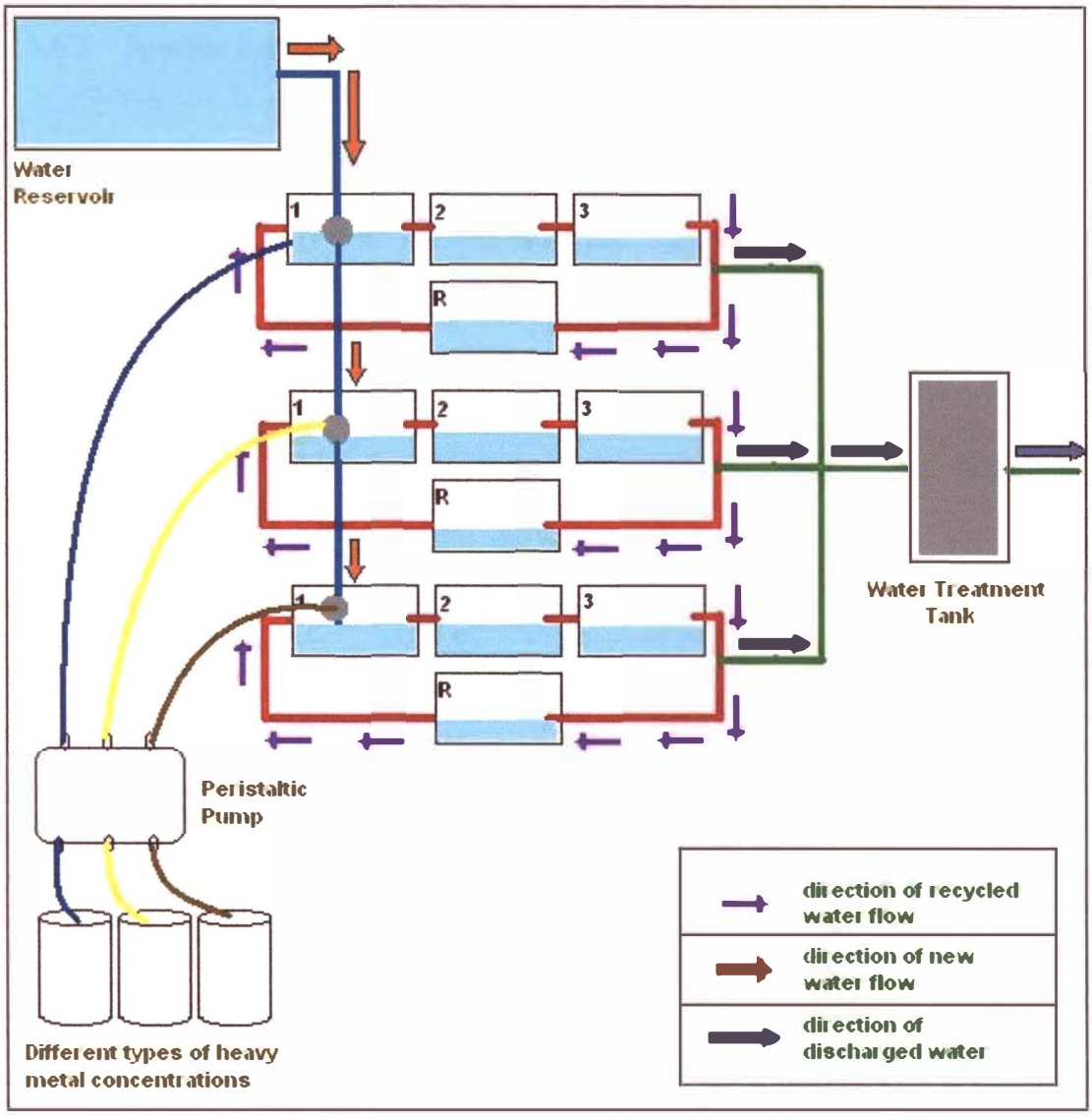


Figure 3.1 Overview of 21 days sub-lethal toxicity test

3.5 Water Treatment

Fly Ash and charcoal were used as a non- biological compound to treat the exposed water with heavy metals before the water being discharged to the environment. The water hyacinth (*Eichornia crassipes*) was used as a biological compound in pollution monitor for the simultaneous accumulation of heavy metal (Chigbo *et al.*, 2003).

3.6 ICPMS Analysis

3.6.1 Sample Preparation

Fingerlings were harvested at day 7, 14 and 21 of the test period. Harvested tilapia fingerlings were stored in -70 ^{O}C to evade contamination and samples at all times reserved in thermos flask containing ice during analysis in laboratory (Yilmaz, 2005). The harvested fingerlings were cleansed with deionized water and their wet weight, body length and whole length is measured. Using a clean dissecting tool, gills, viscera and muscle tissues were dissected and were then dried in oven at 60 ^{O}C till a constant dry weight is obtained. This dry weight is recorded.

3.6.2 Open Acid Digestion

The dried gills, muscle tissue, viscera and fingerling were digested using 1 mL of concentrated hydrochloric acid (HCL) and 1 mL of nitric acid (HNO_3 65 %) in a 100 mL Teflon beaker. The mixtures were left for preliminary digestion before complete digestion was done on hot plate at 100 ^{O}C. 1mL of hydrogen peroxide (H_2O_2) were added to the samples after cool down to oxidize any leftover organic matters. This final mixture were heated up to more or less to dryness before the contents were transferred to digestive tubes and topped up to 50 mL with de-ionized double distilled (Milli-Q) water. Additional blank runs were run con-currently to exclude any error which may occur due to contamination.

3.6.3 Detection of Heavy Metal through ICPMS

Inductively coupled plasma mass spectrometry (ICP-MS) was used to analyze the amount of Cadmium concentration in the digestions.

3.6.4 Calculation and Statistical Analysis

Readings acquired from ICP-MS, were used in calculating the value of heavy metal concentrations based on the dry weight, as this discounts the variability due to the inter-station differences in the moisture content of organisms.

Statistical analysis were done using SPSS version 10.0 software using two-way ANOVA method and tests were run on data obtained to investigate the possible significance of time and concentrations on measured parameters. Post hoc multiple comparisons were run and all statistical significance was set to $P< 0.05$ in all tests (Rogers *et al.*, 2003).

3.6.5 Recovery Test

Standard certified reference material; Dogfish Liver (DOLT-3) was used in recovery test. It is mainly used as a quality assurance to assure levels of contaminant are controlled during experiments and their effects were made negligible (Yilmaz, 2005).

3.7 Single Cell Electrophoresis Study

3.7.1 Protocol

The alkaline Comet assay was performed under alkaline conditions. 15 μL of the cell suspension was mixed with 90 μL of 0.75 % low melting point agarose in Phosphate Buffered Saline (PBS) at 37 ^{0}C. One hundred micro liters of the cell suspension was spread on a glass slide previously coated with one layer of 1.5 % normal melting point agarose in PBS, covered with a glass cover slip and placed at 4 ^{0}C for 15 min. The cover slip was gently removed and the slide was submerged into ice-cold lysing solution (2.5 M NaCl, 10 mM Tris, 0.1 M MEDTA, 1% sodium sarcosinate, 1 % Triton X-100 and 10 % DMSO, pH 10) at 4 ^{0}C for 1 h. After lysis, the slides were placed in a horizontal gel electrophoresis chamber with freshly made alkaline buffer (300mM NaOH and 1 M EDTA, pH >13.0). The slides were kept in that solution for 30 min at 4 ^{0}C to allow unwinding of the DNA and expression of alkali-labile sites. Following unwinding, the samples were subjected to electrophoresis in the same solution at 300 mA, 0.81 Vcm^{-1} for 30 min at 4 ^{0}C. After electrophoresis, the slides were rinsed gently three times (5 min each time) with 0.4 MTris–HCl (pH 7.5). Each slide was stained with 50 μL of ethidium bromide (20 μg mL^{-1}) and was covered with a cover slip. The analysis of the cells was performed by a visual scoring system (Andrighetti-Frohner *et al.*, 2006).

For alkaline Comet assay, hydrogen peroxide (30 % stock solution, Nuclear, Diadema, Brazil) was used as a positive control; it was kept at 4 ^{o}C and diluted immediately before use (Andrighetti-Frohner *et al.*, 2006).

3.7.2 Microscope Examination

Single cell images was captured and analyzed using an Olympus AX70 immunofluorescent microscope (Olympus, Japan) and tail length was measured by ImagePro Plus software (Yanke *et al.*, 2006).

3.7.3 Statistical Analysis

Data are expressed as mean values ± standard deviation (SD) of three individual experiments, each performed in triplicate. Differences in tail-length between solvent-treated (control) and Cd-treated cells were assessed using a one-way ANOVA. Comparison to the controls was done using a post-hoc tukey test. For all tests, a probability value $P < 0.05$ was considered as statistically significant (Doorten *et al.*, 2005).

3.8 Genome Study

3.8.1 DNA Isolation

Phenol–Chloroform Protocol was applied in extracting the genomic DNA of tilapia fingerlings. Approximately 50 mg of tissues and gills were excised out and transferred to 1.5 ml digestive tubes. Then, 500 µL of digestion buffer solution [1 M NaCI, 0.5 M ethylenediaminetetracetic acid (EDTA), 0.1 M Tris-HCI (pH 8.0)] and 50 µL of 10 % Sodium dodecyl sulfate (SDS) was added into the tubes, followed by manual homogenization. Before the samples being incubated at 55 ^{0}C for one and half hours, 80 µL of proteinase-k was added to breakdown protein components of the cells (Simon *et*

al., 1991). After incubation, 50 µL of Rnase was added and the samples were left in room temperature for 30 minutes. Rnase and Proteinase-k solutions were used to ensure the purity of the DNA (Saez *et al.*, 2003).

Thereafter, 50 µL of phenol: chloroform: isomyl alcohol (25:24:1) was added and samples were vortex at high speed for 2 minutes, followed by centrifugation (13, 000 rpm for 5 minutes) and precipitated sediment in the tubes were discarded. Next, supernatant was discarded and deposits were washed with cold 70 % ethanol. Following centrifugation (6000 rpm for 15 minutes), DNA pellet was resuspended in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and stored in -20 ^{0}C for term use (Ferreira *et al.*, 2004).

3.8.2 Purification and Quantification of DNA

Bio-photometer was used to measure concentration and purity of yielded DNA at OD 260 and by the absorbance ratio at 260/280 nm (Ferreira *et al.*, 2004). A Yielded DNA was decanted into 490 µL of distilled water (deionized water) and the purity of the DNA was measured. Blank was measured first before other new samples.

Quality of extracted DNA was viewed by running an agarose gel electrophoresis, whereby, 7.0 µL of DNA mixed with 3.0 µL of loading dye (6x) loaded into 0.8 % agarose gel and electrophoresis was runned for 40 minutes at 70 volts in 1xTAE buffer (Tris base, Glacial acetic acid, .5 M EDTA; pH 8.0). Marker (λ-Hind III ladder plus) was used to mark bases up to 3000 bp (base pair). Agarose gel was stained in 0.5 µl mL^{-1}

ethidium bromide for 30 minutes before bands was visualized with a UV lamp and documented using an Image VDS system (Pharmacia, Sweden).

3.8.3 Primer

RAPD-PCR analysis was performed using four universal primers; OPA9 (5'-gggtaacgcc-3'), OPA16 (5'-tgcgcccttc-3'), OPB1 (5'-ggtgacgcag-3') and OPB8 (5'-gtccacacgg-3') sequence given from 5' to 3' were obtained from Operon Technologies. Primers selected were short fragment of oligonucleotides (10 mers) with melting point temperature more or less at 40 ^{0}C and GC content approximately 60–70 %. Primers were purchased from 1st BASE Laboratories Sdn Bhd, Selangor Malaysia.

3.8.4 DNA Amplification by RAPD-PCR

The following conditions were used for RAPD : 25 µL reactions were prepared using 0.2 µL of Taq polymerase, 2.5 µL 10X PCR buffer, 2.5 Mm $MgCI_2$, 0.5 Mm of dNTP, 2.5 µL of primer, 1 µL genomic template and dd_2O water. One control (absences of DNA template) was performed during each set of amplifications. RAPD-PCR reactions were carried out in thermal cycle (Mastercycler gradient, MJ research Inc, USA).

The amplification cycles consisted of 44 cycles with pre-denaturation at 94 ^{0}C for 3 minutes, denaturation at 94 ^{0}C for 1 minute, annealing at 36 ^{0}C for 1 minute, elongation at 72 ^{0}C for 1 minute and final extension elongation 72^{0}C for 4 minutes. The reaction mixtures were stored at 4 ^{0}C prior to use. Amplified PCR products were resolved by electrophoresis on 1.2 % agarose gel at 60 V for 2 hours and submerging gel in 1x TAE buffer. Gels were stained with ethidium bromide and the bands were viewed and

photographed on a ultra-violet transilluminator (Vilber Lourmat, France) and an image VDC system (Pharmacia, Sweden).

3.8.5 Database Establishment

The electrophoresis band's molecular weight was determined according to the PCR marker, GeneRulerTM 100 bp DNA Plus marker (MBI Farmantsa, USA). The data was collected and was arranged in by molecular weight with each primer. The bands that are at about the same molecular weight size among the individual of the tilapia fingerlings in the identical primer was categorized in one unit. A binary matrix (1 for band presence and 1 for absence) was made for every sample to detect the presence and absence of each band (Zhiyi and Haowen, 2004).

3.8.6 Data Analysis

The analysis for results was carried out in two steps; (1) Numerical analysis step: the band pattern obtained scored was compared to the familiar database via hierarchial cluster analysis. Dendograms were constructed by the between group linkage method. Genotoxicity judgments were then made on the basis of the distance between the specimens (Zhiyi and Haowen, 2004). Similarity coefficient between samples was calculated using the formula $S_{XY} = 2n_{XY} / n_X + n_Y$, where S_{XY} is the similarity coefficient of taxa X and Y, n_{XY} is the number of common bands for taxa X and Y, and n_X or n_Y is the number of specific bands for taxon X and Y (Ferreira et al., 2004); (2) statistical analysis: the genomic template stability (%) was calculated as 100 – (100a/n) where 'a' is

the average number of changes in DNA profiles and 'n' the number of band selected in control DNA profiles (Atienzar *et al.*, 2000).

CHAPTER 4

RESULTS

The research was divided into 3 different parts; Part A focused on determining the 96 hour median lethal concentration (96h LC_{50}) of cadmium and detecting of cadmium concentration accumulated in tilapia fingerlings; Part B focused on determining the effects on the genome of tilapia using RAPD-PCR; and Part C focused on determining the degree of nucleus damage in cells of exposed tilapia fingerlings via Comet Assay.

4.1 Part A: Determination of 96-h LC_{50} Cd^{2+} and detection of Cadmium concentrations accumulated in exposed tilapia fingerlings through ICP-MS.

4.1.1 Toxicity Tests Data

4.1.1.a Physio-chemical Parameters

Table 4.1 Physio chemical properties of test water

No	Characteristics	Unit	Mean	Range
1	Water Temperature	°C	25.84	25.49-26.19
2	pH	-	7.3	7.1-7.5
3	Salinity	mg L^{-1}	0.0	0.0
4	Dissolved Oxygen	mg L^{-1}	5.83	5.45-6.21
5	Total Hardness	mg L^{-1} as CaCO3	19.8	19.1-20.5
6	Total Alkalinity	mg L^{-1} as CaCO3	30.5	30.0-31.0

4.1.1.b 96-hLC_{50} Value of Cadmium

Table 4.2 Median lethal concentration value (LC_{50}) cadmium using Spearman Karber and Probit method

	Spearmann Karber (ppm)	Probit (ppm)
96h LC_{50}	4.8176 ± 0.5664^	4.688±0.5649^
Variance	0.3208	0.3191

^ replicates of 3

By comparing results from Spearmann Kaber and Probit Method, it was clear that the lower variance value of Probit method showed much more consistency in 96h LC_{50} values obtained compared to Spearmann Kaber. Thus, 96 hour median lethal concentration value (96h LC_{50}) of cadmium was chosen as 4.688 ppm, calculated using probit analysis method.

4.1.1.c Concentration Design for Continuous Flow Through Recycled System

Table 4.3 Concentration design for continuous flow through recycled system

	96-h LC_{50} value of Cd^{2+} 4.688 ppm	
Numbers of concentrations	**Portion of 96-h LC_{50} value, (%)**	**Concentrations, ppm**
1 (control)	0	0.0000
2	10	0.4683
3	20	0.9366
4	40	1.8552
5	60	2.8098

4.1.2 Cadmium Analysis in Tilapia Fingerlings through ICP-MS

4.1.2.a Intensity Graph of ICP-MS Analysis

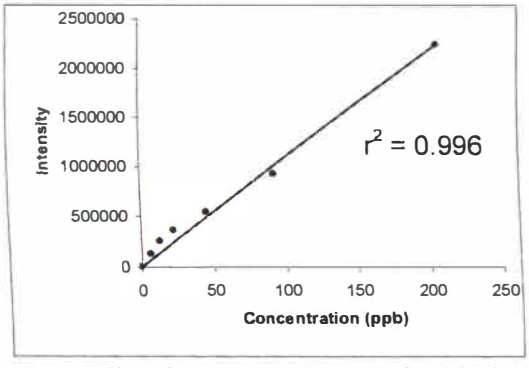


Figure 4.1 Calibration curve prepared using blank and standard.

4.1.2.b Metal Recovery Test

Table 4.4 Metal Recovery percentage for Cadmium

Certified Value (ug g^{-1})	Value Obtained (ug g^{-1})	Percentage of recovery (%)
18.8	17.8	94.68

4.1.3.c Accumulation of Cadmium in Exposed Tilapia Fingerlings' parts for 7, 14 and 21 days.

