GENOTOXIC EFFECTS OF SOME HEAVY METALS ON FROGS (AMPHIBIANS)

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It is certified that:

i. the thesis entitled “Genotoxic effects of some heavy metals on Frogs (Amphibians)” embodies the work of the candidate, Mrs. Preetpal Kour.

ii. the candidate has worked under my supervision for the period required under statutes.

iii. the candidate has put in the required attendance in the department during that period.

iv. the thesis being submitted is worthy of consideration for the award of Ph.D degree in Zoology and has not been submitted for any degree elsewhere.

v. the conduct and character of the candidate remained excellent during the period of research.

(Prof. Kuldeep K. Sharma)  (Prof. N.K. Tripathi)
Head of the Department  Supervisor
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Chapter 1

Introduction
The advancement of human civilization has largely depended upon the utilization of naturally occurring elements and the creation of new chemical compounds. Bronze brought tools and weapons to a new level, with iron and steel came technology and industrialization, and pesticides and fertilizers were dominant players in the green revolution. The benefits of such utilization were clearly seen and embraced for improving the human condition. However, as 16th century European physician Paracelsus observed, “All things are poison and not without poison; only the dose makes a thing not a poison” (Institute of Medicine, 2004). The Earth contains 92 natural elements, and populations of organisms have evolved to withstand or require the levels present in their habitats. But when concentrations increase due to human extraction, individuals and species are not always capable of tolerating the exposure. Many compounds were created to serve as poisons for particular organisms, but toxicity is rarely limited to targeted species. It was not until Rachel Carson’s book *Silent Spring* was published in 1962 that a large public audience was made aware of the potential for chemical contamination to have harmful and complex effects on the environment (Carson, 1962). The rich flora and fauna of the Earth were identified as innocent bystanders whose fate can be documented by their silence and absence. Following World War II, toxicology was expanded to include studies of toxic effects on non-human animals. A further progression
came in the form of ecotoxicology, which was coined in 1969 by Truhaut as an extension of toxicology that includes the effects of contaminants on populations and their habitats. Since then, there has been much research on the effects of contaminants on wildlife, particularly fishes and birds (Sparling et al., 2000). However, given the number of chemicals in the world today, the complexity of the environment, and the potential for interactions between chemicals and abiotic and biotic factors, the gap between what we know and what we should know is tremendous. In 1992, the UNCED meeting in Rio de Janeiro stated that a sustainable development was desired and that a precautionary principle should guide human activity. This statement is in addition to the concern for protecting individuals of endangered species as well as populations in ecosystems where humans are not present.

Animals, the silent sentinels, stand watch over the world's environmental health. Every day, animals demonstrate intricate connections between them, us and our surroundings. In 1993, O'Brien et al., defined animals as indicators or sentinels. Sentinels are "organisms whose known characteristics can be measured to assess the extent of environmental implications and to provide early warning of those implications." Amphibians, a unique group of animals found world-wide, are apparently being affected by direct and indirect human activities. Amphibians have received relatively little attention by the toxicology community, which may be due to the secretive nature of amphibians. However, amphibians are important components of trophic food webs, and in some ecosystems amphibian biomass equals or exceeds that of small mammals or birds (Burton and Likens, 1975). Also amphibians have their central place in the food chain (being prey, predators and herbivores) and can have different feeding ecologies at different stages of their life cycles making them important components of many ecosystems. Most adult frogs and toads feed on invertebrates, so they are important energy-trophic link between invertebrates and other vertebrates (Sparling et al., 2000). Due to their clear contribution to tropical dynamics, loss of amphibian populations will surely affect other organisms (Blaustein et al., 1994).

Amphibians can be important indicators of environmental contamination, because they are present in both aquatic and terrestrial environments. Aquatic and terrestrial habitat quality can determine the demographics and persistence of amphibian populations (Kucken et al., 1994; Rothermel, 2003), since they have permeable skin, eggs without shell (not amniotic eggs), and a complex life cycle that expose them to changes in both the aquatic and terrestrial environment (Blaustein and Wake, 1990; Blaustein et al., 1994; Blaustein, 2001). Furthermore in water quality criteria protective of fish may also protect amphibians, since in most cases fish are generally considered to be more sensitive to pollutants (Hall and
Swineford, 1980). Exceptions may occur in wetlands and ephemeral bodies of water where the use of a chemical would suggest a high degree of exposure to amphibians, especially where natural or constructed wetlands are used for waste treatment. Amphibians have been found in farm ponds, mining sites, monsoon feed pools and other contaminated aquatic sites. Amphibians (adults) are also unlikely to escape environmental degradation because they have high breeding-site fidelity and low mobility. Amphibians are often described as highly site-philopatric and demonstrate poor dispersal capabilities, even in natural environment (Sinsch, 1990; Blaustein et al., 1994). Frogs have a small home range (200 m² for Rana sp.; Loman, 1994) and they return every year to the same watershed or pond to breed (Duellman and Trueb, 1986). Moreover they are poikilothermic or exothermic animals and have thin skin consisting of a living cell layer for cutaneous respiration but makes them sensitive to contaminants.

The number of toxicological studies on amphibians has increased dramatically in the last fifteen years, a likely result of concern about global declines in their populations (Sparling et al., 2000). Amphibians appear to be disappearing at a higher rate than mammals or birds (Stuart et al., 2004). There are many proposed causes for the declines and it appears that there is no single culprit. Chemical contamination of habitats is one of the proposed causes (Wake, 1998) and is thought to cause deformities (Ouellet et al., 1997), intersex gonads and sex-ratio reversal (Reeder et al., 1998). Contaminants have also been implicated in the loss of amphibian abundance or diversity at some field sites (Beyer et al., 1985; Kucken et al., 1994; Lambert, 1997; Bishop et al., 1999). The majority of the literature on amphibian ecotoxicology has focused on early life stage responses to aquatic contamination. However, regardless of relative sensitivities, population viability depends on the success of all life stages. Assessments should be conducted on multiple life stages to increase the understanding of the effects of particular contaminants on amphibian species. Post-metamorphosis studies are particularly needed when metamorphic traits such as mass are affected, or when the contaminant bioaccumulates in amphibian tissue. Toxicology studies of the effects of specific contaminants on amphibian should therefore assess multiple life stages, both during and after exposure.

Chemical contaminants can adversely affect behavior, reproduction, growth, morphology, physiological processes, and organ function. Expected environmental concentrations cause sub lethal effects more often than they cause lethality. Finally, it is important to conduct tests using native species, especially when there is concern that
contamination is affecting a particular species in the wild. The majority of amphibian toxicology research has been on metals, non-chlorinated pesticides, and acidification (Sparling et al., 2000). Metals are of interest because they bioaccumulate and affect physiological processes such as enzyme function. Uptake may occur by oral, dermal, and pulmonary exposure. Metals are also persistent in the environment and unlike many pesticides, do not degrade or transform. Metals are among the most frequently cited pollutants present at contaminated sites and can cause habitat degradation and alteration. Considerable work has been done on metal toxicity and bioaccumulation with a vast array of amphibian species (Khangarot and Ray, 1987; Birge et al., 2000).

Amphibians are particularly sensitive to changes in the environment. Amphibians have been described as ‘biomarker species’ or the equivalent of “canaries of the coal mines” meaning they provide an important signal to the health of biodiversity; when they are stressed and struggling, biodiversity may be under pressure. When they are doing well, biodiversity is probably healthy. Unfortunately, amphibians are threatened and declining world-wide at an alarming rate representing the greatest mass extinction of land vertebrates since the dinosaurs and are therefore of global biodiversity concern (Collins and Storfer, 2003; Stuart et al., 2004). Wilbur and Collin (1973) described the ecological significance of amphibians. Amphibians are often referred as ‘farmer friends’ as they occupy the critical position in the ecological pyramids as they are the major predators of the insects and pests (Heyer et al., 1975). Decline in their population from a particular habitat can have drastic consequences by increasing the insects/pests population (Chakarbati, 1987). Presence of a good population of amphibians in a region is indication of healthy environment (Gururaja et al., 2008). Tadpoles eat algae and therefore act as a natural filtration system that keeps our drinking water clean whereas frogs eat mosquitoes, ticks and flies that carry vector-borne diseases such as malaria, West Nile virus, dengue fever, lyme disease and leishmaniasis. Also frogs serve as an important food source to a diverse array of predators, including dragonflies, fish, snakes, birds, centipedes and even monkeys. Disappearance of frog disturbs an intricate food web and results in negative impacts that cascade through the ecosystem. Frogs are exploited as food by humans, both for local consumption and commercially for export, with thousands of tons of frog legs harvested annually.

Frogs have served as experimental animal throughout the history of science in embryology, anatomy, physiology and cytogenetic studies. The skin secretions of various tropical anurans are known to have hallucinogenic effects and effects on the central
nervous and respiratory systems in humans. Some secretions have been found to contain magainin, a substance that provides a natural antibiotic effect. Other skin secretions, especially toxins, have potential use as anesthetics and painkillers. More than 73 amphibian species are known to have some kind of medicinal values. Because frog toxins are extraordinarily diverse, they have raised the interest of biochemists as a "natural pharmacy". The alkaloid epibatidine, a painkiller two hundred times more potent than morphine, is found in some species of poison dart frogs, a synthetic derivative of the peptide, named magainin from the skin of Xenopus laevis are drugs to treat ovarian cancer and malignant melanoma, the deadliest form of skin cancer. Other chemicals isolated from the skins of frogs may offer resistance to HIV infection. A sticky adhesive (frog glue: strong, flexible and sets in water) secreted from the skin of crucifix frog (Notaden bennetti) have unique property to be used as an adjunct to rotator cuff repair in humans. The frog's capability to survive frozen environment has drawn the attention of scientist involved in human organ transplantation. For the first time an amphibian (Xenopus tropicalis) genome has been sequenced, and scientists say it represents a big hop forward in understanding not just frogs but Earth's whole tree of life. This genome encodes more than 20,000 proteins coding genes, including orthologues of at least 1700 human disease genes. Approximately 10% of Nobel Prizes in Physiology and Medicine have resulted from investigations that used frogs. When a frog species disappears, so does any promise it holds for improving current and future human health.