Table 4.5 Total accumulation of Cadmium in Tilapia Fries throughout 21 days of exposure to various Cadmium concentrations

Samples	Nominal concentrations, mg L^{-1}	Average Cd^{2+} accumulation, ug g^{-1}		
		7 Days	14 Days	21 Days
Gills	Control	0.5794	0.5636	0.5712
	0.4683	1.8844	1.5161	3.9212
	0.9366	1.3640	4.1956	4.7382
	1.8552	3.7847*	0.6329*	9.0201*
	2.8098	1.1498*	5.4667*	467.31*
Viscera	Control	0.7567	0.7551	0.7562
	0.4683)	5.042	5.3408	9.7203
	0.9366	4.3187	27.8419	8.6473
	1.8552	9.4511*	14.2889*	292.7846*
	2.8098	13.0204*	6.5858*	204.3091*
Muscle Tissue	Control	Trace	Trace	Trace
	0.4683	8.6721	1.1173	3.1087
	0.9366	2.0656	1.5583	24.6685
	1.8552	2.9214*	2.4859*	358.8997*
	2.8098	4.9122*	2.0172*	1551.189*
Whole Body	Control	0.0030	0.0027	0.0033
	0.4683	0.0338*	0.0818*	0.1618*
	0.9366	0.0665*	0.1065*	0.3487*
	1.8552	0.1626*	0.1718*	0.1657*
	2.8098	0.2293*	0.2372*	0.1707*

*shows significant mean difference ($p<0.05$) when compared to control.

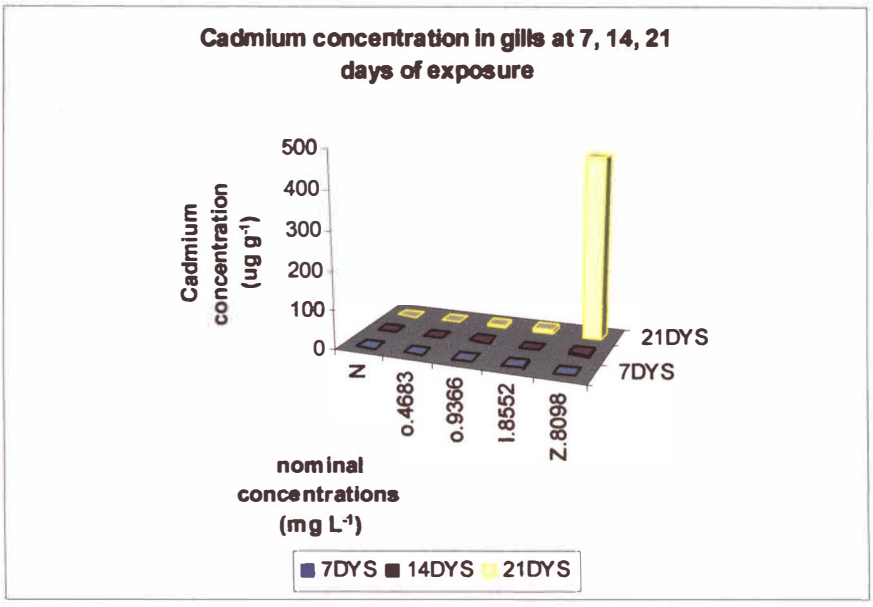


Figure 4.2 Cd^{2+} concentration in gills of exposed tilapia fingerlings in different Cd^{2+} concentration for 7, 14 and 21 days.

Highest uptake of Cadmium was noted on day 21 for exposure concentration 2.0898 mg L^{-1}, which was 467.31ug g^{-1}, while lowest was noted on day 7 for the same concentration, which was 1.1498ug g^{-1}. There was a fluctuating pattern (increase and decrease) in the uptake of cadmium in the gills in the first and second week as the exposure concentration level increases. However, in the 3rd week, there was a definite rise in the uptake of cadmium by gills from 3.9212ug g^{-1} in concentration 0.4683mg L^{-1} to 467.31ug g^{-1} in concentration 2.8098 mg L^{-1}.

Except for exposure concentration 0.9366 mg L^{-1} and 2.8098 mg L^{-1}, other two nominal concentrations did not indicate any continuous rise in the uptake of cadmium in gills. Instead, they showed an increase in the uptake of cadmium from week 1 to 2 followed by decrease in week 3.

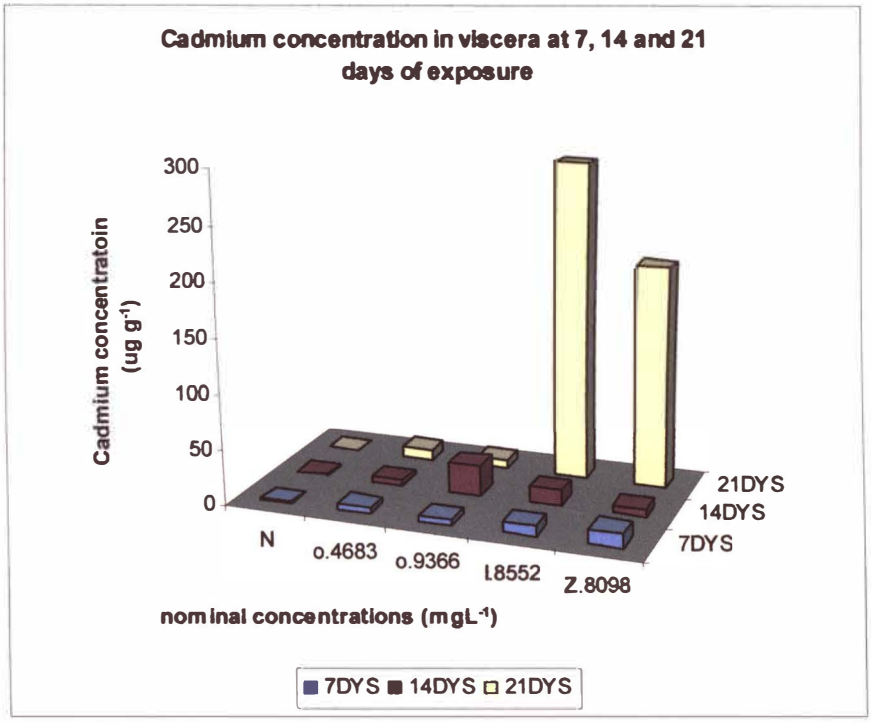


Figure 4.3 Cd^{2+} concentrations in viscera of exposed tilapia fingerlings in different Cd^{2+} concentration for 7, 14 and 21 days.

Cadmium was highest on day 21 for exposure concentration 1.8552mg L^{-1}, which was 292.7846ug g^{-1}, while lowest was noted on day 7 at exposure level of 0.9366mg L^{-1}, which was 4.3187ug g^{-1}. Unlike in gills, the uptake level trend was different in week. For instant, the cadmium concentration in viscera for week 1 decreased from exposure concentration 0.4683 mg L^{-1} (5.042 ug g^{-1}) to 0.9366 mg L^{-1} (4.3187ug g^{-1}) but started to increase from thereon to exposure concentration 2.8098(13.0204 ug g^{-1}). However week 2 showed a totally opposite trend of week 1. The concentration of Cd^{2+} in viscera rises from exposure level 0.4683 mg L^{-1} (5.3408ug g^{-1}) to 0.9366 mgL^{-1} (23.8719ug g^{-1}) but started to decrease afterward till exposure level 2.8098 mg L^{-1} (6.5858ug g^{-1}). Week 3, on the other hand, did not show any increase in uptake as occurred in gills but rather showed a fluctuating trend.

When the concentration level in stomach was compared against increasing time intervals, only exposure level 0.4683 mg L^{-1} and 1.8552 mg L^{-1} indicated an elevation in the cadmium concentration while concentration 0.9366 mg L^{-1} (increased and decreased) and concentration 2.8098 mg L^{-1} (decreased then increased) between the given time intervals.

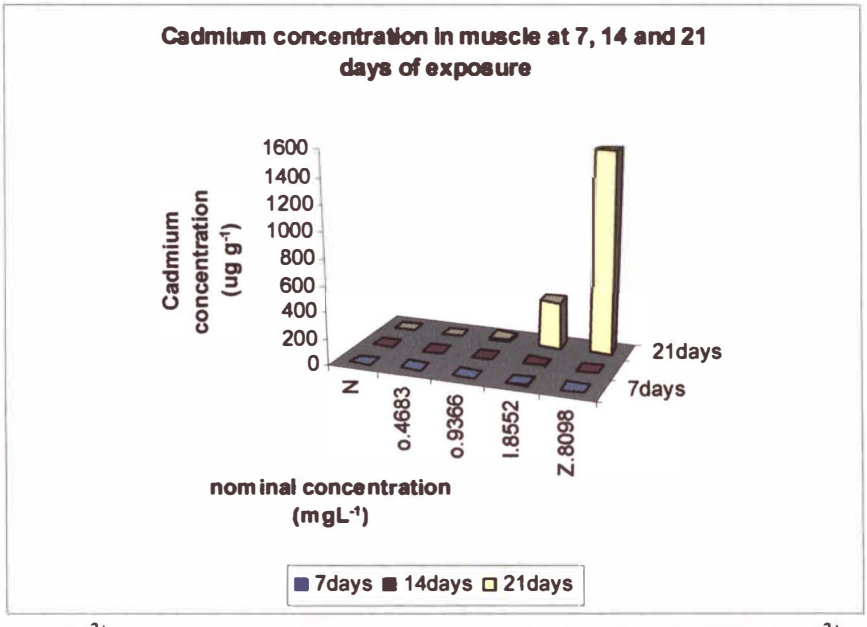


Figure 4.4 Cd^{2+} concentration in tissue of exposed tilapia fingerlings in different Cd^{2+} concentration for 7, 14 and 21 days.

The highest cadmium level in muscle tissues (1551.189 ug g^{-1}) of fingerlings was observed at day 21 in the highest exposure level to cadmium while lowest Cd^{2+} level in tissues (1.1173 ug g^{-1}) were observed in exposure concentration 0.4638 mg L^{-1} at week 2. The concentration of Cd^{2+} in tissue showed a decrease from concentration 0.4638 mg L^{-1} (8.6721 ug g^{-1}) to 0.9366 mg L^{-1} (2.0656 ug g^{-1}) and an gradual increase in both exposure level of 1.8552 mg L^{-1} (2.9214 ug g^{-1}) and 2.8098 mg L^{-1} (4.9122 ug g^{-1}) respectively.

Week 2, however, indicated a gradual rise in the Cd^{2+} uptake of muscle tissue from concentration 0.4683 mg L^{-1} to concentration 1.8552 mg L^{-1} followed by a slight drop in concentration 2.8098 mg L^{-1}. Only week 3 indicated an increase in the accumulation of Cd^{2+} in the muscle tissue, and it was a sharp one. Across the increasing time intervals, all exposure level showed a fluctuating pattern of decrease followed by an increase except for concentration 0.4683 mg L^{-1}, which decreased with increase of time of exposure. Cadmium was not recovered in controls due to low levels in the standard which would have required parts per billion testing to recover it instead of, as this study conducted, parts per million.

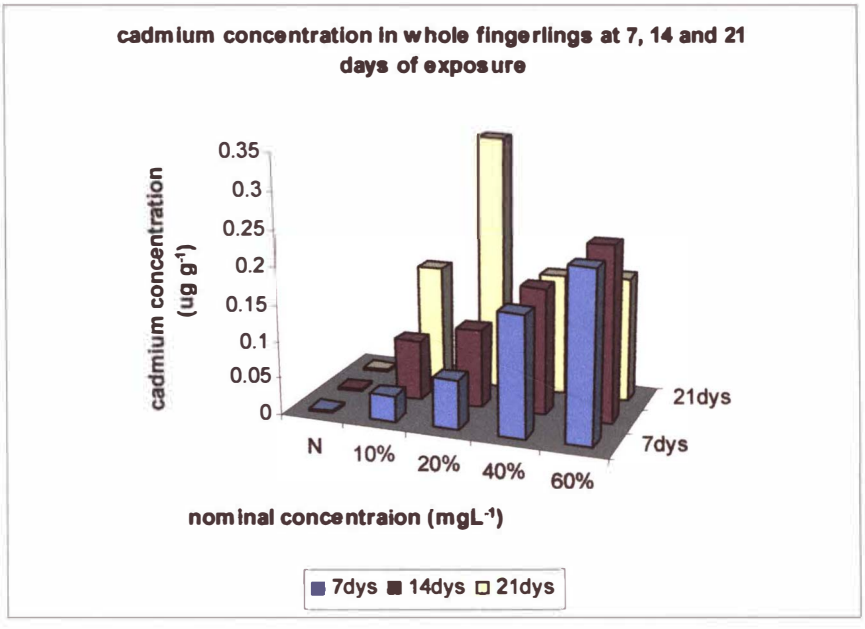


Figure 4.5 Cd^{2+} concentration in whole tilapia fingerlings in different Cd^{2+} concentration for 7, 14 and 21 days.

For the whole fingerlings, the highest concentration was found at day 7 in exposure concentration of 2.8098mg L^{-1}, which is 0.229333ug g^{-1} and the lowest in day 7 in

exposure concentration 0.4683 mg L^{-1}, which was 0.033867 ug g^{-1}. Unlike the other parts investigated, whole fish showed a gradual increase of concentration with increasing exposure concentration for week 1 and week 2. This trend, however, is not applicable for week 3 which showed an increase from exposure concentration 0.4683 mg L^{-1} to 0.9366 mg L^{-1} and then decreased from there onward.

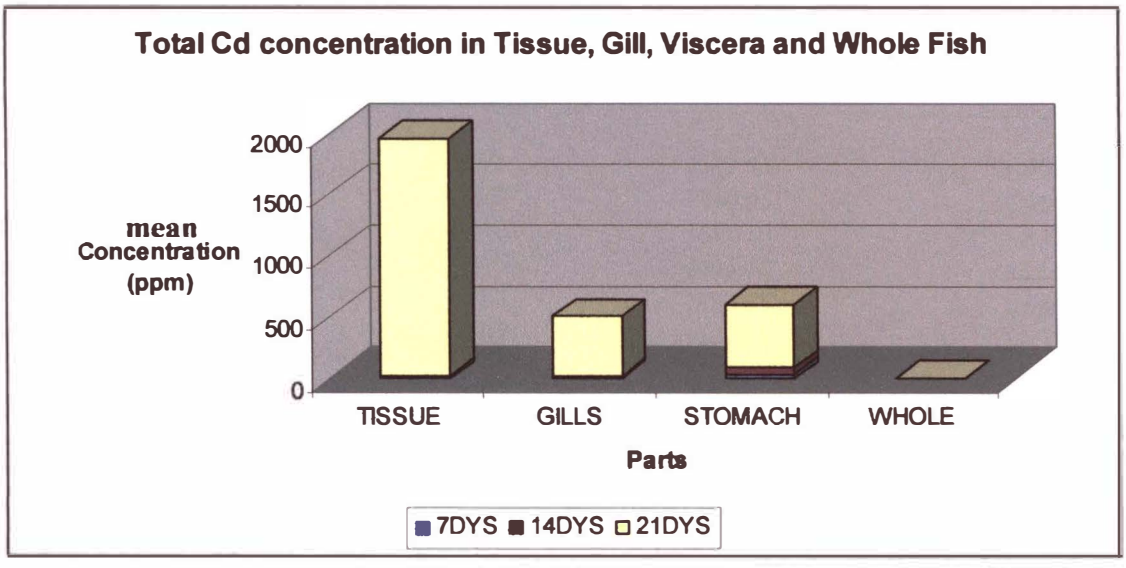


Figure 4.6 Total Cd^{2+} concentrations in parts of tilapia fingerlings at different time intervals.

When all the parts were compared, it was found that muscle tissue collected the highest amount of Cd^{2+} followed by viscera, gills, and last in whole fingerlings. Although, whole fingerlings' concentration was expected to be higher than the rest, however, the amount/weight of tissue used for detection greatly influences the result. During digestion, an approximate of 5 mg of tissues was derived from all of the parts so that the homogeneity in weight will not interfere with the outcome. However, for whole fish, the amount of tissue used was far much greater than the rest. The uneven proportion of weight used will result in the metal detection of uneven proportion.

4.2 Part B: Effects in the genome of exposed tilapia fingerlings using Randomly Amplified Polymerase Chain Reaction (RAPD-PCR).

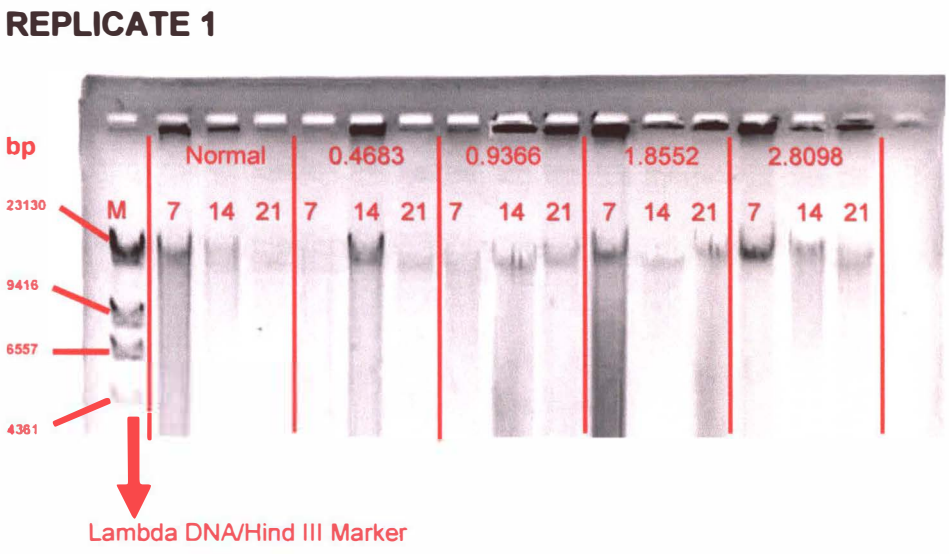
4.2.1 DNA Purification and Quantification

Table 4.6 Concentration and quality of extracted DNA, measured using Bio-photometer at 260 nm/ 280 nm absorbance ratio.

Exposure concentration	Day/Replicate	Concentration, μgml^{-1}	Optical density, A260/280
Normal	7/ 1	10524.5	2.002
	7/ 2	4175.50	2.052
	14/1	3316.50	1.933
	14/ 2	4093.60	2.032
	21/ 1	4939.55	1.673
	21/ 2	6809.80	1.695
(0.4683 ppm)	7/1	3573.55	1.992
	7/2	7260.95	1.954
	14/1	3641.10	1.859
	14/2	1495.00	1.806
	21/1	3353.75	1.685
	21/2	11097.5	1.687
(0.9366 ppm)	7/1	4653.15	1.988
	7/2	1716.50	2.431
	14/1	6370.90	1.806
	14/2	2637.95	1.805
	21/1	13114.0	1.807
	21/2	7633.55	1.796
(1.8552 ppm	7/1	3331.90	1.992
	7/2	8314.00	1.978
	14/1	2812.65	2.019
	14/2	2110.45	2.25
	21/1	1907.45	1.898
	21/2	17555.0	1.816
(2.8098ppm)	7/1	13602.0	1.949
	7/2	8651.75	1.907
	14/1	7827.60	1.983
	14/2	4717.80	2.023
	21/1	3533.15	1.812
	21/2	6132.95	1.784

4.2.2 DNA extraction

A)



B)

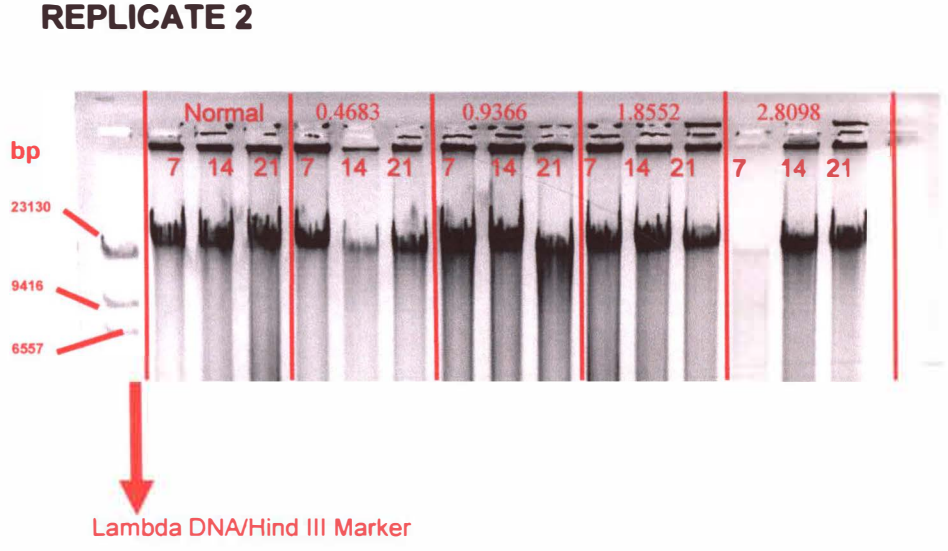


Figure 4.7 Replicate 1(A) and 2(B) of the electrophoresis pattern of *Oreochromis niloticus* genomic DNA. Normal specimen extraction in lane 2,3 and 4, fish exposed to 0.4683 ppm Cd^{2+} in lane 5, 6 and 7, fish exposed to 0.9366 ppm in lane 8, 9 and 10, fish exposed to 1.8552 ppm in lane 11, 12 and 13 and fish exposed to 2.8098 ppm in lane 14, 15 and 16. The DNA-HindIII marker was in lane 1. In all samples, the genomic bands showed the same size, about 23.0 kbp.

The suitability of the extraction methods was evaluated on the basis of the extract purity and integrity. Concentration and purity of the DNA extracted were measured at OD 260 and by the 260 nm / 280 nm absorbance ratio. The purity grade obtained from the tilapia *niloticus* tissue extraction is in the range of 1.65-2.5 and the yield obtained is about 1000-10,000 ug DNA mL^{-1} tissue. This indicates the high DNA purity grade of the extraction. The integrity of the genomic DNA extracted by this method is illustrated in Fig.4.7. The purity and integrity of the template DNA are crucial for good RAPD analysis (Zhou *et al.*, 1997).

4.2.3. RAPD fingerprinting pattern

Under the selected conditions, the fingerprinting obtained was clear. Of each primer tested there exist several legible amplified bands from 300 to 2000 bp in size. Among them, three bands, the 400bp in OPA 9 and 900bp, 700bp in OPB 8 appear in the amplified product of all the normal *O.niloticus*, as shown in Fig.4.8-Fig.5.1.

The result that all the primers can generate some distinct bands indicates that this set of primers have a high efficiency to amplify tilapia *niloticus* genomic DNA under this PCR condition and they are available as genetic markers. This also guaranteed the reliability of the database established. When each band is analyzed individually, it is noted that the results for the control and exposed data are greatly dispersed. Fortunately, after the data were arranged and grouped according to their molecular weights, this variability decreased and it became easy for us to establish and RAPD fingerprinting database. The

stable bands that appear in all normal *O.niloticus* are species-specific. They represent the common characteristics of the total normal *O.niloticus*. The familiar bands with a frequency of emergence above 60 % stand for the features of most normal *O.niloticus*.

In total four 10-mer priming oligonucleotides were used to analyze the results. In all cases, RAPD patterns generated by Cd-exposed tilapias were different from those obtained using control DNA. The results obtained from four primers are presented in Fig4.8., Fig.4.9., Fig.5.0. and Fig 5.1. DNA patterns generated by each treatment were reproducible, although each RAPD profile was obtained from individual *O.niloticus*. The principal events observed following the Cd^{2+} exposure were a variation in the band intensity, as well as the disappearance and appearance of new bands (Fig.4.8.-Fig.5.1.). The decrease and increase in band intensity were observed in several bands throughout the RAPD profiles of different specimens. According to Atienzar *et al.*, (1999), the changes in band intensities were not due to a variation in template DNA concentration nor in PCR reagent concentration (e.g. Taq DNA polymerase, since a master mix was performed).

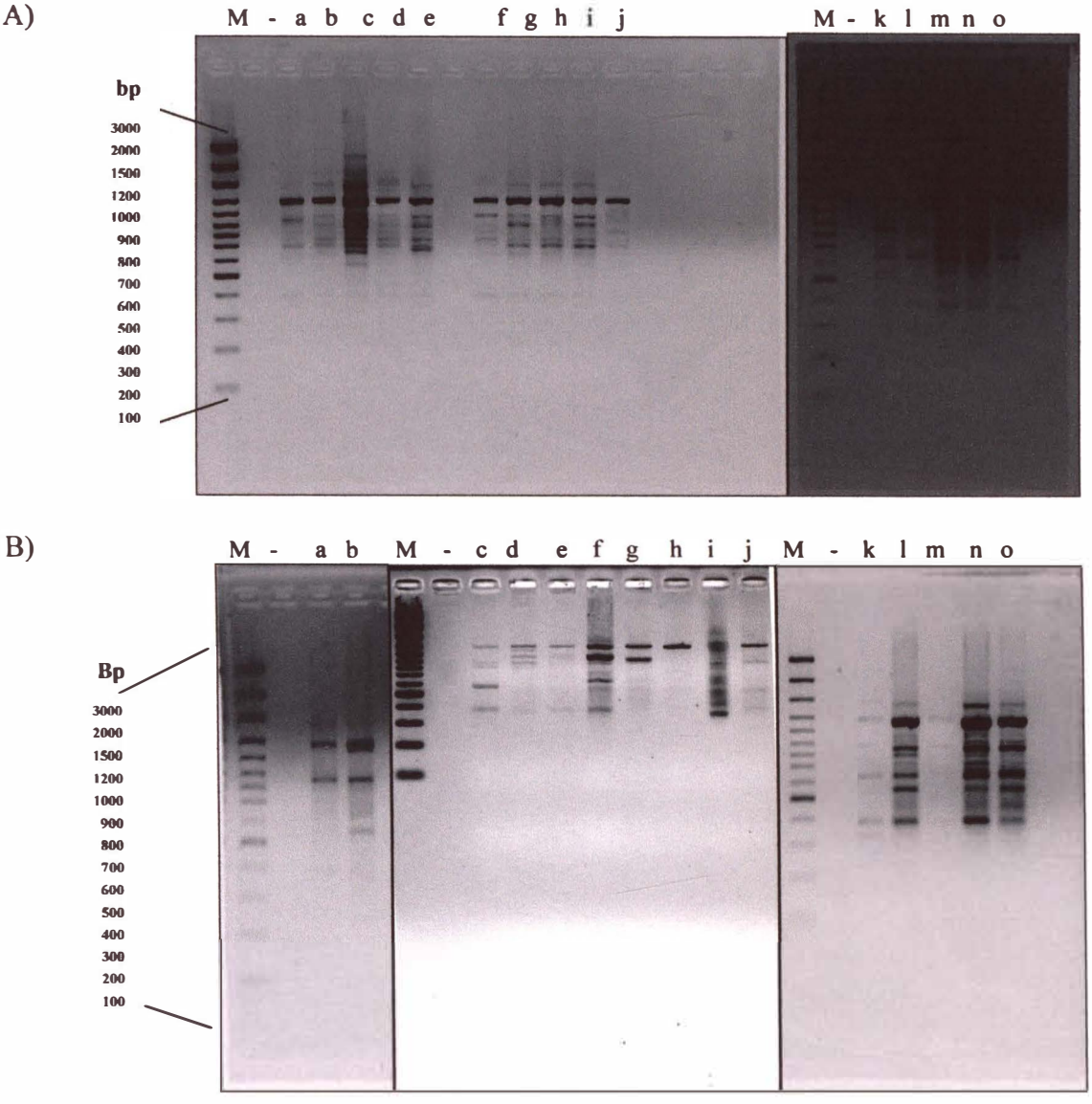


Fig.4.8. RAPD profiles of genomic DNA from *Oreochromis niloticus* exposed to varying Cd^{2+} concentrations. M, DNA molecular size marker (1 kb ladder, BRL; the molecular sizes (in kilobases) are indicated on the left).—, no DNA control. RAPD reactions were performed using oligonucleotide primer OPB 1 with Replicates 1(A) and 2(B). Each small letter represents and individual tilapia. Lanes a-o: tilapia exposed to varying Cd^{2+} concentrations; a-e: 7 days of exposure with increasing nominal concentration, f-j: 14 days of exposure with increasing nominal concentration, k-o: 21 days of exposure with increasing nominal concentration. (Increasing nominal concentration set as follows: Normal (0.00 mgL^{-1}), 0.4683 mg L^{-1}, 0.9366 mg L^{-1}, 1.8552mg L^{-1} and 2.8098 mg L^{-1}). The weights of the Markers' fragments are shown on the left in descending order in accordance with the descending fragments.

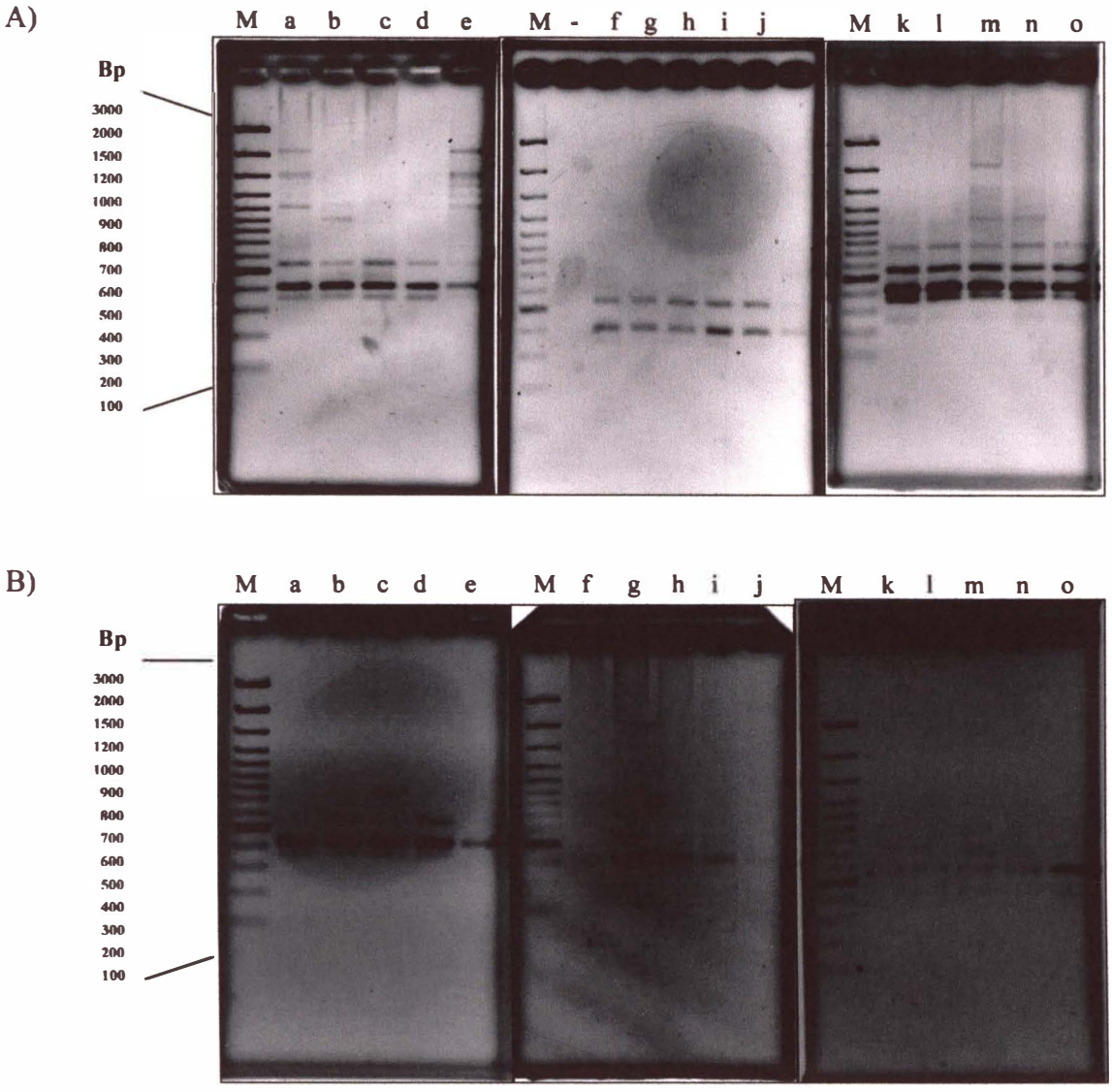


Fig.4.9. RAPD profiles of genomic DNA from *Oreochromis niloticus* exposed to varying Cd^{2+} concentrations. M, DNA molecular size marker (1 kb ladder, BRL; the molecular sizes (in kilobases) are indicated on the left).—, no DNA control. RAPD reactions were performed using oligonucleotide primer OPA 9 with Replicates 1(A) and 2(B). Each small letter represents and individual tilapia. Lanes a-o: tilapia exposed to varying Cd^{2+} concentrations; a-e: 7 days of exposure with increasing nominal concentration, f-j: 14 days of exposure with increasing nominal concentration, k-o: 21 days of exposure with increasing nominal concentration. (Increasing nominal concentration set as follows: (Increasing nominal concentration set as follows: Normal (0.00 mg L^{-1}), 0.4683 mg L^{-1}, 0.9366 mg L^{-1}, 1.8552mg L^{-1} and 2.8098 mg L^{-1}). The weight of the Markers' fragments is shown on the left in descending order in accordance with the descending fragments.