There are growing evidences that the chemical contaminants are in some way responsible for the amphibians decline (Blaustien et al., 2003). The chemical stressors especially the heavy metals are known for their accumulation features and high toxicity at very low concentrations. The term heavy metal refers to any metallic chemical element that has relatively high density and is toxic or deleterious at even low concentrations. Metals and metallic compounds are natural constituents of all ecosystems, moving between the atmosphere, the hydrosphere, the lithosphere and the biosphere (Bargagli, 2000). These cannot be degraded and destroyed. Their distribution in the environment is a result of natural processes (volcanoes, erosion, spring water, bacterial activity) and anthropogenic activities (fossil fuel combustion, industrial and agricultural processes) (Florea et al., 2004). They are quite literally the pillars of all major past and present civilizations, on one hand and the other; they are the environmental pollutants of global concern owing to their non-biodegradability. Although, nature tries to treat, recycle and make good use of the pollutants that are dumped into it, it has its own limit.
Water bodies are the ultimate recipients of all the water soluble toxicants from agricultural and industrial source, municipal wastes and anthropogenic activities. Among the myriad of organic and inorganic substances released into the aquatic ecosystems, heavy metals have received considerable attention due to their toxicity and potential to bioaccumulate in aquatic species (Szefer et al., 1990). While some of the pollutants or toxicants decompose or volatilize others form insoluble salts get precipitated and incorporated into the sediments (Bowen, 1979). Uptake of such toxicants by aquatic organisms is usually followed by metabolism of toxicants into toxic derivatives (Webb, 1975).

Heavy metals, among important classes of pollutants, constitute a core group of aquatic pollutants (Vutukuru, 2005). According to Mason and Jenkins (1995), heavy metal pollution is one of the five major types of toxic pollutants commonly present in surface waters. Metals are regarded as “conservative pollutants” because they are either not broken down at all or take such a long period of time to degrade, that essentially become toxic (Wittmann, 1979; Rainbow, 1985). Intense mining operations and processing of the minerals, potential use of agrochemicals (fertilizers, pesticides, herbicides) to meet the ever increasing demands of escalating population has led to a pervasive release of metals in aquatic environment. Besides these operations, augmentation of heavy metal engineering and chemical industries together with increased heavy metals (Mn, Ni, Cr, Zn, As, Cd, Pb, Fe and Cu) and their salts by industries definitely pose a threat to fresh water bodies (Yousafzai, 2004). The quandary of environmental pollution due to toxic metals has therefore really become a cause of concern in most of the metropolitan cities today.

Cadmium is one of the PBTs (persistent, bioaccumulative and toxic) chemicals identified as a primary toxicant. It is a non essential element, with no known biological function, naturally found at low concentrations in natural waters (Viarengo, 1985). The metal is widely distributed in natural waters due to industrial discharge but it also occurs naturally in earth’s crust and is released with the natural weathering of rocks (DWAF, 1996). The metal is released into the air, land and water by human activities (Linder, 1985; WHO report, 1992) and very steep increases in contamination by this metal has been documented (Cabrera et al., 1998).

Anthropogenic activities such as mining, production and consumption of cadmium and non-ferrous metals have accelerated the rate of mobilization and distribution of cadmium from non bioavailable geological matrices into biologically accessible situations far in excess
of natural cycling process (Suru, 2008). It is a fairly common pollutant of aerosol fall-out from motor vehicles, lead mining and smelting operations, fossil fuel consumption, paint manufacture, electroplating plants, chemical industries and even sewage (McCarty and Shugart, 1990). It is also widely used in the production of nickel-cadmium batteries, as a stabilizer in PVC products, in metallic pipes, as a color pigment and an anti corrosive agent, in agriculture (phosphate fertilizers and pesticides), and as a neutron absorber in nuclear power plants. (Cuthbert et al., 1976; Ezemonye and Enuneku, 2012). Effluents from these activities are sources of cadmium in aquatic environments (Kaoud et al., 2011). These have predisposed animal and human populations to both subtle and direct exposure pathways with an attendant increase in cadmium related pathologies (Satarug and Moore, 2004) and guidelines have been established for cadmium levels in public water supplies and other designated uses (DWAF, 1996).

Cadmium occurs primarily in fresh waters as divalent forms including free cadmium (II) ion, cadmium chloride and cadmium carbonate (DWAF, 1996). Cadmium does not break down in the environment but can change form. Adult frogs can acquire cadmium through their skin or orally by consumption and respiration. Once absorbed, it can be found in numerous amphibian tissues especially the liver, intestine, kidney, gonads, placenta, brain and bones (Ezemonye and Enuneku, 2011a,b,c; Sobha et al., 2007). Sources of human exposure to cadmium include food (when sewage sludge used as a food-crop fertilizer), cigarette smoke and alcoholic beverages (Jarup et al., 1998). Cadmium can remain in the body for long period of time and can bioaccumulate for many years after exposure to low levels of this metal. Cadmium has been shown to stimulate free radical production, deplete antioxidant levels resulting in oxidative deterioration of lipids, proteins and DNA and initiating various pathological conditions in animals and humans (Sarkar et al., 1997; Shaikh et al., 1999). It promotes an early oxidative stress and afterward contributes to the development of serious pathological conditions because of its long retention in some tissues (Bagchi et al., 2000). Cadmium may cause the deterioration of cell membranes by binding to metallothionein or glutathione and consequently interfere with the ability of these proteins to avoid oxidative stress. Cadmium can also replace essential metals such as copper and zinc in several metalloproteins, altering the protein conformation and affecting their activity because this element interacts ubiquitously with sulphydryl groups of amino acids, proteins and enzymes (Serafim and Bebianno, 2007). Thus, the toxic effects of cadmium are related to changes in natural physiological and biochemical processes in organisms. Cadmium is defined by the United States Environmental Protection Agency (USEPA) as potential hazards
to most forms of life and is considered to be relatively accessible to aquatic organisms (DWAF, 1996). The symptoms associated with Cd poisoning are diarrhea, nausea, vomiting and acute abdominal pain. This metal causes damage to the proximal convoluted tubules of kidney. The disease Itai-Itai (characterized by osteomalacia and renal tubular dysfunctions) resulted from consumption of cadmium contaminated rice in Toyama (Japan) in 1935, is a well known case of cadmium toxicity (Hodgson et al., 2004).

Chromium is virtually omnipresent in the environment. It enters the environment through natural and several anthropogenic sources (Abbasi et al., 1991). Chromium naturally occurs in air, rocks, soil, water and biological materials (Adriano, 1980; Abbasi et al., 1988; Soni, 1990). It is more abundant in the earth crust than other hazardous metals viz., cobalt, copper, zinc, lead, nickel and cadmium; ranking 21st in abundance among all elements. Chromium is widely used in industrial process viz. production of ferrochromium, chromates, refractory materials, chromium steels, cement, fungicides, dyes, pigments, oxidants, catalysts and fertilizers, in chromate plating, leather tanning, as wood preservative, petroleum refining and manufacturing of automobile parts, etc. and is released into aquatic environments largely by electroplating, tannery and textile industries (Abassi and Soni, 1984; Goyer, 1996). The other anthropogenic sources for introduction of chromium in environment include burning of oil and coal and oil well drilling (Soni, 1990; Abbasi et al., 1991). In freshwater, instances of anthropogenically introduced chromium contamination are occurring with increasing frequency.

Like other metallic pollutants, chromium not only alters the quality of water by changing the physico-chemical equilibrium but also affect the normal functioning of the vital activities of the body of aquatic organisms (Thaker et al., 1996; Steinhagen et al., 2004). The discharge of chromium containing wastewater on land surface may result in greater than natural or permissible levels of chromium in the tissues of receiving organisms resulting in toxicological insults of varying degree. The toxicity of chromium to aquatic life is strongly influenced by the chemical speciation of chromium and water quality (Abbasi and Soni, 1985; Soni, 1990) and considerably varies between and within groups of organisms.

Chromium is a transition metal and is present in the environment in several oxidation states from Cr (0) to Cr (VI). The most common are Cr (0), Cr (III) and Cr (VI). Trivalent chromium occurs naturally in the environment and is essential for life and must be supplemented as a trace element in the diet of humans and animals. Chromium dietary deficiencies have been clinically correlated with abnormalities of glucose metabolism and
insulin malfunction. Hexavalent chromium is produced in industrial processes is less stable and more biologically reactive form, highly toxic, genotoxic and carcinogenic (Csuros and Csuros, 2002) and may come to have lethal effects if absorbed through the skin, ingested and inhaled (Harmel, 2004). Chromium (VI), as a metallic ion or in the chromate and dichromate salt, acts by inducing mutations through oxidative damages, DNA damages and is said to be a strong clastogen or mutagen (Gayatri et al., 1997; Stearns 2000; Zhitkovich et al., 2001; Quievryn et al., 2002). Cr(VI) produces a variety of DNA lesions such as single-strand breaks, alkali-labile sites and DNA-protein cross links as well as selective inhibition of the activity of enzymes such as glutathione reductase (Langard, 1990). Hexavalent chromium is more toxic and carcinogenic than trivalent form as it readily gains entry into cells by the sulphate transport system.

Copper is an essential trace metal for living organisms and present in all natural waters and sediments (Linder, 2001). This is one of the 26 essential trace elements occurring naturally in plants and animal tissue. As an essential trace element, copper is third in abundance in the human body after iron and zinc. It functions as a cofactor and is required for structural and catalytic properties of a variety of important enzymes and hormones (Uauy et al., 1998; Turnlund, 1999; Gaetke and Chow, 2003). Its importance in health and disease is well documented (Evans, 1973; Venugopal and Lucky, 1978; Berman, 1980; Owen, 1981; Linder, 1983; Aaseth and Norseth 1986; Prohaska, 1986). Copper displays four oxidation states: Cu (0), Cu (I), Cu (II) and Cu (III). Copper (II) or the cupric ion is the most important oxidation state of copper and is generally encountered in water. If copper is present in relatively high concentration in the environment, toxicity to aquatic organisms occur.

Natural source of copper include windblown dust, volcanic activity and spray from ocean waves whereas anthropogenic sources are mining, refining, smelting and incineration of copper and related metals that are mixed or alloyed with copper in the ores and processed forms (ATSDR,1990). Although some contents of the metal are removed in the sewage treatment plants of industries because of its sediment binding properties still sewage is a major source of Cu input to the water bodies, where it is a well known environmental hazard associated with toxicity to a variety of aquatic organisms (ATSDR, 2004). It has also been established in the literature that lead and copper reach drinking water through the dissolution of plumbing materials (Murphy, 1993). Cu is used today as a chemotherapeutic agent in aquaculture but increased levels of Cu in aquatic environments is because of industrial and agricultural wastes.
Copper sulphate, commonly known as blue stone or blue vitriol is the best known and most commonly used among all copper salts. It is also used as a mordant in dyeing process and as a broad-spectrum inorganic pesticide having many-fold uses as molluscicide, herbicide, algaecide and weedicide. In combination with lime and water, it is used as a protective fungicide, referred to as Bordeaux mixture. Copper sulfate is classified within the pesticides for general use, as defined by the EPA. It is stated that this pesticide, labeled as class I (highly toxic) in terms of toxicity, may have dangerous effects, particularly on species under threat of extinction in aquatic ecosystems, due to its potential risk of being mixed with surface waters (Extoxnet, 1996). Copper occurs in virtually all of human contact, including air, water and soil. The usual routes by which human receive toxic exposure to copper sulphate are through skin and eye contact, as well as by inhalation of powder and dust (ATSDR, 1990).

The toxicity of copper has been linked with reactive oxygen species (ROS) whose formation is catalyzed by free copper ions (or certain complexes) that can occur when the ability of the cells to store excess copper in a benign form has been exceeded (Linder, 2001). Copper acts as a catalyst in the formation of ROS and catalyzes the peroxidation of membrane lipids (Chan et al., 1982); it is known to be as effective as or even more effective than iron in causing DNA damage (Ozawa et al., 1993). Studies on aquatic organisms exposed to polluted waters or sediments have implicated DNA strand breakage, formation of chromosome aberrations and micronuclei (Bhunya and Pati, 1987; Fahmy, 2000; Georgieva et al., 2013).

Lead is a heavy metal that due to its distribution and abundance on the earth’s crust is considered ubiquitous (ATSDR 2007). This metal has been used for a great variety of purposes during the last three centuries since the Industrial Revolution. Because of its broad industrial usage in the manufacture of batteries, fuel additives, pipes, pigments, paints, solders, shielding, etc., Pb is a common occupational and environmental health hazard throughout the world. (U.S. EPA, 1998). Activities such as mining, refining, and casting of lead have moved great amounts of this metal from its natural deposits to the environment (Gloag, 1981). It is not an essential element and it can be absorbed by many means; all of its forms are toxic, in some cases, having harmful effects exceed those of other inorganic toxicants resulting in poisoning, poor performance and death in animals (McDowell, 1992; Gurer-Orhan and Ercal, 2000; Babu et al., 2007). Lead is a toxic element of high risk for the biota and human beings; it is classified as a carcinogen in animals and known as a possible carcinogen (group 2B) in humans (ATSDR, 2007). In addition, it gradually accumulates in the
body affecting the circulatory, nervous, renal, and reproductive system (ATSDR, 2007). A disease associated with lead accumulation is Plumbism. Lead may also contribute to high blood pressure and heart disease (U.S. EPA, 1998).

Toxicity of Pb has been studied extensively in fish, birds, and mammals, but information related to amphibians is relatively sparse (Linder and Grillitsch, 2000). A few field studies have related Pb levels in amphibians to those in their ambient environment (Birdsall et al., 1986). Although without correlating Pb levels in amphibians with negative effects on amphibians, these studies did suggest that amphibians living at contaminated sites might face the risk of Pb toxicity. However, field studies alone may not be able to establish causes of effects because of the potential confounding influences of other contaminants or environmental stressors (Linder and Grillitsch, 2000). Thus, laboratory-based dose–response toxicity tests are essential to confirm the effects caused by this metal.

Manganese (Mn) is the twelfth most abundant element in the earth’s crust and is naturally present in rocks, soil, water, and food. Mn is an essential element for humans, animals, and plants, and is required for growth, development, and maintenance of health. There are inorganic and organic Mn compounds, with the inorganic forms being the most common in the environment. Inorganic manganese compounds are used in the production of steel, dry cell batteries, glass, matches and fireworks, tanning of leather, ceramics, and dietary supplements. These manganese compounds are also generated as combustion products from motor vehicles and coal-burning industrial plants. Organic forms of manganese are used as fungicides, in some pesticides, fertilizers, as fuel-oil additives, smoke inhibitors, an anti-knock additive in gasoline, and a medical imaging agent. Manganese compounds can be present as dust particles in the air, and dissolved in ground water or drinking water (ATSDR, 2000).

It has long been known that Mn, an essential element for humans and the fourth most widely used metal in the world, is a neurotoxic substance. But just as in the case of any chemical substance, the dose makes the poison; therefore, Mn toxicity has primarily been observed in occupational settings where there is the potential for chronic exposure to high levels or following the accidental ingestion of large quantities (e.g. in Mn mining and smelting, battery manufacturing, and steel production; ATSDR, 2000). In 1837, John Couper was the first to report neurological effects associated with exposure to Mn, which was later recognized as “manganism”. In chronic situations, the neurological sign of manganism resembles Parkinson’s disease and dystonia (Santamaria, 2008).
Mercury is a naturally occurring metal whose primary store is within the planet. It is regarded to most toxic, non-essential, persistent, immutable and non-biodegradable heavy metal. Mercury contamination of aquatic environment through natural weathering processes and anthropogenic activities is reported to be increasing on a significant scale (WHO, 1971). It is a xenobiotic metal which was found to be instrumental in Minamata biological disaster of Japan in 1956, where a factory manufacturing acetaldehyde used mercury as catalyst and discarded the waste sludge containing it into Minamata bay. Aquatic biota converted mercury into methyl mercury and also accumulated toxic amount of it in their bodies and the inhabitants after consuming them, got affected by heavy metal poisoning (Kitamura, 1968).

Mercury is used in a variety of consumer, industrial and medical products and processes. Product examples are fluorescent light bulbs and batteries, medical devices (e.g. thermometers, blood pressure instruments), laboratory chemicals, pharmaceutical and dental products, and various temperature and moisture measurement and sensing devices (barometers, hygrometers, flame sensors). Metallic mercury is used to produce chlorine gas and caustic soda and Mercury salts are sometimes used in skin lightening creams and as antiseptic creams and ointments. Mercury emissions come from a range of human activities, primarily coal burning, but also from incineration or disposal of mercury-containing products, cremation, and from natural sources (Joy and Kirubagaran, 1989).

High doses can be fatal to humans, but even relatively low doses of mercury containing compounds can have serious adverse neurodevelopment impacts, and have recently been linked with possible harmful effects on the cardiovascular, immune and reproductive systems (Peraza et al., 1998; Silva et al., 2005). Studies of the sources, accumulation, and toxicological effects of Hg span most taxa and include a growing body of research on wildlife (Wolfe et al., 1998; Eisler 2006; Scheuhammer et al., 2007). What little data are available; indicate that metal can significantly reduce viability in amphibians through their actions on metabolism, development and gametogenesis (Byrne et al., 1975; Goyer, 1986; Kanamadi and Saidapur, 1991, 1992; Punzo, 1993).

Nickel is a ubiquitous, one of the five ferromagnetic elements naturally occurring in soil and a recognized, environmental and industrial pollutant. Nickel is used in a wide variety of metallurgical processes such as electroplating and alloy production as well as in nickel-cadmium batteries. There is evidence suggesting that nickel may be an essential trace element for mammals (Goyer, 1991). Human activities that contribute to nickel loadings in aquatic and terrestrial ecosystems include mining, smelting, refining, alloy processing; scrap metal
reprocessing, fossil fuel combustion, and waste incineration (NAS, 1975; WHO, 1991). Nickel enters natural waterways from waste water because it is poorly removed by treatment processes (Cain and Pafford, 1981). Nickel is a known haematotoxic, immunotoxic, neurotoxic, genotoxic, reproductive toxic, pulmonary toxic, nephrotoxic, hepatotoxic and carcinogenic agent. The adverse health effects of nickel depend on the route of exposure (inhalation, oral, or dermal) and can be classified accordingly. The most common harmful health effect of nickel in humans is an allergic skin reaction in those who are sensitive to nickel (Das et al., 2008). Out of the five priority substances which are selected by WHO for the nickel risk assessment, NiSO₄, NiCl₂, NiCO₃ and NiNO₃ are classified as carcinogen class I (by inhalation). Carcinogenicity of nickel compounds are being invested by many investigators (Kasprzak et al., 1987; Hass et al., 1996). Toxic and carcinogenic effects of nickel compounds are associated with nickel-mediated oxidative damage to DNA and proteins and to inhibition of cellular antioxidant defenses (Rodriguez et al., 1991).

Zinc is an essential nutrient for living organisms, representing the 23rd most abundant element on earth (Broadley et al., 2007) and the 2nd most abundant transition metal, after iron (Jain et al., 2010). As a constituent of more than 200 metalloenzymes and other metabolic compounds, zinc assures stability of biological molecules such as DNA and of biological structures such as membranes and ribosomes (Vallee, 1959; Casey and Hambidge, 1980; Llobet et al., 1988; Mason et al., 1988; Leonard and Gerber 1989). Zinc plays a central role in cellular growth and differentiation and the effects of its deficiency are especially pronounced in tissues and organs with a rapid turnover, including immune system and during period of rapid growth both pre- and post-natally (Varin et al., 2008). Uses of zinc include the production of noncorrosive alloys, galvanizing steel and iron products, and the therapeutic treatment of zinc deficiency (Elinder 1986).