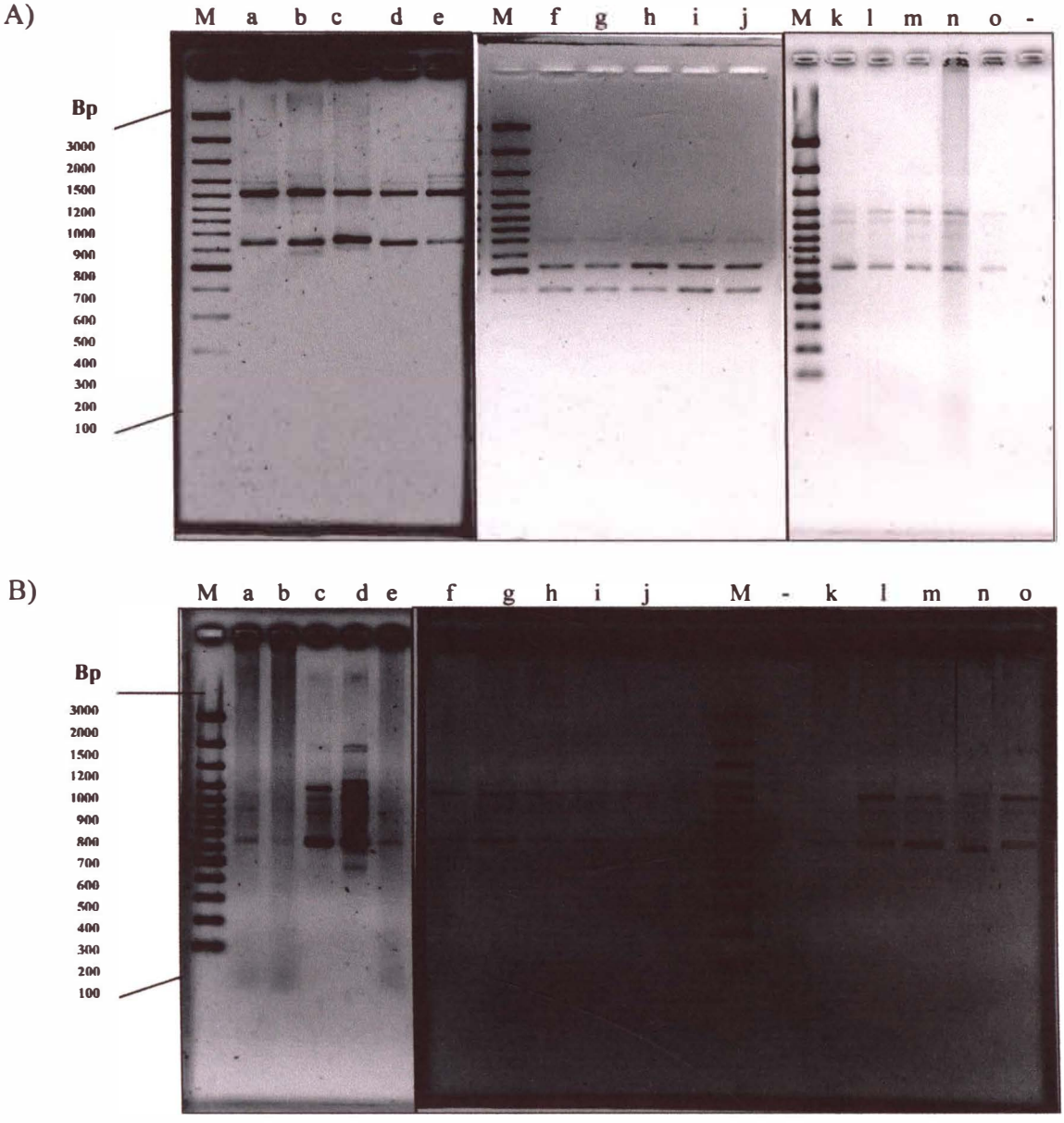


Fig.5.0. RAPD profiles of genomic DNA from *Oreochromis niloticus* exposed to varying Cd^{2+} concentrations. M, DNA molecular size marker (1 kb ladder, BRL; the molecular sizes (in kilobases) are indicated on the left).—, no DNA control. RAPD reactions were performed using oligonucleotide primer OPA 16 with Replicates 1(A) and 2(B). Each small letter represents and individual tilapia. Lanes a-o: tilapia exposed to varying Cd^{2+} concentrations; a-e: 7 days of exposure with increasing nominal concentration, f-j: 14 days of exposure with increasing nominal concentration, k-o: 21 days of exposure with increasing nominal concentration. (Increasing nominal concentration set as follows: Normal (0.00 mg L^{-1}), 0.4683 mg L^{-1}, 0.9366 mg L^{-1}, 1.8552mg L^{-1} and 2.8098 mg L^{-1}). The weight of the Markers' fragments is shown on the left in descending order in accordance with the descending fragments.

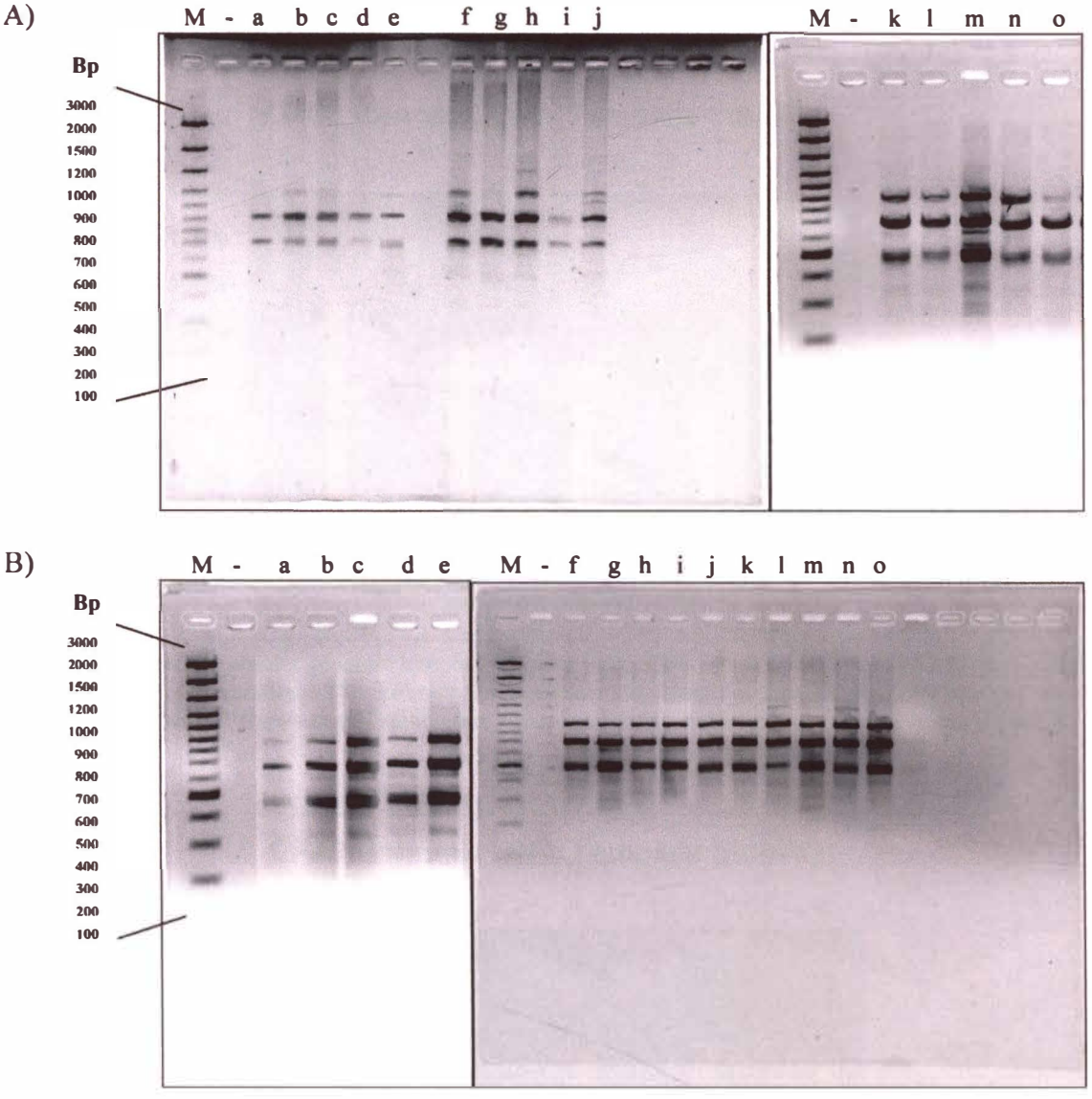


Fig.5.1. RAPD profiles of genomic DNA from *Oreochromis niloticus* exposed to varying Cd^{2+} concentrations. M, DNA molecular size marker (1 kb ladder, BRL; the molecular sizes (in kilobases) are indicated on the left).—, no DNA control. RAPD reactions were performed using oligonucleotide primer OPB 8 with Replicates 1(A) and 2(B). Each small letter represents and individual tilapia. Lanes a-o: tilapia exposed to varying Cd^{2+} concentrations; a-e: 7 days of exposure with increasing nominal concentration, f-j: 14 days of exposure with increasing nominal concentration, k-o: 21 days of exposure with increasing nominal concentration. (Increasing nominal concentration set as follows: Normal (0.00 mg L^{-1}), 0.4683 mgL^{-1}, 0.9366 mg L^{-1}, 1.8552mg L^{-1} and 2.8098 mg L^{-1}). The weight of the Markers' fragments is shown on the left in descending order in accordance with the descending fragments.

4.2.4 Genomic DNA Template Stability

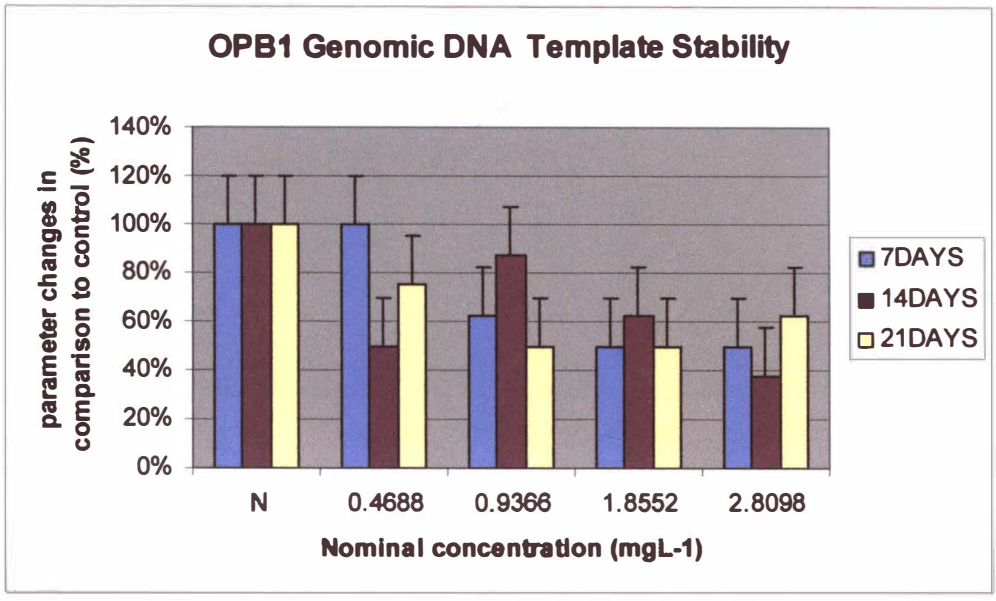


Fig.5.2. Comparison among genomic template stability using OPB1 in population of *O.niloticus* exposed to various Cd^{2+} concentrations. The different times represent the length of time (days). Error bars represent standard deviation. All the measured parameters were significantly different from control values ($P<0.05$).

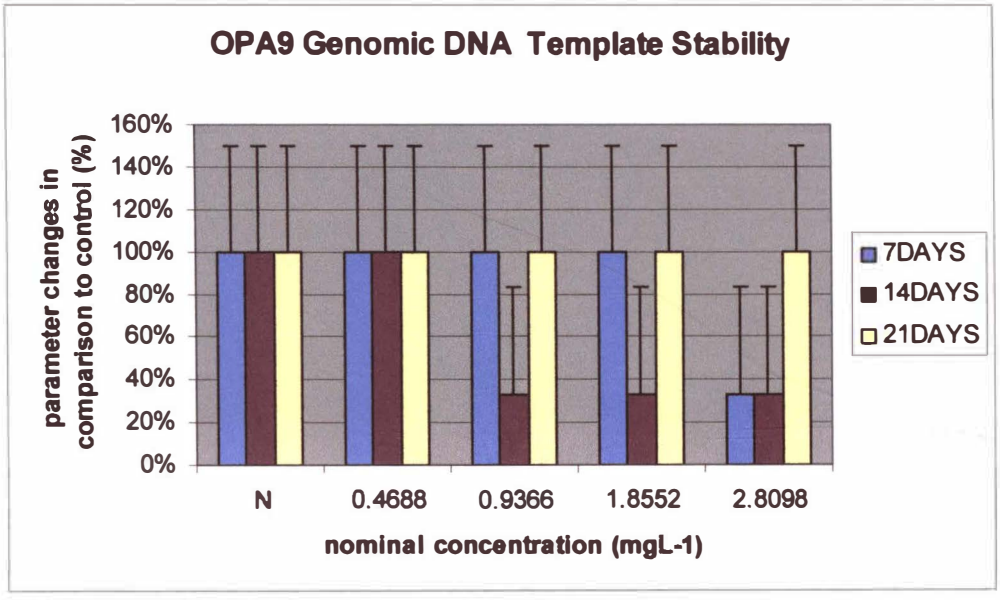


Fig.5.3. Comparison among genomic template stability using OPA9 in population of *O.niloticus* exposed to various Cd^{2+} concentrations. The different times represent the length of time (days). Error bars represent standard deviation. All the measured parameters were significantly different from control values ($P<0.05$).

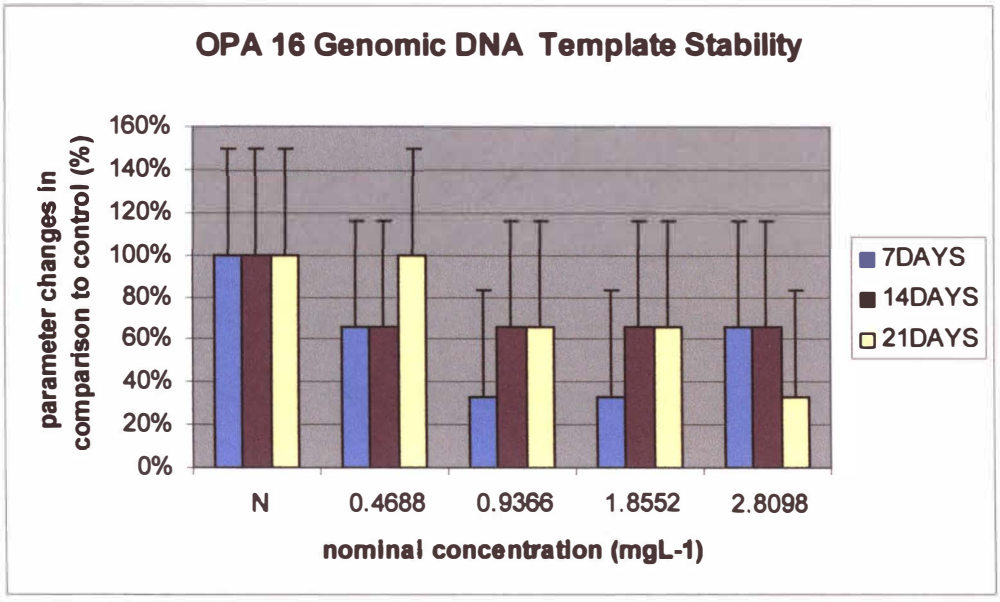


Fig.5.4. Comparison among genomic template stability using OPA 16 in population of *O.niloticus* exposed to various Cd^{2+} concentrations. The different times represent the length of time (days). Error bars represent standard deviation. All the measured parameters were significantly different from control values ($P<0.05$).

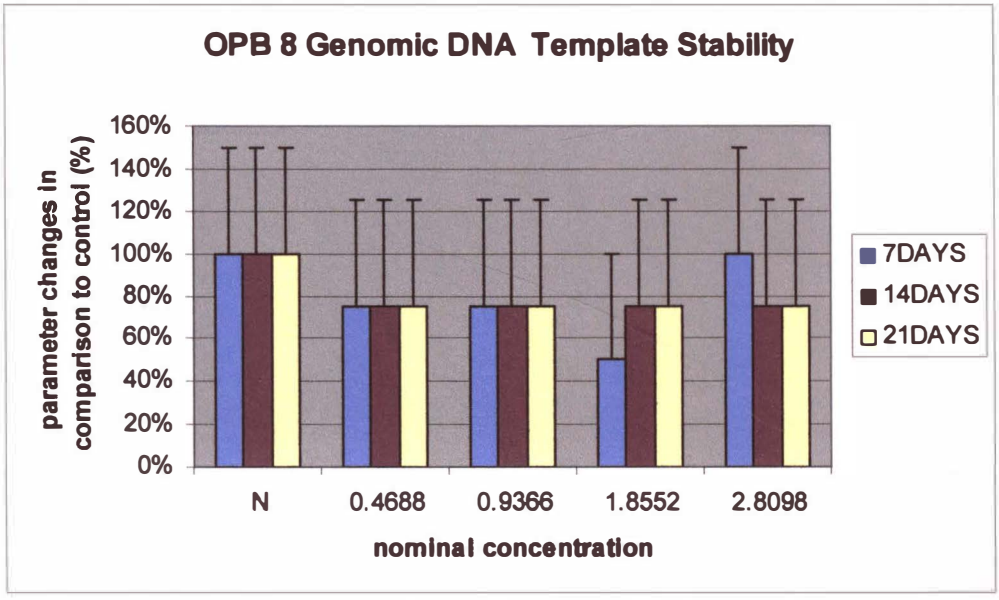


Fig.5.5. Comparison among genomic template stability using OPB8 in population of *O.niloticus* exposed to various Cd^{2+} concentrations. The different times represent the length of time (days). Error bars represent standard deviation. All the measured parameters were significantly different from control values ($P<0.05$).

In Fig.5.2., Fig.5.3., Fig.5.4. and Fig 5.5, the genomic template stability, a qualitative measure reflecting changes in RAPD patterns, was used to compare the modifications in RAPD profiles with the increase in Cd^{2+} concentration. The genomic template stability decreased after exposure to Cd^{2+} of various concentration, although some genomic seem to stabilize with increasing concentration while other remain unchanged (OPA 9, 21days). According to Atienzar *et al.*, (1999), the plateau effect is ascribed to multiple changes in RAPD profiles (appearance, disappearance of bands, etc.) which tend to counterbalance each other. In other words, for the highest Cadmium concentration, the high frequency of disappearing bands was compensated by the low frequency of newly appearing bands.