Environmental Zn accumulation is due to mining and refining of nonferrous metals, chemical industry, burning of fossil fuels, and agricultural utilization of fertilizers and herbicides (Zn chloride and Zn sulphate) (Paivoke, 2003) which is often discharged into aquatic systems and therefore poses a great threat to aquatic organisms (Holcombe et al., 1979). In many types of aquatic plants and animals, growth, survival and reproduction can be adversely affected by elevated zinc levels (Eisler, 1993). Zinc did not induce DNA damage in normal cells, but did so in cancer cells as it helps the growth of rapidly proliferating cells such as tumors. Zn is redox-stable under physiological conditions, being considered as one of the least toxic heavy metals (Steinkellner et al., 1998; Codina et al., 2000). It is a structural stabilizing factor of cell membrane and DNA-linking proteins (Salama and El Fouly, 2008).
and it plays a significant role in the control of gene expression and DNA transcription (Paivoke, 2003). However, elevated zinc levels can cause mortality, pancreatic degradation, reduced growth, and decreased weight gain in birds (NAS, 1980; Eisler, 1993) and elevated zinc can cause a wide range of problems in mammals such as cardiovascular, developmental, immunological, neurological, haematological, pancreatic, and reproductive defects (Domingo, 1994).

By adversely affecting the self-purification process of water bodies, heavy metals induce devastating effects in the ecological balance of the recipient environment (Ashraj, 2005; Vosyliene and Jankaite, 2006; Farombi et al., 2007). These being non-biodegradable become irritant in lieu of the fact that once they exceed their normal limits; adversely affect not only the normal equilibrium of water but also vital activities of organisms inhabiting the aquatic habitat. No living being has developed any homeostatic mechanism for non-essential metals having no biological role. It is in spite of the fact that although heavy metals like Cu, Fe, Cr, Zn, Ni etc are essential for the proper functioning of biological systems but their surfeit could lead to severe disorders (Ward et al., 1995). Beijer and Jernelov, 1986 and Diagomanolin et al., 2004 reported that heavy metals are critical because of their easy uptake into the food chain and bioaccumulation processes. Some of the most common pollutant heavy metals listed by Environmental Protection Agency (EPA) are: Cd, Cr, Cu, Hg, Ni, Pb and Zn. The toxicity of a metal depends upon the chemical form of the metal. (Bryan and Langston, 1992). Some inorganic substances such as iron, zinc, copper, selenium, chromium, nickel and cobalt are known to be essential micronutrients. Several of these heavy metals are mutagenic in nature. They cause heavy mortality when present in lethal concentrations; however, when present in sublethal concentrations they exert undesirable alterations in genetic content of a living cell. Further, these mutagens may adversely affect the fertility and fecundity of living organisms. Heavy metals such as chromium, mercury, arsenic, copper, lead, zinc, cadmium, iron, manganese and nickel are responsible for playing havoc with the environment balance. It is therefore, not surprising that in recent years there has been increasing concern regarding the genotoxic hazards and reports on the occurrence of malignancies in aquatic organisms especially amphibians. The occurrence of malignancies in aquatic organisms has also been correlated with the higher incidence of tumours in human population (Beneden, 1994). Therefore, natural biota might be used as sentinel or surrogate species for the evaluation of genotoxic chemicals in the environment and their risk to human health. Exposure of aquatic organisms to genotoxic contaminants could pose a risk to human health via the food chain. Moreover, there is an ecological risk that may lead to heritable
mutations and loss of total genetic diversity (either intra or inter species), with significant implications for long term survival of natural populations (Anderson et al., 1994). According to the studies of Hartwig, 1995 and Knasmuller et al., 1998 toxic heavy metals cause DNA damage and their carcinogenic effects in animals and humans are most probably caused by their mutagenic ability (Kovalchuk et al., 2001). Agents that produce alterations in the nucleic acids and associated components at sub toxic exposure level, resulting in modified hereditary characteristics or DNA inactivation, are classified as ‘Genotoxins’. Genetic toxicology or Genotoxicology is a new division of toxicology that identifies and analyses the action genotoxins directed towards the heredity components of living systems. Genotoxicology has played a dual role in safety evaluation programs. It can be widely implemented for testing the impacts of genotoxic agents found in the environment, whose presence may alter the integrity of the gene pool of a wide range of organisms including human beings. Further, it can be applied in genetic methodologies for the detection and mechanistic understanding of carcinogenic chemicals.

Reporting on the mutagenic activity of heavy metals by using different assays is important in the environmental studies. Within the last decade, chromosomal aberration test (Hooftman and De Raat, 1982; Al-Sabti et al., 1994) and micronuclei test (Al-Sabti, 1995) have played an important role in assessing exposure to water pollutants and have proved as appropriate tools to provide an early warning of genotoxic threat to amphibian and other aquatic organisms, their ecosystem and finally to man. Induction of cytogenetic damage in frogs could serve to monitor not only selected genotoxic agents in the laboratory, but also the genotoxic potency of surface water and aquatic ecosystems. Thus, genotoxicity studies have a great importance as genotoxic pollutants damage the DNA which affects the biological system at different organization levels.

World-wide, there are believed to be 7145 species of amphibians, out of which anurans are 6300 species, which include frogs and toads i.e. about 90% of all amphibians (McDiarmid and Mitchell, 2000). Therefore they are important link between humans and ecosystem health (Hayes et al., 2002) and are main components of aquatic and terrestrial ecosystems (Unrine et al., 2007). Amphibians are not evenly distributed throughout India and the highest concentration is in western peninsula followed by northeast. According to IUCN Red List of 2013 of threatened species, the global status of Indian amphibians is 23.68%. Out of the 342 species of known Amphibians from India, 75 species are yet to be evaluated and 81 species are still under the data deficient category. The first comprehensive list of Indian
amphibian was provided by Inger and Dutt (1986) which include 181 species. Subsequently Dutta (1997) revised the list and reported 216 species occurring in India. Of these 11 species are found in Jammu and Kashmir State (Sahi and Duda, 1984) which forms just 5.3% of the total amphibian population of the country, 6 species are found in Jammu. Anurans are the most neglected group of vertebrates of the state. Out of the 6 species of anurans, there species i.e. *Euphlyctis cyanophlyctis, Hoplobatrachus tigerinus, Chrysopaa sternosignata*, are of frogs belonging to family Dicroglossidae. This family is perhaps the most widely distributed family having 186 species in 13 genera (Frost, 2013). Out of the three, *H. tigerinus* is more secretive in nature, larger in size and is witnessed after a heavy monsoon shower in water logged areas whereas *Chrysopaa sternosignata* is found in the hilly districts of Jammu. *Euphlyctis cyanophlyctis*, also known as common skittering frog is one of the mostly widely distributed oriental frog and is highly aquatic and littoral frog. It extends from Thailand to Nepal throughout India, Sri Lanka, almost throughout Pakistan, westward to Iran and Afghanistan. It remains permanently resident in most fresh water bodies that sustain submerged aquatic plants, paddy fields, canals and rainwater ditches. The adults, largely aquatic, floating amongst plants with the lower half of the body and hind limbs submerged in water. This green frog dives under water when disturbed often staying beneath for long durations. Female lay eggs after the first rains and throughout the rainy season and a female may lay 2000 eggs in ponds or paddy fields. The frog can tolerate a wide range of pH variations, from fresh water to considerably brakish and polluted refuse water; it thrives equally well in sewer systems of towns and cities.

*E. cyanophlyctis* is also claimed to be the native frog of Kashmir and is reported declining at an alarming rate especially in the urban and semi-urban areas of the valley, warning environmentalists. The research report regarding the native frog of Kashmir in the paddy field environment, which appeared in the Journal of Centre of Research for Development, University of Kashmir reveals that native population of frog – *Rana cyanophlyctis* – is sparsely found in the valley. The research, done in four different paddy fields and a perennial spring site in northeastern suburbs of Srinagar, found population of native frog to be woefully inadequate. The report has given several reasons for its decline, the major one being the frequent and indiscriminate use of pesticides and other chemicals in paddy fields. The study highlights that toxins accidentally destroy numerous species of frogs every year. The chemical fertilizers used in agriculture and horticulture, contain nitrogen and
phosphorous which has negative impact on the growth of these species (Kashmir frogs croaking towards extinction; Well, 2007).

The use of frogs and toads, as biological indicators of metal pollution is becoming common (Burger and Snodgrass 1998). Frogs have proved to be a valuable bioindicators and a sensitive model for environmental and ecotoxicological studies (Jaylet et al., 1986; Krauter et al., 1987; Zoll et al., 1990; Gauthier, 1996; Ferrier et al., 1998; AFNOR, 2000; Djomo et al., 2000; Mouchet et al., 2005, 2006).

The present study has been undertaken to biomonitor the genotoxic potential of mercury as HgCl$_2$, lead taken as Pb(COOH)$_2$, cadmium taken as CdCl$_2$.H$_2$O, chromium taken as K$_2$Cr$_2$O$_7$, copper taken as CuSO$_4$.5H$_2$O, nickel taken as NiCl$_2$, zinc taken as ZnCl$_2$, manganese taken as MnCl$_2$, arsenic taken as As$_2$O$_3$ and cobalt taken as Co(C$_2$H$_4$O$_2$)$_2$ by utilizing chromosome aberration test and micronucleus test as genotoxic assays.

In the present study, out of three frog species prevalent in Jammu division, *E. cyanophlyctis* has been selected as a model for the genotoxic experiments and evaluations. The main objectives of present study are as follows:

- Assessment of frog, *Euphlyctis cyanophlyctis* as a model animal for genotoxic studies.
- Assessment of genotoxic potential of above said heavy metals by using frog as test model.
- Preparation of standard karyotype of the frog *Euphlyctis cyanophlyctis* by using various banding techniques (C and NOR banding).
- Evaluation of genotoxic effects of heavy metals by using chromosome aberration test (CAT), micronucleus test (MNT) and by determination of mitotic index (MI).
- Comparative evaluation of genotoxicity of heavy metals in different tissues of test frog (intestine, kidney, bone marrow and red blood cells).
- Determination of effect of dose and duration of exposure on the toxicity of heavy metals.
Chapter 2

Review of Literature
Aquatic toxicology or toxicity testing is the qualitative and quantitative study of toxic effects of pollutants on aquatic system and organisms (Adams, 1995), with pollutants being various chemicals and anthropogenic substances (insecticides and herbicides) (Rand and Petrocelli, 1985; Roux, 1994). Aquatic organisms have played important roles as early warning and monitoring systems (bioindicators) for pollutant burdens in our environment (Pritchard, 1993). Aristotle conducted the first aquatic toxicity test over 2000 year ago by placing freshwater animals in sea water and determining the effect it had on the organisms (Buikema et al., 1982). The formal discipline known as toxicology only arose in the early 1800’s in response to the development of organic chemistry where man would produce chemicals and then their effects.

The field of genetic toxicology began its development before the biochemical basis of heredity was understood. Early investigators observed that physical and chemical agents could cause heritable mutations. The role of radiation in producing heritable changes in a living organism was first reported by Muller in 1927. Auerbach et al., 1947 were the first to report the ability of chemicals to cause mutations. These early observations of induced change in heritable traits formed a core of study that evolved into the field of genetic toxicology. Genetic toxicology was recognized as a discipline in 1969 when the
`Environmental Mutagen Society' was formed under the leadership of Dr. Alexander Hollaender and several other geneticists who were concerned about the potential genetic impact associated with the proliferation of man-made chemicals in the environment (Naismith, 1980). Genetic toxicology investigates the interaction of chemical and physical agents with genetic material, in relation to subsequent adverse effects, such as cancer (in the case of alterations in somatic cells) or genetic diseases in future generations (in the case of alterations in germ cells). The changes in genetic material of organisms can be detected at specific levels by various genotoxicity assay systems (like chromosomal aberrations, sister chromatid exchange, micronucleus assay, comet assay etc) having different end points. These assays can detect relatively greater damage to genetic material manifested at cellular, chromosomal and DNA levels.

Review of Literature has been discussed under following headings:

- **Genotoxicity in Animals (in general).**
- **Genotoxicity in Amphibians.**

### 2.1 GENOTOXICITY IN ANIMALS (IN GENERAL)

Mouw *et al.*, (1978) tested the hypothesis that an acute dose of lead reduces tubular electrolyte reabsorption and alters the secretion of renin. In sodium pentobarbital-anesthetized dogs, acute intravenous administration of lead increased the excretion of sodium, potassium, calcium and water, despite a constant glomerular filtration rate; therefore lead reduced the tubular reabsorption of these substances. Lead also caused an increase in plasma renin activity. The threshold dose of acutely administered lead necessary to elicit these responses was determined in dose-response experiments on unanesthetized rats; a dose of 0.1mg of lead/kg was sufficient to cause significant increases in plasma renin and the renal excretion of sodium. Lead concentrations in the tissues of the rats were measured in samples taken immediately at the conclusion of the study; the threshold dose of lead was associated with very low blood lead (5μg/100 ml) and kidney lead (1.2μg/g wet wt).

Hahn and Kim (1979) studied the effect of various concentrations of four alkylating agents (MMS, EMS, DMN and DEM) on mouse bone marrow erythrocytes by means of the micronucleus test. The lethal doses on mice obtained were MMS=130mg/kg/b.w., EMS=300mg/kg/bw, DMN=50mg/kg/bw, and DEM=70mg/kg/bw. The micronuclei obtained
in controlled were a little, over 0.1% where in other there was a dose-effect relationship. In the MMS and EMS treated groups incidences of micronucleus were 0.45 to 2.56.

Noel-Lambot et al., (1980) evaluated cadmium, zinc and copper accumulation in limpets (*Patella vulgata*) from the Bristol Channel with special reference to Metallothioneins. The study of metal concentrations in *Patella vulgata* caught from a polluted environment reveals a direct relationship between Cd concentration and body size and an inverse relationship in regard to Zn and Cu. Most of the Cd present in limpets with heavy Cd loads was bound to thioneins, but this was not the case for Zn and Cu. In young limpets, Cd-thioneins were not detected. All results indicated that during long-term Cd intoxication under field conditions, Cd deposition and increase in metallothioneins in limpets were directly linked. The induced production of metallothioneins may thus be considered as the main mechanism responsible for the cumulative absorption of Cd in limpets living in the Bristol Channel. Isolation and characterization of limpet metallothioneins were also performed and the result indicated that mollusc’s metallothioneins were very similar to those from vertebrates.

Sunila (1981) studied the effects of short term (24h) exposure to copper and cadmium on the survival and subsequent physiology of the common mussel, *Mytilus edulis* in brackish water in aquaria. The LC 50 (6°C) for copper was 0.4ppm and for cadmium 4.0ppm. Activity was defined as the rate of opening of the valves under exposure (EC 50: Cu 0.2ppm, Cd 0.5ppm) and the beating of the cilia (EC 50: Cu 1.4 ppm, Cd 5.1ppm). In copper solutions, but not in cadmium, the rate of opening of the valves showed a negative regression with the concentration. The proportion of mussels that opened their valves under cadmium exposure correlated with the survival rate. In copper solutions the correlation was not significant, since already very low concentrations resulted in closure. There were differences between the tolerances of different populations to these metals. Small individuals were more resistant to cadmium and more sensitive to copper than large ones. No changes in histological sections from the digestive glands of exposed bivalves could be observed in light microscopic studies. Mucus accumulated on the surface of the siphons of mussels exposed to copper. Cadmium caused loosening of epithelial cells. Gill filaments parted from each other after breaking of the interfilamentar junction.
Bogaert et al., (1984) determined the toxicity of four heavy metal compounds and three carcinogens using two nematode species, *Monhystera microphthalmia* and *Diplolaimelloides bruciei*.

Gomez-Arroyo et al., (1985) determined the potential cytogenetic effects of Heptachlor, Malathion and Methyl Parathion, meristems of broad bean root tips, treated with several concentrations of these insecticides for different periods of treatment and recovery. Heptachlor and Malathion induced chromosomal alterations in anaphase cells as fragments and bridges, chromosomes with inactivated centromeres and isochromosomes and as micronuclei in interphase cells. Multipolar anaphases also appeared when the mitotic spindle was damaged. Heptachlor induced pycnosis but Malathion did not. Chromosomal aberrations caused by Methyl Parathion were observed in cells only during metaphase, since a strong c-mitotic effect was induced mostly in the form of fragments. Longer recovery periods (42 and 44hrs) revealed tetraploid and pycnotic cells. Only in the case of Malathion, there was an increase in chromosome alterations with higher concentrations. However, no such dose response relationship was observed for micronuclei frequency.

Lee and Hong (1985) studied the apparent effectiveness of 1-naphthyl-N-methyl carbamate (carbaryl) against a wide variety of insects. In this toxicological study of carbaryl, mature male rats inhaled carbaryl at a mean concentration of 112mg, 168mg and 224mg/m$^3$ for one hour. After inhalation, pentobarbital sleeping time, NADPH-cytochrome c reductase activity, cytochrome p-450 and protein content in liver microsomes, various tissue residues, cholinesterase inhibition in plasma and histopathological findings at autopsy were observed. The pentobarbital sleeping time was prolonged in rats inhaled with carbaryl for one day while the sleeping time was shortened in the 3 days inhaled group. The changes of cytochrome p-450 content and NADPH-cytochrome c reductase activity exhibited biphasic response showing the decrease in the one day inhaled group and the increase in the three day inhaled group. The marked depression of plasma ChE activity was observed in rats inhaled with carbaryl at 112 mg/m$^3$, however no more progressive effect was observed at the higher concentration of the compound. The main observations in histopathological findings were ciliary detachment, epithelial swelling and sub epithelial inflammatory cellular infiltration in trachea due to the irritation.

Malhi and Grover (1987) assessed the genotoxic effects of four organophosphorus pesticides, i.e. ekatin, fenitrothion, methylparathion and phorate employing *in vivo*
chromosomal aberration bioassay in bone marrow cells in rat. Methylparathion and phorate were found to be mutagenic while ekatin was weekly mutagenic. The frequency of chromosomal aberration induced by fenitrothion did not differ significantly from that observed in the negative control.

Agarwal et al., (1990) studied the clastogenic effects of coppersulphate on the bone marrow chromosomes of mice in vivo. Intraperitoneal administration of coppersulphate to Swiss albino induced a significant increase in the frequency of chromosomal aberrations in bone marrow cells at all concentrations used (1.1-6.6mg/kg b.w), when compared to the negative control. Statistical analysis indicates that the degree of clastogenicity was directly related to the concentrations used and indirectly to the period of exposure. The effect was maximal at 6hr after treatment as compared with 12 and 24 hrs.

Ribeiro et al., (1993) investigated the inter-laboratory calibration program for the mouse micronucleus test. The frequency of micro nucleated polychromatic erythrocytes (MNPCES) in the bone marrow of mice treated with cyclophosphamide (CP), bleomycin (BLEO) and benzidine (BZN) was determined in three different laboratories. Although the experiments were conducted independently, the results were quiet similar for all three laboratories. The mean MNPCE frequencies induced by CP and BLEO were not statistically different. Only for the group treated with a single BZN dose were the mean frequencies statistically different between laboratories two and three. The homogeneous results obtained by the three laboratories for the micronucleus test indicate the efficacy of the method and its applicability for the identification of clastogenic agents.

Cai et al., (1995) reported cadmium exposure among the residents in an area contaminated by irrigation water in China. River water used to irrigate arable land in Dayu County, Jiangxi Province, China, got polluted with cadmium from tailings and the wastewater of tungsten ore dressing plants. From information about the date of construction of ore dressing plants and an analysis of the annual growth rings of trees, they deduced that local residents have been exposed to cadmium for at least 25 years. Cadmium exposure was estimated based on a meal survey, which indicated that 99.5% of the oral cadmium intake came from rice and vegetables grown locally. The average oral intake of cadmium was calculated to be 367-382μg/day. Smokers had additional exposure from locally grown cadmium-containing tobacco. Cadmium concentrations in samples of urine (11μg/g
creatinine), blood (12μg/l) and in the hair (0.11μg/g) of persons in the exposed area were high.