4.2.5 Dendogram

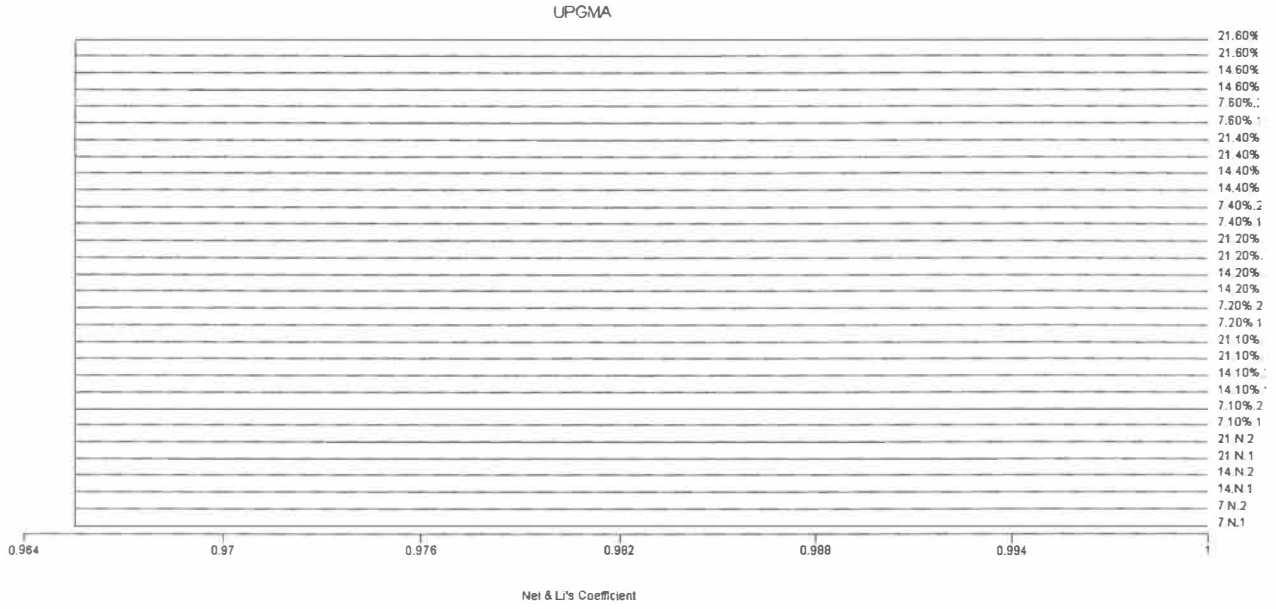


Fig.5.6. UPGMA (unweighted pair-group method using arithmetic averages) dendogram for tilapias exposed to various concentration of Cd^{2+} at various time intervals based on nie and li cluster analysis using OPB1.

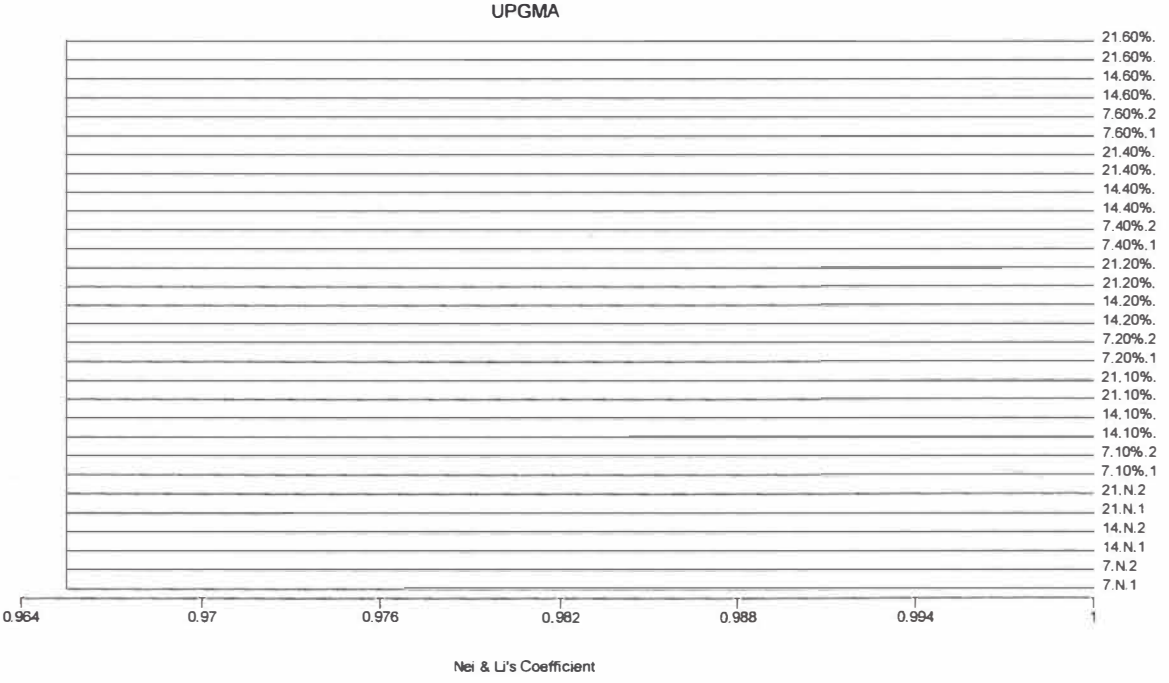


Fig.5.7. UPGMA (unweighted pair-group method using arithmetic averages) dendogram for tilapias exposed to various concentration of Cd^{2+} at various time intervals based on nie and li cluster analysis using OPA 9.

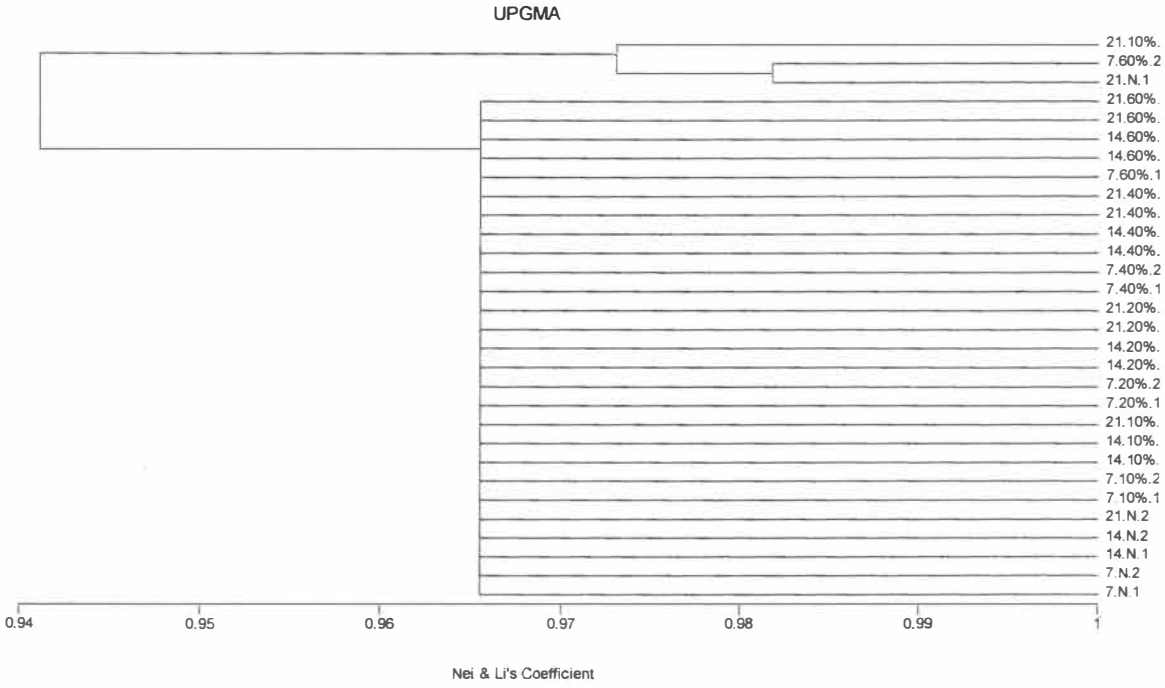


Fig.5.8. UPGMA (unweighted pair-group method using arithmetic averages) dendogram for tilapias exposed to various concentration of Cd^{2+} at various time intervals based on nie and li cluster analysis using OPA 16.

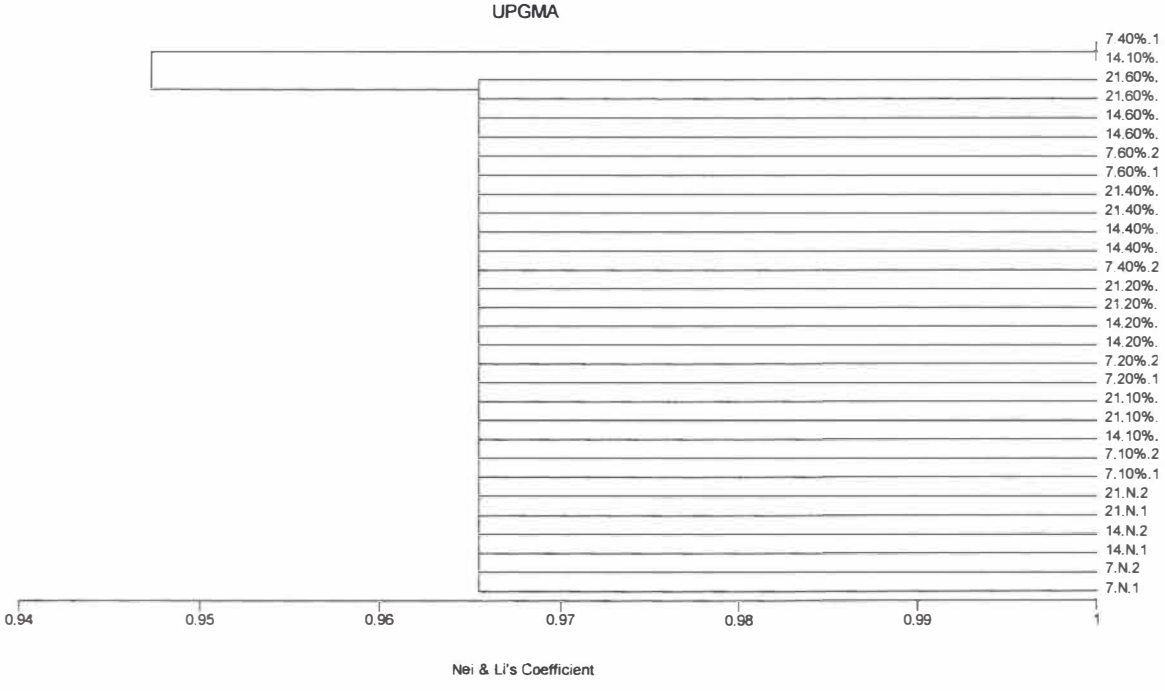


Fig.5.9. UPGMA (unweighted pair-group method using arithmetic averages) dendogram for tilapias exposed to various concentration of Cd^{2+} at various time intervals based on nie and li cluster anlaysis using OPB 8.

For OPA 9 (Fig 5.6.) and OPB 1 (Fig 5.7.), there were no multiple clusters formed. Instead, only one distinct cluster was formed for all the samples, with all samples showing similarity of 0.9666 at nei and li coefficient index.

For OPA 16 (Fig 5.8.), there were two distinct clusters dividing at nei and li similarity index of 0.941. This is the furthest similarity observed. All samples in first cluster showed similar similarity at 0.9666 nei and li similarity index. The second cluster however divided again in two distinct clusters at 0.973 and 0.982 nei and li coefficient index, which was also the nearest similarity observed among the samples.

For OPB 8 (Fig 5.9.), there were two main clusters which divided at 0.947 nei and li coefficient index. This was the furthest similarity value observed for the samples experimented using this primer. All the samples in the first cluster, like OPA 16, showed same nei and li coefficient similarity value of 0.966. However, another cluster formed showed nei and similarity index of 1.0, which was the nearest similarity observed for this primer.

4.3 Part C: Effects in the genome of exposed tilapia fingerlings by using Single Cell Gel Electrophoresis / Comet Assay

4.3.1 Microscopic Analysis

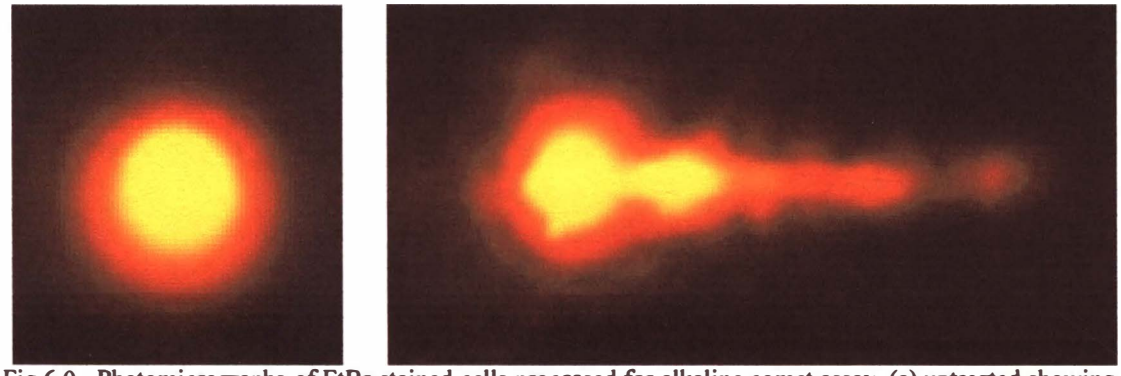


Fig.6.0 Photomicrographs of EtBr-stained cells processed for alkaline comet assay. (a) untreated showing no DNA damage, (b) treated showing DNA damage. Scoring was done using TriTek corp. CometScore Software.

4.3.2 Statistical Analysis

Table.4.7. Effect of various Cd^{2+} concentrations at various time intervals on the mean tail length.

	Tail Length (microns)		
Concentration (mg L^{-1})	7days	14days	21days
0.0000	1.07 ± 0.013	1.07 ± 0.013	1.06 ± 0.0008
0.4683	12.55 ± 0.015*	20.34 ± 0.073*	13.56 ± 0.017*
0.9366	4.010 ± 0.011*	5.650 ± 0.014*	20.42 ± 0.020*
1.8552	10.58 ± 0.017*	18.17 ± 0.032*	19.96 ± 0.016*
2.8098	11.35 ± 0.016*	11.65 ± 0.015*	16.97 ± 0.017*

*shows significant difference ($p<0.05$) when compared to control

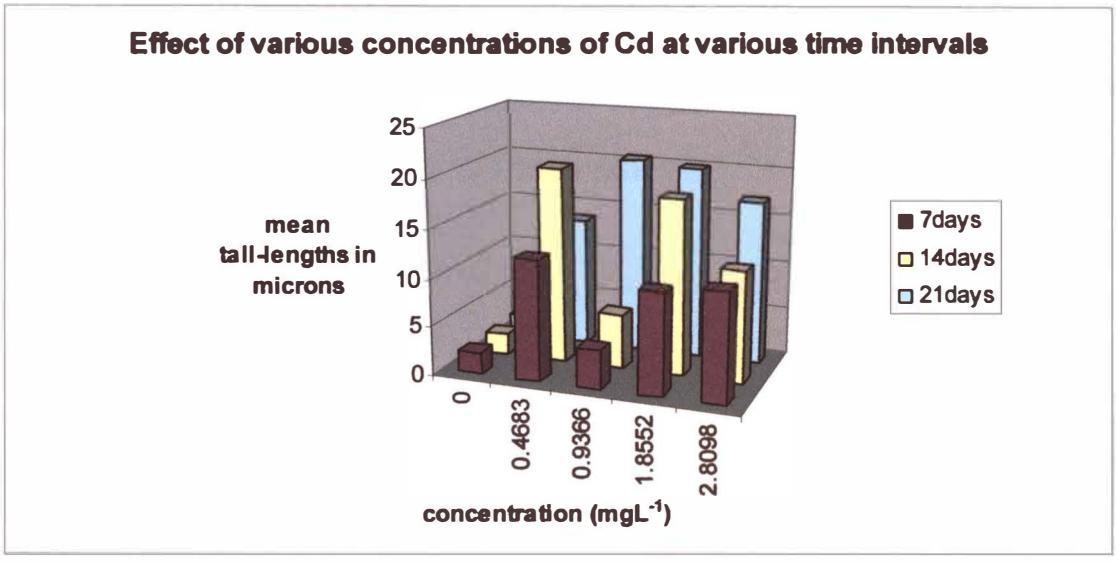


Fig.6.1. Effect of various Cd^{2+} concentrations at various time intervals on the mean tail length (microns).

The results obtained after treatment by Cadmium are summarized in Table 4.6 and Fig 6.1. The mean comet tail-length induced by Cadmium were significantly ($P<0.005$) higher than that of controls (0.00 mg L^{-1}) at all time. At day 7 and 14, the DNA damage was found to be decreasing from concentration 0.04683mgL^{-1} to 0.9366 mg L^{-1}. But at day 21, there is an increase in mean comet tail length between those same concentrations. Thereafter at week 1, there was and gradual increase in DNA damage from concentration 0.9366 mg L^{-1} to 2.8098 mg L^{-1} while day 21 displayed a gradual decreasing pattern for those same concentrations. Meanwhile, the DNA damage increased from concentration 0.9366 mg L^{-1} to 1.8522 mg L^{-1} and decreased at concentration 2.8098 mg L^{-1} for week 2. From 7 days onwards, an increase in mean tail lengths was observed in all the concentrations except for concentration 2 which showed a significant decrease from day 14 to day 21, indicating a repair of the Damaged DNA was occurring.