Carey and Bryant (1995) worked out the possible interrelations among various toxicants and the potential role of environmental xenobiotics in: a) affecting the susceptibility of young to disease; b) retarding growth and development of amphibian young; c) affecting the ability of larvae to avoid predation; d) affecting the development of physiological, morphological, or behavioral processes in a manner that subsequently impairs the ability of the young for future reproduction; and e) directly causing mortality of young leading to the decline in amphibian population.

Ma et al., (1995) used the simplified in vivo Mouse-Erythrocyte-Micronucleus (Mus-EMN) assay to monitor the chronic clastogenicity of industrial wastewater throughout one year in the current study. Twenty four young (2 month old) CD-1 white mice were divided into 3 groups of 8, with 4 males and 4 females in each group and caged individually for this study. Two treated groups of animals were fed with industrial wastewater collected weekly from the downstream of the Arena Canal (wastewater disposal system) from the Benito Juarez Industrial District of the Queretaro City of Mexico. In order to reduce the toxicity, wastewater samples were diluted with tap water at 1:5 ratio (wastewater: tap water) for treated group 1 and a 1:2 ratio for treated group 2. Animals of the control group drank the tap water. Red blood samples were collected monthly from the tail and blood smears were double stained with hernatoxylin and Giemsa, and about 10,000 mature red blood cells were scored from each of the 8 slides of the experimental groups to derive the means and standard errors. Results of this year-round study showed a significant increase in MN frequencies (5.08 - 9.80 MN/1000 cells) in the treated groups during the months of October through January of the following year, the dry season of this area of Mexico. The MN frequencies of the treated mice declined to the control level (1.29 - 3.20 MN/1000 cells) after 6-7 months of continuous exposure. Results of this study indicated that the Mus-EMN assay was adequate for chronic clastogenicity tests of water pollutants with the maximum time limit around 6 months which is about 20% of the youthful life of the mouse.

Bhunya and Jena (1996) evaluated the clastogenic potential of copper sulphate in chicks, employing chromosome aberration (CA) and micronucleus test (MNT) assays. For CA dose, route, time-response and acute vs. sub acute studies have been done while only route and dose-response studies were done for MNT. Three different doses were administered
intraperitoneally, and only the highest dose was administered per oral. Neonatal chicks were killed after different time intervals. One-fifth of the highest dose was injected repeatedly with a gap of 24 h in-between for sub-acute regimen. A statistically significant (p<0.05) increase of CA was observed by the two higher doses in intraperitoneally route and by the highest dose in per oral route. In time-response studies, significant (p<0.05) results were obtained after 24 and 48 h of exposures. A significant increase in micronucleus count was observed with the two higher doses in both bone marrow and peripheral blood erythrocyte by the intraperitoneal route and only by the highest dose in bone marrow erythrocyte by oral route.

Lutz et al., (1996) studied the concentrations of mercury (Hg), cadmium (Cd) and lead (Pb) in brain (cerebrum) and kidney during fetal (second trimester terminations or abortions, n=20) and postnatal (infants deceased before three months of age, n=15) development. Information on possible sources of exposure was obtained from the mothers of the fetuses, but not from those of the infants. The median concentration of Hg in the brain was 4μg/kg wet weights in both fetuses and infants (total range ≤ 2-23μg/kg). The concentrations of Hg in the kidneys were significantly higher than in brain, median of Hg 6μg/kg (range ≤5-34μg/kg) in fetuses and 10μg/kg (≤7-37) in infants. There was a tendency of increasing concentration of Hg in the fetal kidney, but not in the brain, with increasing number of amalgam fillings in the mothers. The concentration of Cd in the brain was less than 1μg/kg in most cases, both in fetuses and infants. The concentration of Cd in the kidneys was significantly higher, with a median of about 2μg/kg (1-8) μg/ kg) in both groups. There was no detectable association between tissue Cd concentrations and the smoking habits of the mothers. The concentration of Pb in brain was below 10μg/kg in most cases. In the kidneys, the concentrations of Pb were significantly higher, with a median of 12μg/kg in the fetuses (range ≤ 6-20μg/kg) and 15μg/kg (≤ 9-36 μg/kg) in the infants.

Mersch et al., (1996) studied the induction of micronuclei in haemocytes and gill cells of Zebra mussels, Dreissena polymorpha, exposed to four directly acting reference clastogens (mitomycin C, bleomycin, dimethylarsinic acid and potassium chromate). The mean MN frequencies in treated mussels ranged between 3.2 and 6.9% in haemocytes and between 5.4 and 6.7% in gill cells. The MN induction capacity of the different chemicals was equivalent in both tissues, except for the treatment with dimethylarsinic acid which generated a significantly higher MN rate in gill cells than in haemocytes.
Licht and Grant (1997) discussed the fundamental physics of UV and types of biological damage after exposure. A deleterious change in DNA, especially the production of pyrimidine dimers, is a main effect of UVB exposure. Damaged DNA can be repaired by enzymes such as photolyase when organisms are irradiated with UVA or visible light. Field studies in which embryos were exposed to natural sunlight or sunlight with UVB removed has shown conflicting results: some show increased embryonic mortality after UVB exposure, whereas others show that current levels of UVB are not detrimental to amphibian embryos. The abiotic factors such as water depth, water color, and dissolved organic content of aquatic oviposition sites effectively reduces UVB penetration through water and reduces exposure to UVB of all life history stages. Biotic factors such as jelly capsules around eggs, melanin pigmentation of eggs, and color of larvae and metamorphosed forms further reduce effectiveness of UVB penetration. Therefore, the hypothesis of the causal connection between current UVB levels and amphibian decline was suggested.

Hayashi et al., (1998) developed and evaluated the monitoring systems that use aquatic organisms to assess the genotoxicity of water in the field and in the laboratory. In a field study, it was shown that the micronucleus assay is applicable to freshwater and marine fishes and that gill cells are more sensitive than hemopoietic cells to micronucleus-inducing agents. Gill cells from Carassius sp. (Funa) and Zacco platypus (Oikawa) collected upstream on the Tomio River (Nara, Japan), tended to have lower micronucleus frequencies than gill cells from fish collected at the midstream of the river. Leiognathus nuchalis (Hiiragi) and Ditrema temmincki (Umitanago), small marine fishes collected periodically at Mochimune Harbor (Shizuoka, Japan), showed seasonal differences in the frequencies of micronucleated gill cells and erythrocytes; they were highest in summer. For laboratory studies, a method for analyzing chromosomal aberrations and micronuclei using Rhodeus ocellatus ocellatus (rose bitterling) embryo was developed. One day after artificial insemination (gastrula stage), structural chromosomal aberrations and micronuclei were observed in the cells of embryos grown in water containing trichloroethylene.

Bat et al., (1999) performed acute toxicity tests on Echinogammars olivii (Amphipoda), Sphaeroma serratum (Isopoda) and Palaemon elegans (Decapoda), from the Sinop Peninsula in the Black Sea. 96-h LC50 values were estimated for copper, zinc and lead in these species using the static bioassay method. The LC50 values of Cu for E. olivii, S. serratum and P. elegans were 0.25, 1.98 and 2.52mg/l, respectively. The LC50 values of Zn for E. olivii, S. serratum and P. elegans were 1.30, 6.12 and 12.3mg/l, respectively. The
LC50 values of Pb for *E. olivii*, *S. serratum* and *P. elegans* were 0.62, 4.61 and 5.88mg/l, respectively. The results indicated that Cu was more toxic to the species followed by Pb and Zn. *E. olivii* was more sensitive to the metals than *S. serratum* and *P. elegans*.

Mathew and Jahageerdar (1999) assessed the effect of lead on the karyotype of *Channa punctatus*. The effects of lead in the form of lead nitrate were studied for 96, 120 and 144 hours at 0.012, 0.025 and 0.050mg/l of water. The model number of chromosome was found to be 2n = 32 out of which 14 were metacentric, 8 submetacentric, 6 telocentric and 4 acrocentric. The lead nitrate induced structural chromosomal aberrations were breaks, fragments, dicentric and ring type chromosomes. Lead nitrate at a very low concentration of 0.012mg/l and for just 96 hours of exposure can induce chromosomal aberrations in fish.

Sharma and Talukder (2000) analyzed the available data on the clastogenic effects of metals and their compounds on higher organisms and indicated some general trends. Following chronic exposure to sub toxic doses, a decrease in mitotic frequency and an increase in the number of chromosomal abnormalities were observed. These effects were usually directly proportional to the dose applied and the duration of treatment within the threshold limits. Recovery after acute treatment was inversely related to the dosage. The ultimate expression of the effects depended on certain factors, including the mode and vehicle of administration; the test system used; the rate of detoxification, distribution and retention in the different tissues; and interaction with foreign and endogenous substances as well as the mode of action with the biological substances as well as the mode of action with the biological macromolecules. In mammals, the clastogenic activity of the metals within each vertical group of the periodic table was directly proportional to the increase in atomic weight, electropositivity and solubility of the metallic cations in water and lipids, expect for lithium and barium. This pattern of inherent cytotoxicity increased with successive periods in the horizontal level. It is enhanced by the formation of covalent and coordinate covalent complexes by heavy metals with the biological macromolecules. In plants, the solubility of the metals in water is of much greater importance. The degree of dissociation of metallic salts and the rate of absorption affect significantly the frequency of chromosomal aberrations. Therefore in assessing the effect of environmental metal pollution, the presence of other metals and toxic chemicals and the level of nutrition should be taken into account, since in nature metals occur in combination and these factors modify the cytotoxic effects to a significant extent.
Yadav and Chhillar (2001) analyzed the mitotic index, chromosomal aberrations, sister chromatid exchanges and satellite associations in peripheral blood lymphocytes of 40 workers exposed to polyvinyl chloride in the plastic industry. All the parameters showed a significant increase (p<0.01) in the exposed sample compared with the controls. The occurrence of the DG type of satellite association was highest and that of 3D type lowest. The frequency of all the parameters increased with the duration of exposure, but MI declined after 15 years of exposure.

Yadav et al., (2001) concluded that the welding fumes, containing chromium (Cr IV) and nickel, cause considerable chromosomal damage. Therefore, welders, in their occupational settings are prone to high genetic risk.

Seoane and Dolout (2001) evaluated the aneugenic and clastogenic ability of cadmium chloride (II), cadmium sulphate (II), nickel chloride (II), nickel sulphate (II), chromium chloride (III) and potassium dichromate (IV) by the kinetochore staining in the cytokinesis-block micronucleus by employing the Human diploid fibroblasts (MRC-5). Staining kinetochores in the cytokinesis-block micronucleus assay is a useful way to discriminate between clastogens and aneuploidogens and may allow a rapid identification of aneuploidy-inducing environment compounds. The work demonstrated the genotoxic ability of cadmium and chromium salts. Nickel salts showed lower genotoxic effects than the other salts because of their weak mutagenicity.

Afonne et al., (2002) explained that zinc protects chromium- induced testicular injury in mice. Male mice (CD-1) were exposed to 150 ppm potassium dichromate and 500 ppm zinc chloride either individually or in combination for 12 weeks. After the exposure, the animals were sacrificed and epididymal sperm number counted. The testes were processed for histological examination. The result reveal that zinc had no effect on food intake and epididymal sperm number compared to control, while it significantly reduced the fluid intake, body weight gain and testis weight of mice. Chromium VI on the other hand, significantly decreased the body weight gain, food and fluid intake, and epididymal sperm number, but had no effect on testis weight compared to control. Concomitant exposure to both metals significantly increased the epididymal sperm number compared to chromium. Histological examination showed that chromium exposure induced severe pathologic changes on the mouse testis, which were reduced significantly by the combined metal exposure. Zinc had no deleterious effect on the testicular histology.
Chu and Chow (2002) illustrated the complexity of toxicity tests in biological systems and show that physical–chemical monitoring of toxicants may underestimate biohazards in environmental samples. They also demonstrate that a transgenic derivative nematode strain, KC136, carrying a heat shock promoter driven \textit{gfp} reporter gene could be used to reduce the duration of an assay so that the synergistic effects among toxicants could be revealed. This derivative strain allows rapid and frequent monitoring of environmental hazards, which usually requires the handling of a large number samples.

Kessel \textit{et al.}, (2002) examined the effects of free radical scavenging enzymes on the cytotoxic and mutagenic potential of arsenic using the AL cells. Concurrent treatment of cells with either superoxide dismutase or catalase reduced both the cytotoxicity and mutagenicity of arsenite by an average of 2–3 folds, respectively. Using immunoperoxidase staining with a monoclonal antibody specific for 8-hydroxy-2′-deoxyguanosine (8-OHdG), they demonstrated that arsenic induced oxidative DNA damage in AL cells. This induction was significantly reduced in the presence of the antioxidant enzymes. Furthermore, reducing the intracellular levels of non-protein sulfhydryls (mainly glutathione) using buthionine S-R-Sulfoximine increased the total mutant yield by more than 3-fold as well as the proportion of mutants with multilocus deletions.

Duez \textit{et al.}, (2003) investigated the alkaline Comet assay is a widely used single cell gel electrophoresis technique for the quantification of DNA strand breaks, cross links and alkali-labile sites induced by a series of physical and chemical agents. DNA migration in an electric field, supposed proportional to strand breakage is a proposed estimation of genotoxicity. Breaks are quantified from geometric and fluorescence measurements by image analysis of comet-shaped DNA, often reported parameters being tail DNA and tail moment. Although a variety of statistical approaches have been used in the literature, most of these do not take into account the distribution patterns of comet data. In order to investigate, a methodology for statistically demonstrating a comet effect, two different experiments, a reproducibility study and a trend analysis, were undertaken on a murine lymphoma cell line (P388D1) photo dynamically stressed after induction of porphyrins with δ-aminolaevulinic acid. This treatment results in significant heterogeneity of DNA damage, producing values ranging from 0 to 100% tail DNA in the same sample. The comparison of distribution curves for stressed and non-stressed samples shows that none of the application conditions are verified, either for parametric tests (which require normal distributions), or non-parametric tests (which assume essentially similar distributions). Meaningful statistics (median and 75th
percentile) were consequently extracted from repeated experiments and found suitable for comparing stress conditions in an ANOVA and in a trend analysis; the 75th percentile is theoretically more sensitive but tends to more rapidly saturate at extensive stress levels. It was concluded that a trend analysis of median comet metrics from repeated experiments at different stress levels is certainly an efficient way to statistically demonstrate a genotoxic effect. Whether the considered comet parameter was tail DNA or tail moment had no influence on the conclusions of our experiments, which were carried up to stress levels leading to a median 70% tail DNA.

Saotome and Hayashi (2003) improved the sea urchin micronucleus assay for aquatic samples and used it to evaluate marine pollution. It was found that the water samples collected for 2 years from the Tokyo bay coast near Tokyo, an industrial megalopolis, were positive due to the water samples being hypo-osmotic rather than to chemical pollutants. The evidence was as follows: (i) the osmolality and salinity of the samples were about half that of sea water; (ii) the micronucleus frequency induced in the water sample decreased to the control level when the osmolality was increased to that of sea water; (iii) artificial sea water diluted with distilled water induced micronuclei dilution dependently. Since micronucleus induction in the sea urchin assay is influenced by sample osmolality, the osmolality must be adjusted to that of sea water for the assay and osmotic pressure must be considered when evaluating water pollution.

Winter et al., (2004) evaluated genotoxic effects in feral and caged chub (Leuciscus cephalus) from three rivers with different water quality (due to presence of organics, metals and pesticides) around Birmingham, UK. In general, evaluated levels of DNA damage were recorded with a decrease in chemical water quality, in both feral and caged animals indicating an impact of chronic pollution. Recorded seasonal DNA adduct data suggested a higher degree of damage in the feral compared with caged animals.

Celik et al., (2005a) investigated the genotoxicity of lambda-cyhalothrin (LCT), a pyrethroid insecticide, in bone marrow cells and in colonic crypt epithelial cells of groups of four Swiss albino rats (Wister rats) per dose treated in vivo by gavage at doses of 0.8, 3.06 and 6.12 mg/kg body weight (body wt) using the micronucleus (MN) assay, scoring 2000 polychromatic erythrocytes (PCEs) per animal for bone marrow and 1000 colonic crypt epithelial cells per animal for the colon. Cytotoxicity in the bone marrow was assessed by calculating the ratio of PCEs to normochromatic erythrocytes, and in the colonic crypt
epithelium by observing the frequency of binucleated cells and the mitotic index in 1000 cells. Apoptosis in colonic crypt epithelial cells was measured by observing the frequency of karyorrhexis and karyolysis in 1000 cells. They found that LCT induced a statistically significant dose-related increase in MN formation in the bone marrow and the colonic crypt. The colonic epithelium was more sensitive to the clastogenic effects of LCT than the bone marrow as judged by the significantly higher frequencies of MN in the colon than in the bone marrow at doses of 3.06 and 6.12mg/kg body wt.

Celik et al., (2005b) investigated the possible cytotoxic and genotoxic effects of lead acetate on peripheral blood reticulocytes of rat using the micronucleus test following chronic exposure. Outbred female Wistar rats were treated by gavage once per week for 10 weeks with cumulative doses of 140, 250 and 500mg/kg body weight (body wt) of lead acetate. Mitomycin C (MMC) 2mg/kg body wt was used as a positive control. They evaluated the frequency of micronuclei in the peripheral blood of female rats and found that the effects are both cytotoxic and genotoxic because of a decrease in the number of polychromatic erythrocytes in the peripheral blood and an increase in frequency of micronucleated reticulocytes, respectively.

Silva-Pereira et al., (2005) studied cytotoxicity and genotoxicity of low doses of mercury chloride and methylmercury chloride on human lymphocytes culture in vitro. Short-term human leukocyte cultures from 10 healthy donors (5 females and 5 males) were set-up by adding drops of whole blood in complete medium. Cultures were separately and simultaneously treated with low doses (0.1 to 1000μg/l) of HgCl$_2$ and CH$_3$HgCl and incubated at 37ºC for 48h. Genotoxicity was assessed by chromosome aberrations and polyploid cells. Mitotic index was used as a measure of cytotoxicity. A significant increase (P<0.05) in the relative frequency of chromosome aberrations was observed for all concentrations of CH$_3$HgCl when compared to control, whether alone or in an evident synergistic combination with HgCl$_2$. The frequency of polyploid cells was also significantly increased (P<0.05) when compared to control after exposure to all concentrations of CH$_3$HgCl alone or in combination with HgCl$_2$. CH$_3$HgCl significantly decreased (P< 0.05) the mitotic index at 100 and 1000μg/l alone, and at 1, 10, 100, and 1000μg/l when combined with HgCl$_2$, showing a synergistic cytotoxic effect. The data showed that low concentrations of CH$_3$HgCl might be cytotoxic/ genotoxic.

Brender et al., (2006) examined maternal exposure to arsenic, cadmium, lead, and mercury and neural tube defects in offspring. Using a case–control study design, the relation
between exposure to these heavy metals and neural tube defects (NTDs) in offspring of Mexican–American women was investigated. A total of 184 case-women with NTD-affected pregnancies and 225 control-women with normal live births were interviewed about their environmental and occupational exposures during the periconceptional period. Overall, the median levels of these biomarkers for heavy metal exposure did not differ significantly (P=0.05) between case and control-women. However, among women in the highest income group, case-women were nine times more likely (95% confidence interval (CI) 1.4–57) than control-women to have urinary mercury X5.62mg/L. Case-women were 4.2 times more likely (95% CI 1.1–16) to report burning treated wood during the periconceptional period than control-women. Elevated odds ratios (ORs) were observed for maternal and paternal occupational exposures to arsenic and mercury, but the 95% CIs were consistent with unity. The 95% CIs of the ORs were also consistent with unity for higher levels of arsenic, cadmium, lead and mercury in drinking water and among women who lived within 2 miles at the time of conception to industrial facilities with reported emissions of any of these heavy metals.