CHAPTER 5

DISCUSSION

Genotoxic affects of environment pollutant can threaten the survival of aquatic organisms by modifying their genetic ability for adaptation to variable environment condition (Castano and Becerril, 2004). Studies in natural population are associated with inherent methodological difficulties, particularly when evaluating chronic exposure situation (Castano and Becerril, 2004). Thus this study involves the studies of invitro toxic effect on the fish.

In the present study, metal toxicity of Cadmium was performed using Nile tilapia fingerlings (*Oreochromis niloticus*) to justify and identify the sublethal concentration (exposure to 30 days) used for the genotoxic study. It was found that by using the probit method (computer programme), the 96 hours median lethal concentration falls at 4.688 ppm (Table.4.2.).

The result was far more below than that was reported by Zirong and Shijun, (2006), whereby in their studies the 96 hours median lethal concentration for juvenile *O. niloticus* was 16.8 mg L^{-1}. Another study conducted by (Almeida *et al.*, 2000), using juvenile *O. niloticus*, determined the 96 h-LC_{50} as 18.58 mg L^{-1}.

The differences in the 96 h-LC_{50} of these literatures compared to present study can be attributed to the water quality and parameters involved specifically the alkalinity and water hardness. For instant, study by Zirong and Shijun was done under water condition which has water hardness and alkalinity of respectively 5 and 3 folds higher compared to present study. According to (Penttinen, 1995), the hardness cations (Mg and Ca) compete with heavy metal cations for binding sites within the organisms. These metal complexing agents may reduce the toxicity of heavy metals in water by reducing the number of free metal ions available to interact with organisms. Acidic water, with high concentrations of hydrogen ions, may also compete with metal cations for binding sites in the similar manner as magnesium and calcium in hard water (Penttinen, 1995). These literatures gave a possibility that the 96 hours median lethal concentration which was obtained from this study can be accepted.

In accordance to Canli *et al.*, (1997), present study shows that heavy metal concentrations in different tissues tended to vary significantly and the concentrations of Cadmium in some tissues exceeded the acceptable levels for a food source for human consumption.

Studies have shown that fish are able to accumulate and retain heavy metals from their environment and it has been shown that accumulation of metals in tissues of fish is dependent upon exposure concentration and duration, as well as other factors such as salinity, temperature, hardness, interacting agents, food, metabolism of the animals (Canli *et al.*, 1997), metallothioneins and other metal detoxifying proteins in the body of the

animal, bio-magnification and/or bio-diminishing of a particular metal, transport of metal across the membrane and the metabolic rate of the animal and physical and chemical properties of the water (Shah, 2003). Although factors such as temperature, salinity, DO, pH etc were maintained during the course of our study, metabolism and physiology of the fingerlings were beyond our control and the differences of it among the individuals might have lead to such results.

Besides that Sharif *et al.*, (1993), stated that the variations were most likely due the geographical locations of catch, season, nature of diet, and the size of fish used for analyses could lead to different metal concentration in the same fish species. Excluding the other factors, sizes of fishes (length) and weight of fishes analyzed did vary from one individual to another in present study (Appendies IV). This might explain the unclear concentration-accumulation relationship in each part of the fingerlings analyzed.

Compared to all time intervals, the highest recorded concentration for all four parts was observed on week 3 in the highest exposure concentration. Previous study by Camusso *et al.*, (1995) also found that two different experimental stations of the River Po showed different metal concentrations after 30 days, indicating stations contaminated heavily caused the fish to accumulate heavy metals in greater levels.

In contrast to study by Wu *et al.*, (2006), using *Oreochromis mossambicus*, present study however indicated that the mean Cadmium concentration was highest in muscle followed by viscera, gill and whole fingerling at the end of the 21 days of experiment. However,

the distribution of accumulated Cadmium in organs differs among studies (Cattani *et al.*, 1996). The inconsistencies among previous studies may be ascribed to differences in doses and exposure times of Cd (Wu *et al.*, 1999). However, a few papers have compared Cd accumulation transfer among organs following Cd exposure time.

Previous study by (Shah, 2003), on the other had stated that tissue-wise accumulation for Cd using tinca tinca is testes> gills> muscles> ovaries> liver. The order of metal accumulation in the tissues of this study was also supported by other studies: Dallinger and Kautzky, (1985) found that highest concentrations of Zn, Pb, Cd, Cr and Ni were observed in the gill of *Salmo gairdneri*, followed by the liver and muscle. They indicated that different concentrations of heavy metals in different fish species might be a result of different ecological needs, metabolism and feeding patterns. The different concentration in different tissues also indicates that metabolic activities of different fish species may be an important factor in metal accumulation (Canli *et al.*, 1997).

The present data showed that the highest content of Cd was found in tissue. To explain this first we need to understand the nature of the metal studied, the detoxifying agents involved and its degradation rate. According to Suzuki *et al.*, (1993), cadmium detoxification is metallothioneins' putative function, most likely carried out by its metal-free precursor, thionein. Metallothionein (MT) is still known as the only protein to collect appreciable amounts of Cd in vivo (Suzuki *et al.*, 1993). The discovery of metallothionein was the result of a search for biological role of cadmium about which surprisingly, even to this day nothing is known (Suzuki *et al.*, 1993). Being a non-

essential metal, not much biological function, including excretion route in muscle had been revealed of Cd. Few information is available on the turnover rates and degradative pathways of MT. However, earlier studies have suggested that the turnover rate of MT depends on the species of metal bound to it. Thus the turnover rate of Cu, Zn-MT is faster than Cd-MT ($t_{1/2}$ 15-20 hours Vs. 80 hours). Besides this, Suzuki *et al.*, (1993) also mentioned that the susceptibility of MT to proteolytic breakdown is regulated by metals contained in the structure of MT. According to them, CdMT is more resistant than ZnMT degradation in rat liver in vivo, which is consistant with the fact that Cd has a higher binding affinity for MT than does Zn in vitro. They suggested that metal stabilizes MT with respect to intracellular degradation and that the extent of stabilization is inversely related to the dissociation of bound metals. Thus we suggest that the low degradation rate of Cd-MT together with inefficient excretion pathway of CdMT from muscle had lead to the high concentration of Cd in muscle in our study.

Next to tissue, Cd concentration was high in viscera followed by gills. According to (Pelogram *et al.*, 1995), intestine wall serves as a storage organ and possibly excretion route for heavy metals for tilapia. They also mentioned that metal accumulation in the intestinal wall might be a specific mechanism for tilapia, because in general in fish about 95% of the whole body Cu accumulation was allocated into the liver (Stagg and Shuttleworth, 1982). Absorbed cadmium is bound to plasma proteins and transported to the liver, from which it is slowly released and finally accumulated in the kidney. The kidney naturally accumulates cadmium at a concentration 10 times higher than the liver (Nogami *et al.*, 1999). According to (Pelogram *et al.*, 1995), perhaps an efficient

accumulation and excretion route via the intestine contributes to the metal tolerance of tilapia, which is higher than in other species studied. Besides, metals, various kinds of metabolites and yet other organic agents can all induce metallothionein in the liver (Suzuki *et al.*, 1993), a detoxification agent which might further support the tolerance of tilapia to significantly high concentration of Cd in viscera as found in our study.

According to (Wu, 2006), gills are a temporary target organ of Cd accumulation, and then Cd is transferred to digestive organs (such as the liver, kidneys, and intestines) via the circulatory system or the enterohepatic circulation. This might explain the higher concentration of Cd in stomach compared to gill.

The significantly high concentration in both viscera and gills is in consistent as reported (Brouwer *et al.*, 1984). According to them, Cd remains bound to MT in a steady state of biosynthesis and biodegradation for a period of 12 days after Cd exposure in the gills and digestive gland of blue crab. Study by (Roesijadi, 1992), who found that most of the Cd found in the gills and intestinal cells of aquatic animals were bound to MT explain the tolerance of tilapia to Cd throughout the experiment period although the Cd concentrations were significantly higher in those areas.

After suitable optimization of the PCR conditions (Atienzar *et al*, 2000), and the judicious choice of oligonucleotide primers for each species-specific DNA template, the RAPD assay performs well in terms of number of bands, product yield and clarity of the profiles. Although the generation of RAPD profiles has often been criticized as

unreliable, reproducible DNA profiles have been generated from a range of aquatic invertebrates, plants and bacteria species (Atienzar *et al.*, 2000) and successfully used to detect genotoxin induced DNA damage (Atienzar *et al.*, 1999).

Previous studies have shown that changes in band patterns observed in DNA fingerprint analyses reflect DNA alterations from single base changes (point mutation) to complex chromosomal rearrangement (Atienzar *et al.*, 2000). Similarly in present study, DNA damage induced by Cd was reflected by the changes in RAPD profiles, variation in band intensity, disappearance of bands, and appearance of new PCR products occurred in the profiles generated by exposed organisms (Fig.5.2., Fig.5.4. and Fig 5.5).

After exposure to the Cd, the amplified products of genomic DNA reveal some differences from the fingerprinting pattern of normal *O.niloticus*. It is obvious that some stable bands that appear in all normal *O.niloticus* are lost (See Appendices V-VIII). With the increased concentration of Cd, the number of stable bands disappearing is enhanced, too. In other words, the genotoxical effect of the Cd exposed is promoted by concentration .

Each individual is genetically unique. Most DNA varies little between individuals and the difference represents the characteristics of each specimen. But some of the existing variation may result in genetic diseases. As mentioned just before, the fingerprinting of normal tilapia is not the same, but the familiar RAPD pattern expresses the general genomic features of tilapia. However, the exposure organisms to genotoxic chemical

pollutants can induce DNA damage. Mutations that inhibit primer binding or otherwise interfere with amplification can be detected as alterations of the pertinent bands in those individuals (Atienzar *et al.*, 2000), which can lead to the variation under DNA fingerprinting of control and exposed specimens can then be compared and analyzed statistically. In this study, tilapia exposed to Cd at 0.4688 mg L^{-1} - 2.8098mg L^{-1} lost the stable bands that are species specific to some extent (Appendices 4-7). This result means that the chemicals exposed caused damage to the genomic DNA.

Extra bands were also detected in RAPD profiles in present study (Fig.5.2., Fig.5.3., Fig.5.4. and Fig 5.5). New PCR amplification products may reveal a change in the DNA sequence due to mutation [resulting in (a) new annealing event(s), and/or large deletions (bringing two pre-existing annealing sites closer), and/or homologous recombination (juxtaposing two sequences that match the sequence of the primer) (Atienzar *et al.*, 1999).

Variation in band intensity was also observed (Fig.5.2., Fig.5.3., Fig.5.4. and Fig 5.5), where increase/decrease in band intensity was observed for certain bands. Both changes can be ascribed to the structural alterations induced by the DNA photoproducts, which influence the availability of *Taq* DNA polymerase and primers. Alternatively, the increase in band intensity could also be due to a better availability of 10-mer primers. Decrease in band intensity and possibly a disappearance of band (depending on the degree of DNA damage), can be attributed to the extent of DNA lesion, where *Taq* DNA polymerase is more often blocked (Atienzar *et al.*, 1999).

Genomic template stability is related to the level of DNA damage, the efficiency of DNA repair and replication. Therefore, a high level of DNA damage does not necessarily decrease the genomic template stability (in comparison to low level of DNA alterations) because DNA repair and replication are inhibited by the high frequency of DNA damage (Atienzar *et al.*, 1999).

Present study shows that all samples from OPB 1(Fig. 5.6.) and OPA 9 (Fig. 5.7.) show diversity of 0.034 at nei and li coefficient index. The nearest diversity among samples of OPA 16 (Fig. 5.8.), on the other hand, was 0.059 wherelse the furthest diversity for this primer was 0.018. For OPB 8 (Fig. 5.9.), the ranges of diversity was noticed to be in the range of 0.034 – 0.053. Only two samples tested with this primer shows no diversity whatsoever. The appearance and disappearance of the bands in RAPD profiles is reflected in the dendogram. The dendogram shows that the samples treated at different concentrations at different time intervals do show diversity among them to some extent.

Metal accumulation causes oxidative stress in fish by directly increasing the cellular concentration of highly reactive oxygen species (ROS) and by reducing the cellular antioxidant capacity (Pinto *et al.*, 2003). Hydrogen peroxide, (H_2O_2), a non-radical reactive oxygen species, for instant, can penetrate through all biological membranes and directly inactivate few enzymes (Sanchez *et al.*, 2005). This oxidative damage might had caused the significant damage in nucleus in all samples (Table 4.6).

Cadmium is also found to covalently bind with adenine, guanine, AMP and dGMP forming labile adducts which dissociate in the HPLC mobile phase. The changes in retention time for adenine and adenine nucleotides suggest a structural change introduced in the nucleobase or the nucleotide as a result of interaction with Cd. It is believed that the interaction with Cd causes a shift in tautomeric equilibrium of adenine and adenine nucleotides followed by a more permanent change. The shift in tautomeric equilibrium that can result to mispairing, eventually leading to the mutation of DNA (Zahed, 2002). These interactions may relate to the mechanism by which Cd may be capable of inducing single strand breaks, hence leading to significant damage at nucleus level as found by present study.

Present study shows that significant increase in mean tail-lengths clearly gives evidence that Cadmium causes DNA damage effectively (Table 4.6). However, DNA single strand breaks showed no clear concentrations-response between the DNA damage and different concentrations of Cadmium. The mean tail-length did not show a concentration–related increase and the elevation in the mean tail-length was of a fluctuating type.

It has been reported that the increase in the frequency of micronuclei was of a fluctuating type after treatment of mice with various doses (Jagetia and Aruna, 1998). Studies have shown that the total mass of Pb/mg of cell protein was dependent upon, but not directly proportional to the Pb concentration of the medium (Cory Slechta *et al.*, 1985). Experiments have been carried out on Pb $(NO^3)_2$ uptake from ligated gut loops in adult mice which have demonstrated a maximum absolute rate of Pb transfer across the gut

wall which was independent of dose and suggested that the mechanism responsible for Pb absorption might be saturable, if large single doses were administered (Barltrop and Khor, 1975). Bioavailability of lead could be the possible reason for the fluctuating results observed in our study (Jamil *et al.*, 2000).

Pb has been reported to inhibit the enzymes involved in repair and thus leading to the alteration of DNA replication and repair mechanisms (Popenoe and Schmaeler, 1979). The above reports correlate with our results. All samples treated with Cd showed a significant increase in comet tail-length with a mean length of 15.555+4.020 microns compared to control at all time. However, concentration 0.9366 ppm of day 7 and 14 showed a significantly lower damage compared to other samples. In fact the value for these concentrations at that specified days decreased suddenly compared to their preceding concentration. This difference can be attributed to more efficient DNA repairing capacity and lower levels of free oxygen radicals in the harvested fries compared to the rest (Jamil *et al.*, 2000).

From day 7 onwards, increase in the mean comet tail length was observed for all concentrations except concentration 0.4683 ppm which increased from day 7 to 14 and eventually decreased on day 21 (Table.4.6.). This shows that cells of fries in concentration 0.4683 ppm started to adopt defense strategies on the second week to counteract the damaging effects of free radicals (Jamil *et al.*, 2000).

Studies from the literature suggested that some of the toxic effects of Cd may result from alterations in cellular glutathione (GSH) metabolism (Zirong and Shijun, 2006). Glutathione (GSH) system is one of the most important antioxidant systems in fish and is regarded as the first line of defense against oxidative stress (Zirong and Shijun, 2006). According to Zirong and Shijun, The GSSG–GSH ratio, a potential indicator for oxidative stress, increased with the exposure time to Cd. It is reported that the presence of Cd in the body activates glutathione (GSH) peroxidase to metabolise ROS and DNA repairing enzymes to repair the damages caused to DNA (Stohs and Ragchi, 1995). The antioxidant role of GSH in cells relies on its concentrations, rates of turnover and rates of synthesis (Zirong and Shijun, 2006). High concentrations of Cd might accumulate in the body and consume longer time periods to get excreted when compared to low doses (Choie and Richter, 1972). This could be a possible reason for the delayed repair in the higher concentrations compared to concentration 0.4683 ppm which started repairing process in the second week. Interaction of Cd with glutathione peroxidase could be the possible reason for observing gradual repair in the DNA damage caused by Cd in present study.

CHAPTER 6

CONCLUSION

Cadmium is a compound which has no known vital role in the biology of T.Niloticus and is proven toxic by many literatures. However, Present study shows that Cadmium has a potential to accumulate in *T. niloticus* at a level higher than that of median lethal concentration for this species when exposed to longer period of time and that the level exceeds the safety limit for consumption by many folds. These results indicate that the Nile tilapias are relatively tolerant to Cadmium in comparison to other species. Hence, the Cadmium concentrations in aquatic organisms, especially those which are of food source to us, should be monitored.

Present study also revealed that DNA polymorphism detected using RAPD analysis in present study also could be used as an investigation tool for environmental toxicology and as a useful biomarker assay that can be used as an early warning system. Furthermore, the present study suggests that the RAPD assay applied in conjunction with other biomarkers from higher levels of biological organization would prove a powerful ecotoxicological tool.

Present study also conclude that the comet assay is a sensitive, rapid and economic technique for the detection of Strand Breakage, which is ideally suited as a non-specific biomarker of genotoxicity in fish and other aquatic species.

Present study also suggests that there is a need for more comprehensive field studies supported by adequate information on heavy metal exposures. There is also a potential to exploit the assays (both RAPD and COMET) for more detailed cell-specific effects and detection of specific fragments of DNA targeted by Cadmium.

Results from RAPD fingerprinting and Comet Assay also shows that Cadmium caused damage occurs at genomic and cellular level at certain extent. Therefore, from present studies it can be concluded that Cd is a potential genotoxic agent, capable of inducing DNA damage as revealed by comet assay. Hence, this metal should be regarded with more concern.

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APPENDIXES I

Replicate 1

CT-TOX: BINOMIAL, MOVING AVERAGE, PROBIT, AND SPEARMAN METHODS

SPEARMAN-KARBER

TRIM: 30.00%
LC50: 4.375
95% LOWER CONFIDENCE: 3.108
95% UPPER CONFIDENCE: 6.158

CONC. ppm	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(%)
1.60	10.	2.	20.00	.5469D+01
3.20	10.	0.	.00	.9766D-01
4.00	10.	5.	50.00	.6230D+02
6.40	10.	7.	70.00	.1719D+02
8.00	10.	7.	70.00	.1719D+02

THE BINOMIAL TEST SHOWS THAT .00 AND +INFINITY CAN BE USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT CONFIDENCE LIMITS SINCE THE ACTUAL CONFIDENCE LEVEL ASSOCIATED WITH THESE LIMITS IS 100.0000 PERCENT.
AN APPROXIMATE LC50 FOR THIS DATA SET IS 4.000

RESULTS USING MOVING AVERAGE

SPAN	G	LC50	95% CONFIDENCE LIMIT	
4	.412	5.32	3.74	10.36

****** RESULTS CALCULATED BY PROBIT METHOD

ITERATIONS	G	H	GOODNESS OF FIT
6	.428	1.00	.10

SLOPE = 2.60
95% CONFIDENCE LIMITS: .90 AND 4.30

LC50= 5.01
95% CONFIDENCE LIMITS: 3.48 AND 8.84

LC1 = .64
95% CONFIDENCE LIMITS: .02 AND 1.43

DATE: 2nd September 2006 TEST NUMBER: 1 DURATION: 96 hours
SAMPLE: Replicate 1 SPECIES: Oreochromis niloticus

METHOD	LC50	CONFIDENCE LIMITS LOWER	UPPER	SPAN
BINOMIAL	4.000	.000	*******	*******
MAA	5.316	3.741	10.364	6.623
PROBIT	5.014	3.482	8.839	5.357
SPEARMAN	4.375	3.108	6.158	3.049

NOTE: MORTALITY PROPORTIONS WERE NOT MONOTONICALLY INCREASING.
ADJUSTMENTS WERE MADE PRIOR TO SPEARMAN-KARBER ESTIMATION.

**** = LIMIT DOES NOT EXIST

APPENDICES II

Replicate 2

CT-TOX: BINOMIAL, MOVING AVERAGE, PROBIT, AND SPEARMAN METHODS

SPEARMAN-KARBER

TRIM: 10.00%
LC50: 5.456
95% LOWER CONFIDENCE: 4.528
95% UPPER CONFIDENCE: 6.575

CONC. ppm	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(%)
1.60	10.	1.	10.00	.1074D+01
3.20	10.	1.	10.00	.1074D+01
4.00	10.	3.	30.00	.1719D+02
6.40	10.	5.	50.00	.6230D+02
8.00	10.	10.	100.00	.9766D-01

THE BINOMIAL TEST SHOWS THAT 3.20 AND 8.00 CAN BE USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT CONFIDENCE LIMITS SINCE THE ACTUAL CONFIDENCE LEVEL ASSOCIATED WITH THESE LIMITS IS 98.8281 PERCENT.
AN APPROXIMATE LC50 FOR THIS DATA SET IS 6.400

RESULTS USING MOVING AVERAGE

SPAN	G	LC50	95% CONFIDENCE LIMIT	
4	.130	4.80	3.95	6.04

****** RESULTS CALCULATED BY PROBIT METHOD

ITERATIONS	G	H	GOODNESS OF FIT
5	.286	1.00	.06

SLOPE = 4.01
95% CONFIDENCE LIMITS: 1.87 AND 6.15

LC50= 5.02
95% CONFIDENCE LIMITS: 3.89 AND 6.79

LCI = 1.32
95% CONFIDENCE LIMITS: .29 AND 2.14

DATE: 2nd September 2006 TEST NUMBER: 2 DURATION: 96 hours
SAMPLE: Replicate 2 SPECIES: Oreochromis niloticus

METHOD	LC50	CONFIDENCE LIMITS LOWER	UPPER	SPAN
BINOMIAL	6.400	3.200	8.000	4.800
MAA	4.796	3.951	6.040	2.089
PROBIT	5.015	3.885	6.790	2.905
SPEARMAN	5.456	4.528	6.575	2.046

**** = LIMIT DOES NOT EXIST

APPENDICES III

Replicate 3

CT-TOX: BINOMIAL, MOVING AVERAGE, PROBIT, AND SPEARMAN METHODS

SPEARMAN-KARBER

TRIM: 20.00%
LC50: 4.622
95% LOWER CONFIDENCE: 3.867
95% UPPER CONFIDENCE: 5.526

CONC. ppm	NUMBER EXPOSED	NUMBER DEAD	PERCENT DEAD	BINOMIAL PROB.(%)
1.60	10.	2.	20.00	.5469D+01
3.20	10.	2.	20.00	.5469D+01
4.00	10.	3.	30.00	.1719D+02
6.40	10.	9.	90.00	.1074D+01
8.00	10.	9.	90.00	.1074D+01

THE BINOMIAL TEST SHOWS THAT .00 AND 6.40 CAN BE USED AS STATISTICALLY SOUND CONSERVATIVE 95 PERCENT CONFIDENCE LIMITS SINCE THE ACTUAL CONFIDENCE LEVEL ASSOCIATED WITH THESE LIMITS IS 98.9258 PERCENT.
AN APPROXIMATE LC50 FOR THIS DATA SET IS 4.632

RESULTS USING MOVING AVERAGE

SPAN	G	LC50	95% CONFIDENCE LIMIT	
4	.241	4.00	2.90	5.31

****** RESULTS CALCULATED BY PROBIT METHOD

ITERATIONS	G	H	GOODNESS OF FIT
6	.276	1.00	.11

SLOPE = 3.54
95% CONFIDENCE LIMITS: 1.68 AND 5.40

LC50= 4.04
95% CONFIDENCE LIMITS: 2.93 AND 5.36

LCI = .89
95% CONFIDENCE LIMITS: .15 AND 1.58

DATE: 2nd September 2006 TEST NUMBER: 3 DURATION: 96 hours
SAMPLE: Replicate 3 SPECIES: Oreochromis niloticus

METHOD	LC50	CONFIDENCE LIMITS LOWER	UPPER	SPAN
BINOMIAL	4.632	.000	6.400	6.400
MAA	3.998	2.901	5.307	2.407
PROBIT	4.036	2.933	5.364	2.431
SPEARMAN	4.622	3.867	5.526	1.659

**** = LIMIT DOES NOT EXIST

APPENDICES IV Total body length, length, Dry weight and wet weight of fish whole fish

	Concentration	Replicates	Total Body Length (cm)	Length (cm)	Wet weight (g)	Dry Weight (g)
7 days	10%	1	5.5	4.6	3.0621	0.5112
		2	6	5	4.2867	1.0116
		3	6.4	5	3.4302	0.6023
	20%	1	5.5	4.7	2.9501	0.4732
		2	6.3	5.2	2.9503	0.5363
		3	5.5	3.8	1.4354	0.2215
	40%	1	6	4.9	2.5565	0.3541
		2	5.2	4.4	2.7648	0.4001
		3	5.8	4.8	3.7522	0.7704
	60%	1	6.6	5.1	5.1812	1.2637
		2	6.8	5.3	5.3042	0.9813
		3	6.5	5.2	4.4076	0.8463

	Concentration	Replicates	Total Body Length (cm)	Length (cm)	Wet weight (g)	Dry Weight (g)
14 days	10%	1	5.3	4.4	2.4932	0.4021
		2	6.3	4.8	2.7412	0.3871
		3	4.3	3.5	0.9553	0.1572
	20%	1	5	3.8	1.8234	0.3214
		2	6	4.8	3.7432	0.6472
		3	5.3	4.2	2.8004	0.5621
	40%	1	5.6	4.4	2.156	0.4385
		2	4.1	3.4	1.051	0.1638
		3	5.5	4.8	2.1341	0.3529
	60%	1	5.9	4.5	2.4091	0.4156
		2	6.1	4.7	4.001	0.8802
		3	5.5	4.5	2.8516	0.3908

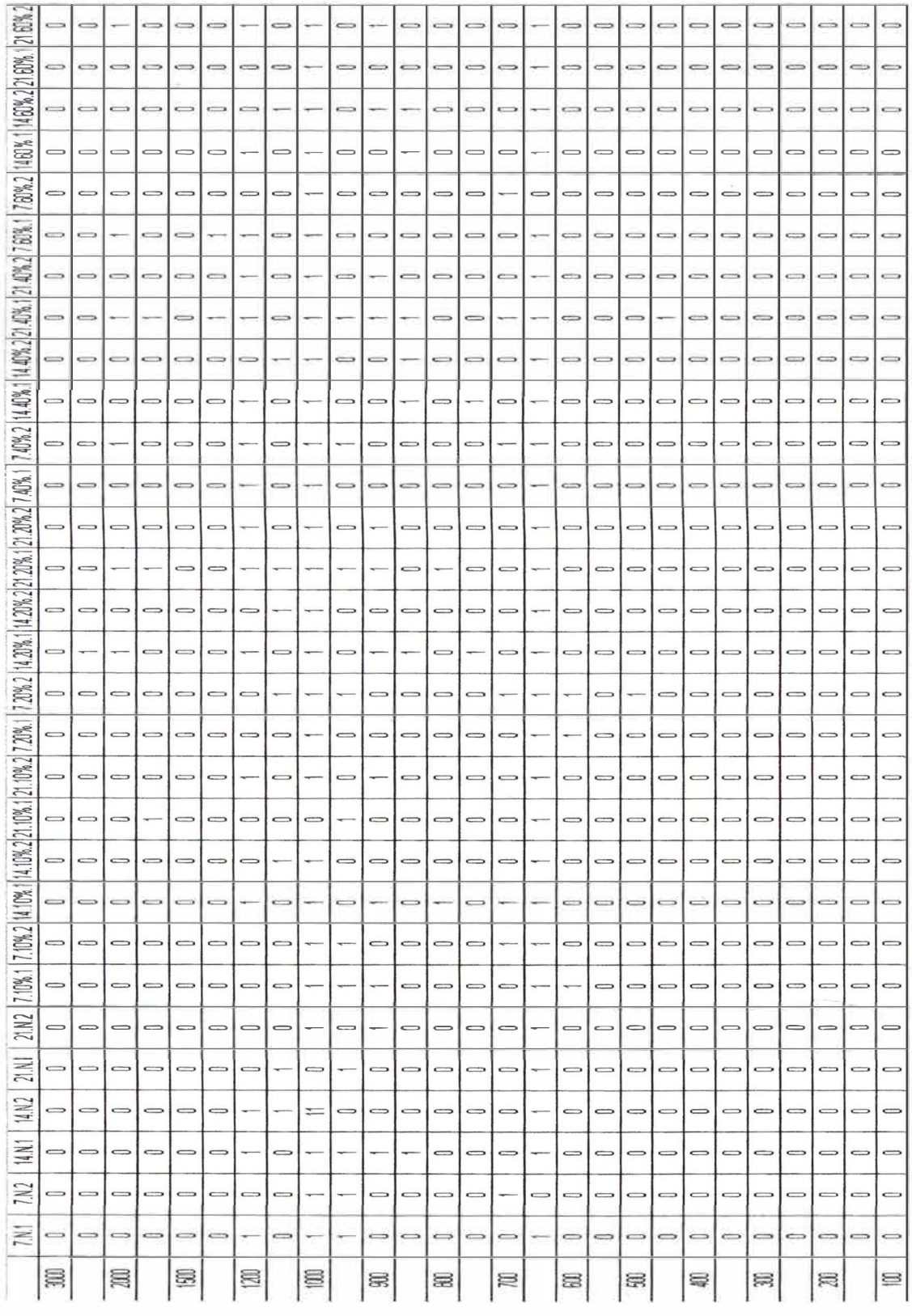
	Concentration	Replicates	Total Body Length (cm)	Length (cm)	Wet weight (g)	Dry Weight (g)
21 days	10%	1	7	5.5	4.0128	0.612
		2	6.2	4.8	3.6604	0.674
		3	5.6	4.4	2.2654	0.3673
	20%	1	6.2	5.2	4.6429	1.2983
		2	7.1	5.8	5.8024	0.8834
		3	7	5.3	0.5901	0.2573
	40%	1	6.4	5.5	4.9711	0.5567
		2	6.3	4.9	3.662	0.7138
		3	4.5	4	1.9539	0.2649
	60%	1	6.7	4.5	3.3301	0.4471
		2	6.1	4.7	3.4472	0.7699
		3	5.8	4.5	2.7211	0.5912

APPENDICES V

Binary Matrix for OPA 9

	7.N.1	7.N.2	14.N.1	14.N.2	21.N.1	21.N.2	7.10%.1	7.10%.2	14.10%.1	14.10%.2	21.10%.1	21.10%.2	7.20%.1	7.20%.2	14.20%.1	14.20%.2	21.20%.1	21.20%.2	7.40%.1	7.40%.2	14.40%.	14.40%.2	21.40%.1	21.40%.2	7.60%.1	7.60%.2	14.60%.1	14.60%.2	21.60%.1	21.60%.2
3000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2000	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1600	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0
1200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1000	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
900	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
800	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	0	0	0	1	0	1	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0
700	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
600	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
400	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	0	1	0	1	1	0	0	1	0
	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1
300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0
200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

APPENDICES VI
Binary Matrix for OPA 16

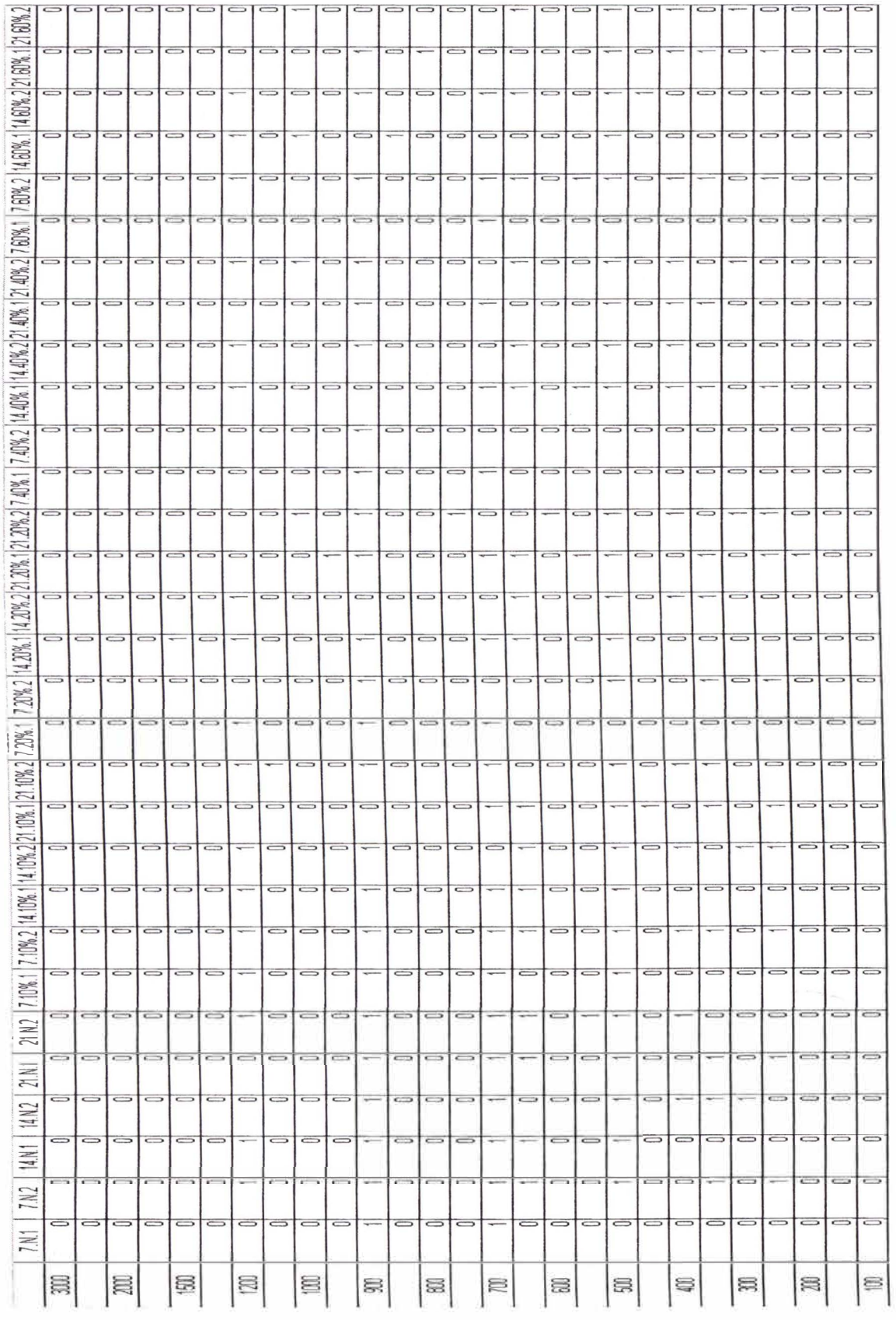


	7.N.1	7.N.2	14.N.1	14.N.2	21.N.1	21.N.2	7.10%.1	7.10%.2	14.10%.1	14.10%.2	21.10%.1	21.10%.2	7.20%.1	7.20%.2	14.20%.1	14.20%.2	21.20%.1	21.20%.2	7.40%.1	7.40%.2	14.40%.1	14.40%.2	21.40%.1	21.40%.2	7.60%.1	7.60%.2	14.60%.1	14.60%.2	21.60%.1	21.60%.2
3000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0	1	0	0	0	0	1
	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0
1500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1200	1	0	1	1	0	0	0	0	1	0	1	0	0	0	1	0	1	1	1	1	1	0	1	1	1	0	1	0	0	1
	0	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0	1	1	1	0	1	0	1	1	1	0	0	0	1	0
1000	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	0	1	1	0	1	1	0	1	1	0	1	0	0	1	0
900	0	0	1	0	0	1	1	0	1	0	0	1	0	0	1	0	1	1	0	0	1	1	1	0	0	0	0	1	0	1
	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
800	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
700	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0
	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
600	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
400	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

APPENDICES VII
Binary Matrix for OPB 1

bdsamole	7.N.1	7.N.2	14.N1	14.N2	21.N.1	21.N2	7.10%.1	7.10%.2	14.10%.1	14.10%.2	21.10%.1	21.10%.2	7.20%.1	7.20%.2	14.20%.1	14.20%.2	21.20%.1	21.20%.2	7.40%.1	7.40%.2	14.40%.1	14.40%.2	21.40%.1	21.40%.2	7.60%.1	7.60%.2	14.60%.1	14.60%.2	21.60%.1	21.60%.2
3000	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
2000	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	0	1	0	0	0	1	0	1	0	0	0	1	0	1	0	0	0	1	0	1	0	1	0	1	0	0	11	0	0
1500	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	0	1	0	1	0	0	1	1	1
	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	1	0	1	0	0	0	11	0	0	0	0	1	11	0
1200	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	1	0	1	0	1	0	1	0	1	0	1	1	11	1
	0	1	0	0	1	0	0	1	0	1	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	1	1	0	0
1000	0	0	1	0	1	0	0	0	1	1	1	0	1	1	1	0	1	0	1	0	1	0	1	0	1	0	1	1	0	1
	1	0	0	0	0	0	1	0	1	1	0	0	1	0	1	0	1	0	1	0	0	0	1	0	1	0	1	1	0	1
900	1	0	1	0	1	1	1	0	1	0	1	1	1	1	1	0	1	0	1	0	1	0	1	0	1	0	1	1	1	1
	0	1	1	1	1	0	1	1	0	1	1	1	1	0	1	0	0	0	1	0	1	0	1	0	0	0	1	1	1	1
800	1	1	1	0	0	1	1	1	1	0	1	1	0	1	1	0	1	0	1	0	1	0	1	0	0	0	0	1	1	1
	1	0	1	0	0	1	1	0	0	0	1	1	1	0	0	0	1	0	1	0	1	0	1	0	1	0	1	1	0	1
700	1	0	1	0	0	0	1	1	1	0	0	1	1	1	1	1	1	1	1	0	1	0	0	0	1	0	1	1	0	0
	1	0	0	1	1	1	1	0	11	0	1	11	1	0	1	0	11	1	0	0	1	0	11	11	1	0	1	1	1	1
600	0	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
	0	1	0	1	1	1	1	0	1	0	1	1	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1	1	1
500	0	0	0	1	1	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0	0	1	0	0	0	0	1	1	1	0
	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	1	1	0	0	0	1	0	1	0	1	0	1	0	0	1
400	1	0	1	1	0	1	1	0	1	0	0	0	1	1	1	0	1	0	1	1	1	1	1	1	0	0	1	1	0	1
	0	1	1	0	1	1	0	1	0	0	1	1	0	0	0	0	1	0	1	0	0	0	1	0	0	0	1	1	0	0
300	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

APPENDICES VIII
Binary Matrix for OPB 8



GILLS
NPar Tests

One-Sample Kolmogorov-Smirnov Test

		DAYS	CONC	GILLS
N		15	15	45
Normal Parameters[a,b]	Mean	2.0000	3.0000	.373864
	Std. Deviation	.8452	1.4639	.812013
Most Extreme Differences	Absolute	.215	.153	.160
	Positive	.215	.153	.160
	Negative	-.215	-.153	-.144
Kolmogorov-Smirnov Z		.833	.592	1.071
Asymp. Sig. (2-tailed)		.492	.875	.201

a. Test distribution is Normal.

b. Calculated from data.

Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: GILLS

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.749[a]	6	.125	10.053	.002
Intercept	4.208E-02	1	4.208E-02	3.390	.103
DAYS	4.996E-03	2	2.498E-03	.201	.822
CONC	.186	4	4.661E-02	3.755	.053
DAYS * CONC	.000	0	.	.	.
Error	9.931E-02	8	1.241E-02		
Total	.904	15			
Corrected Total	.848	14			

a. R Squared = .883 (Adjusted R Squared = .795)

Post Hoc Tests
DAYS

Multiple Comparisons

Dependent Variable: GILLS

Tukey HSD

(I) DAYS	(J) DAYS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-7.156E-02	7.05E-02	.588	-.272914	.129799
	3.00	-.441820*	7.05E-02	.001	-.643176	-.240463
2.00	1.00	7.156E-02	7.05E-02	.588	-.129799	.272914
	3.00	-.370262*	7.05E-02	.002	-.571619	-.168906
3.00	1.00	.441820*	7.05E-02	.001	.240463	.643176
	2.00	.370262*	7.05E-02	.002	.168906	.571619

Based on observed means.

*. The mean difference is significant at the .05 level.

CONC

Multiple Comparisons

Dependent Variable: GILLS

Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.000000	9.10E-02	1.000	-.314292	.314292
	3.00	.000000	9.10E-02	1.000	-.314292	.314292
	4.00	-.486373*	9.10E-02	.005	-.800665	-.172081
	5.00	-.418495*	9.10E-02	.011	-.732787	-.104203
2.00	1.00	.000000	9.10E-02	1.000	-.314292	.314292
	3.00	.000000	9.10E-02	1.000	-.314292	.314292
	4.00	-.486373*	9.10E-02	.005	-.800665	-.172081
	5.00	-.418495*	9.10E-02	.011	-.732787	-.104203
3.00	1.00	.000000	9.10E-02	1.000	-.314292	.314292
	2.00	.000000	9.10E-02	1.000	-.314292	.314292
	4.00	-.486373*	9.10E-02	.005	-.800665	-.172081
	5.00	-.418495*	9.10E-02	.011	-.732787	-.104203
4.00	1.00	.486373*	9.10E-02	.005	.172081	.800665
	2.00	.486373*	9.10E-02	.005	.172081	.800665
	3.00	.486373*	9.10E-02	.005	.172081	.800665
	5.00	6.788E-02	9.10E-02	.939	-.246414	.382170
5.00	1.00	.418495*	9.10E-02	.011	.104203	.732787
	2.00	.418495*	9.10E-02	.011	.104203	.732787
	3.00	.418495*	9.10E-02	.011	.104203	.732787
	4.00	-6.788E-02	9.10E-02	.939	-.382170	.246414

Based on observed means.

*. The mean difference is significant at the .05 level.

APPENDICES X

VISCERA

NPar Tests

One-Sample Kolmogorov-Smirnov Test

		DAYS	CONC	STOMACH
N		15	15	45
Normal Parameters[a,b]	Mean	2.0000	3.0000	.810366
	Std. Deviation	.8452	1.4639	.791027
Most Extreme Differences	Absolute	.215	.153	.123
	Positive	.215	.153	.123
	Negative	-.215	-.153	-.094
Kolmogorov-Smirnov Z		.833	.592	.827
Asymp. Sig. (2-tailed)		.492	.875	.501

a. Test distribution is Normal.

b. Calculated from data.

Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: STOMACH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.616[a]	6	.436	14.461	.001
Intercept	.558	1	.558	18.513	.003
DAYS	7.384E-02	2	3.692E-02	1.225	.344
CONC	.571	4	.143	4.733	.030
DAYS * CONC	.000	0	.	.	.
Error	.241	8	3.015E-02		
Total	3.402	15			
Corrected Total	2.857	14			

a. R Squared = .916 (Adjusted R Squared = .852)

Post Hoc Tests
DAYS

Multiple Comparisons

Dependent Variable: STOMACH

Tukey HSD

(I) DAYS	(J) DAYS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-6.688E-02	.109822	.819	-.380693	.246933
	3.00	-.814625*	.109822	.000	-1.128439	-.500812
2.00	1.00	6.688E-02	.109822	.819	-.246933	.380693
	3.00	-.747746*	.109822	.000	-1.061559	-.433932
3.00	1.00	.814625*	.109822	.000	.500812	1.128439
	2.00	.747746*	.109822	.000	.433932	1.061559

Based on observed means.

*. The mean difference is significant at the .05 level.

CONC

Multiple Comparisons

Dependent Variable: STOMACH

Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.000000	.141780	1.000	-.489823	.489823
	3.00	.000000	.141780	1.000	-.489823	.489823
	4.00	-.824924*	.141780	.003	-1.314747	-.335101
	5.00	-.855335*	.141780	.002	-1.345158	-.365512
2.00	1.00	.000000	.141780	1.000	-.489823	.489823
	3.00	.000000	.141780	1.000	-.489823	.489823
	4.00	-.824924*	.141780	.003	-1.314747	-.335101
	5.00	-.855335*	.141780	.002	-1.345158	-.365512
3.00	1.00	.000000	.141780	1.000	-.489823	.489823
	2.00	.000000	.141780	1.000	-.489823	.489823
	4.00	-.824924*	.141780	.003	-1.314747	-.335101
	5.00	-.855335*	.141780	.002	-1.345158	-.365512
4.00	1.00	.824924*	.141780	.003	.335101	1.314747
	2.00	.824924*	.141780	.003	.335101	1.314747
	3.00	.824924*	.141780	.003	.335101	1.314747
	5.00	-3.041E-02	.141780	.999	-.520234	.459412
5.00	1.00	.855335*	.141780	.002	.365512	1.345158
	2.00	.855335*	.141780	.002	.365512	1.345158
	3.00	.855335*	.141780	.002	.365512	1.345158
	4.00	3.041E-02	.141780	.999	-.459412	.520234

Based on observed means.

*. The mean difference is significant at the .05 level.

MUSCLE TISSUE

NPar Tests

One-Sample Kolmogorov-Smirnov Test

		DAYS	CONC	TISSUE
N		15	15	45
Normal Parameters[a,b]	Mean	2.0000	3.0000	-3.4E-02
	Std. Deviation	.8452	1.4639	1.720999
Most Extreme Differences	Absolute	.215	.153	.241
	Positive	.215	.153	.158
	Negative	-.215	-.153	-.241
Kolmogorov-Smirnov Z		.833	.592	1.619
Asymp. Sig. (2-tailed)		.492	.875	.011

a. Test distribution is Normal.

b. Calculated from data.

Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: TISSUE

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	40.914[a]	6	6.819	216.616	.000
Intercept	36.129	1	36.129	1147.684	.000
DAYS	.759	2	.379	12.055	.004
CONC	15.109	4	3.777	119.990	.000
DAYS * CONC	.000	0	.	.	.
Error	.252	8	3.148E-02		
Total	83.176	15			
Corrected Total	41.166	14			

a. R Squared = .994 (Adjusted R Squared = .989)

Post Hoc Tests
DAYS

Multiple Comparisons

Dependent Variable: TISSUE

Tukey HSD

(I) DAYS	(J) DAYS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-.866867*	.112214	.000	-1.187514	-.546221
	3.00	-3.112606*	.112214	.000	-3.433252	-2.791960
2.00	1.00	.866867*	.112214	.000	.546221	1.187514
	3.00	-2.245739*	.112214	.000	-2.566385	-1.925092
3.00	1.00	3.112606*	.112214	.000	2.791960	3.433252
	2.00	2.245739*	.112214	.000	1.925092	2.566385

Based on observed means.

*. The mean difference is significant at the .05 level.

CONC

Multiple Comparisons

Dependent Variable: TISSUE

Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.000000	.144867	1.000	-.500489	.500489
	3.00	.000000	.144867	1.000	-.500489	.500489
	4.00	-3.623004*	.144867	.000	-4.123492	-3.122515
	5.00	-3.009452*	.144867	.000	-3.509941	-2.508963
2.00	1.00	.000000	.144867	1.000	-.500489	.500489
	3.00	.000000	.144867	1.000	-.500489	.500489
	4.00	-3.623004*	.144867	.000	-4.123492	-3.122515
	5.00	-3.009452*	.144867	.000	-3.509941	-2.508963
3.00	1.00	.000000	.144867	1.000	-.500489	.500489
	2.00	.000000	.144867	1.000	-.500489	.500489
	4.00	-3.623004*	.144867	.000	-4.123492	-3.122515
	5.00	-3.009452*	.144867	.000	-3.509941	-2.508963
4.00	1.00	3.623004*	.144867	.000	3.122515	4.123492
	2.00	3.623004*	.144867	.000	3.122515	4.123492
	3.00	3.623004*	.144867	.000	3.122515	4.123492
	5.00	.613552*	.144867	.018	.113063	1.114040
5.00	1.00	3.009452*	.144867	.000	2.508963	3.509941
	2.00	3.009452*	.144867	.000	2.508963	3.509941
	3.00	3.009452*	.144867	.000	2.508963	3.509941
	4.00	-.613552*	.144867	.018	-1.114040	-.113063

Based on observed means.

*. The mean difference is significant at the .05 level.

WHOLE

NPar Tests

One-Sample Kolmogorov-Smirnov Test

		DAYS	CONC	WHOLE
N		15	15	45
Normal Parameters[a,b]	Mean	2.0000	3.0000	-1.233358
	Std. Deviation	.8452	1.4639	.726178
Most Extreme Differences	Absolute	.215	.153	.189
	Positive	.215	.153	.137
	Negative	-.215	-.153	-.189
Kolmogorov-Smirnov Z		.833	.592	1.269
Asymp. Sig. (2-tailed)		.492	.875	.080

a. Test distribution is Normal.

b. Calculated from data.

Univariate Analysis of Variance

Tests of Between-Subjects Effects

Dependent Variable: WHOLE

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.850[a]	6	1.142	56.494	.000
Intercept	25.309	1	25.309	1252.482	.000
DAYS	3.152E-02	2	1.576E-02	.780	.490
CONC	1.495	4	.374	18.492	.000
DAYS * CONC	.000	0	.	.	.
Error	.162	8	2.021E-02		
Total	33.735	15			
Corrected Total	7.011	14			

a. R Squared = .977 (Adjusted R Squared = .960)

Post Hoc Tests
DAYS

Multiple Comparisons

Dependent Variable: WHOLE

Tukey HSD

(I) DAYS	(J) DAYS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-.919437*	8.99E-02	.000	-1.176336	-.662538
	3.00	-1.445832*	8.99E-02	.000	-1.702731	-1.188933
2.00	1.00	.919437*	8.99E-02	.000	.662538	1.176336
	3.00	-.526395*	8.99E-02	.001	-.783294	-.269496
3.00	1.00	1.445832*	8.99E-02	.000	1.188933	1.702731
	2.00	.526395*	8.99E-02	.001	.269496	.783294

Based on observed means.

*. The mean difference is significant at the .05 level.

CONC

Multiple Comparisons

Dependent Variable: WHOLE

Tukey HSD

(I) CONC	(J) CONC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-1.067792*	.116066	.000	-1.468780	-.666805
	3.00	-1.345726*	.116066	.000	-1.746713	-.944738
	4.00	-1.744540*	.116066	.000	-2.145528	-1.343553
	5.00	-1.905861*	.116066	.000	-2.306848	-1.504873
2.00	1.00	1.067792*	.116066	.000	.666805	1.468780
	3.00	-.277934	.116066	.210	-.678921	.123054
	4.00	-.676748*	.116066	.003	-1.077736	-.275761
	5.00	-.838069*	.116066	.001	-1.239056	-.437081
3.00	1.00	1.345726*	.116066	.000	.944738	1.746713
	2.00	.277934	.116066	.210	-.123054	.678921
	4.00	-.398815	.116066	.051	-.799802	2.17285E-03
	5.00	-.560135*	.116066	.008	-.961123	-.159148
4.00	1.00	1.744540*	.116066	.000	1.343553	2.145528
	2.00	.676748*	.116066	.003	.275761	1.077736
	3.00	.398815	.116066	.051	-2.1728E-03	.799802
	5.00	-.161320	.116066	.650	-.562308	.239667
5.00	1.00	1.905861*	.116066	.000	1.504873	2.306848
	2.00	.838069*	.116066	.001	.437081	1.239056
	3.00	.560135*	.116066	.008	.159148	.961123
	4.00	.161320	.116066	.650	-.239667	.562308

Based on observed means.

*. The mean difference is significant at the .05 level.

CURRICULUM VITAE

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Education:

UPSR (1996) - Sekolah Rendah Jenis Kebangsaan Tamil Jalan Penjara, Kuantan.

PMR (1999) - Sekolah Menengah St. Thomas, Kuantan.

SPM (2001) - Sekolah Menengah St. Thomas, Kuantan.

STPM (2003) - Sekolah Menengah Abdul Rahman Talib, Kuantan.

Bachelor of Science in Marine Science (2004-2007) – University Malaysia Terengganu.

Awards:

Dean's list award (2005-2007)

In-vitro genotoxic effects of cadmium in tilapia fingerlings (Oreochromis niloticus) – Prem Kumar