Kumari and Ramkumaran (2006) conducted a study to assess the cytogenetic changes in an air breathing fish, Channa punctatus inhabiting the polluted water of Hussain Sagar Lake. Several structural chromosomal aberrations such as chromatid breaks and gaps, fragments, sister-chromatid exchanges, dicentric and ring type of chromosomes were observed in these fishes in comparison to the fishes collected from other non-polluted water bodies thus indicating induced mutation in fishes living in Hussain Sagar waters.

Chaurasia et al., (2007) assessed the genotoxic effects of fluoride salts in Swiss albino mice Mus musculus. The increase in frequency of chromosome anomaly was mainly due to significant increase in individual type, viz. chromatid breaks, gaps and acentric fragments. The effect was dose dependent. Fluoride salts present in the ground water might have interfered with the phagocytosis and produced oxygen free radicals that attacked the nucleophilic sites of the DNA leading to the loss of important gene segments responsible for cell growth and ageing.

Saleh and Sarhan (2007) studied the clastogenic effect of chicken food (produced by Animal Food factory located at Asser Region) using the micronucleus test in erythrocytes from peripheral blood of chicken from different farms. Examination of blood smears showed that the formation of micronuclei was 7-8 times folded in the farm chicken (fed on the factory food) as compared to positive control group (fed on natural food) and where more abundant in the same species of chicken according to the farm collection. This increase in the
formation of micronucleus indicates that feeding method and food contents causes clastogenic effects on peripheral erythrocytes of poultry chicken and might generate similar effects on the human population as consumers.

Talapatra *et al.*, (2007) scored the frequencies of micronucleated and binucleated (BN) erythrocytes in gill (peripheral) and kidney (renal) blood of two fishes, *Laboe bata* and *Oreochromis mossambica* inhabited in the pond located at industrial vicinity of thermal power plant, Kolkata, India. Highly significant differences (p<0.001) were noticed for MN frequencies in gill and kidney erythrocytes of experimental fishes, where kidney erythrocytes showed increase value than gill erythrocytes. The frequencies of BN were also counted separately for gill and kidney erythrocytes, in which significantly increased values (p<0.001) were obtained in comparison to control populations.

Tripathi and Dubey (2008) evaluated the protective effect of zinc against Cr (VI) induced genotoxicity using micronucleus test (MNT) in fresh water fish, *Channa punctatus*. Zinc had no effect in causing the formation of micronuclei compared to the respective control. Cr (VI) on the other hand significantly induced the formation of micronuclei compared to the respective control. Concomitant exposure to both metal significantly decreased the frequency of micronuclei compared to the chromium.

Ivanova *et al.*, (2008) investigated the cytotoxic and genotoxic effect of heavy metal and cyanide-contaminated water samples collected from different water sources in the region of “Assarel-Medet” Copper Refinery Works. The contents of heavy metals; copper, arsenic, cadmium, lead, and cyanides (mg.dm-3) were determined using the method of automatic photometry. The *Allium cepa* and *Pisum sativum* plant systems were used for testing of the cytotoxicity and genotoxicity of heavy metals and cyanides. A lower mitotic index and a higher frequency of chromosome aberrations were established in all test samples than in the control ones. Chromosome fragments, anaphase and telophase bridges, micronuclei, lagging chromosomes and C-mitotic effect in cells were observed. It was concluded that the pollution found in the regions for production and processing of ore has cytotoxic and genotoxic effect on cells and it could be a potential threat to water ecosystems and human health.

Reza *et al.*, (2008) evaluated the effects of exposure to 100 ppm of lead in drinking water on blood pressure and some physiologic parameters of isolated beating rat heart using the Langerdroff isolated heart apparatus. The isolated hearts were perfused with Krebs-Henseleit solution. The blood pressure in the 8- and 12- week lead exposed groups was significantly increased as compared to the control group. The results indicated that: 1) low
levels of lead exposure do not significantly affect the ECG in the early phase, 2) low levels of lead exposure causes ECG changes in the late phase of exposure, and 3) this level of lead exposure can increase HR and cardiac contractility but has no effect on coronary flow.

Syarif et al., (2008) studied the effects of lead acetate (0mg/kg bw; 1008mg/kg bw; 1327mg/kg bw; 1747mg/kg bw; 2299mg/kg bw and 3025mg/kg bw) on chromosomal aberration in mouse bone marrow, using Completely Randomized Design method. The result indicated that the maximum chromosomal aberration (62.20%) was showed in 3025mg/kg bw, and the lowest in control (17.80%). The chromosomal aberration analyzed were stickiness, broken fragment, fragment chromosome, gap chromosome, acentric chromosome, ring chromosome, double point chromosome and numeric aberration. The frequency of chromosomal aberration increased along with the lead acetate concentration.

Hong-fei et al., (2010) conducted a field survey concerning lead and cadmium pollution in environment mediums and hair samples around the Chatian mercury mining deposit in western Hunan Province, China, to preliminarily evaluate their health hazard to local inhabitants. The results show that mining wastes, especially tailing, contain high cadmium level with the maximum of 79.92μg/g. High levels of lead and cadmium got accumulated in surface water and paddy soil, respectively, and both metals pollution occurred in brown rice. The average daily intake dose (ADD) of lead for local adults via three routes reaches up to 7.7μg/(kg/day), exceeding the provision tolerable daily intake by JECFA of 3.5μg/(kg/day), and drinking water exposure route contributes the highest daily intake. As an indicator for heavy metal exposure, the hair of local population contains Pb (5.06±3.02)μg/g. The average daily intake dose of cadmium for adults was 0.119μg/(kg/day). More attention must be paid on health risk from lead pollution compared with cadmium.

Missoun et al., (2010) investigate the effect of lead exposure on kidney function. Fourteen male Wistar rats were divided into 2 groups; group 1 was given a tap water diet and group 2 was given 1000 ppm lead acetate in drinking water for 8 weeks. Lead concentration in blood was determined by atomic absorption. The results showed an increase of calcium in blood. The same happened for phosphaturia and calcium in rats administered with Pb compared to control group. The increase of these parameters indicated a renal deficiency which was confirmed by a decrease of creatinine and urea in urine samples and presence of calcium oxalate dihydrate crystals observed in samples of urine of exposed rats. All lead-treated rats showed intranuclear inclusion bodies in kidney proximal tubular. The
determination of the concentration of lead in the blood showed that this factor increases among treated rats and lead administered by oral route causes a renal deficiency to the rats.

Pandey and Agrawal (2010) evaluated the preventive effect of Bauhinia variegate bark extract against cyclophosphamide induced micronucleus formation in the mouse bone marrow cells. The single intraperitoneal administration of B. variegate bark extract the dose of 125, 250 and 375mg/kg body weight, 24 hours prior the administration of cyclophosphamide (at the dose of 50mg/kg) have significantly prevented the micronucleus formations in dose dependent manner in bone marrow cells of mice as compared to group. However, B. variegate bark extract alone has not induced micronucleus formations in bone marrow cells as compared to control group. Therefore seems to have a preventive potential against cyclophosphamide induced micronucleus formation in Swiss mouse bone marrow cells.

Da-Rocha et al. (2011) analyzed the micronuclei (MNs) and other nuclear abnormalities (NAs) frequencies in peripheral blood of Nile tilapia (Oreochromis niloticus) treated with potassium dichromate via contaminated water. Blood samples were collected from caudal vessels after potassium dichromate 12mg/L exposition for 24 and 48 hours. The typical micronuclei were not found in control group. In the exposed groups the frequencies were 1.0 ± 1.15 at 24h and 2.43 ± 0.98 at 48hrs. The nuclear morphological alterations frequencies in control, 24h and 48h were 4.29 ± 4.50, 5.86 ± 3.02 and 11.0 ± 3.74, respectively. The parametric ANOVA showed a very significant difference (p<0.01) in MNs frequencies between control and 48h groups; also there was significant difference (p<0.05) between the two exposition times. In the NAs, there was significant difference only between control and exposed for 48hrs groups (p<0.05). Results confirmed the potentially adverse effects of potassium dichromate. The demonstrated sensitivity to this ion showed that Oreochromis niloticus can be used to monitor the acute effects of pollutants on the basis of hexavalent chromium in freshwater ecosystem

Frohne et al., (2011) used an automated biogeochemical microcosm system allowing controlled variation of redox potential (EH) in soil suspensions to assess the effect of various factors on the mobility of mercury (Hg) as well as on the methylation of Hg in two contaminated floodplain soils with different Hg concentrations (approximately 5mgkg⁻¹ Hg and >30mgkg⁻¹ Hg). The experiment was conducted under stepwise variation from reducing (approximately −350mV at pH 5) to oxidizing conditions (approximately 600mV at pH 5).
Results of phospholipid fatty acids (PLFA) analysis indicates the occurrence of sulfate reducing bacteria (SRB) such as Desulfobacter species or Desulfovibrio species, which are considered to promote Hg methylation. The products of the methylation process are lipophilic, highly toxic methyl mercury species such as the monomethyl mercury ion [MeHg+], which is named as MeHg here. The ln(MeHg/Hg\text{t}) ratio is assumed to reflect the net production of monomethyl mercury normalized to total dissolved Hg (Hg\text{t}) concentration. This ratio increases with rising dissolved organic carbon (DOC) to Hg\text{t} ratio (lnDOC/lnHg\text{t} ratio) (R² = 0.39, p<0.0001, n = 63) whereas the relation between ln(MeHg/Hg\text{t}) ratio and lnDOC was weaker (R² = 0.09; p<0.05; n = 63).

Mitkovska et al., (2012) conducted environmental genotoxicity evaluation using a micronucleus test and frequency of chromosome aberrations in free-living small rodents. An in vivo micronucleus (MN) test in peripheral erythrocytes and frequency of bone marrow cells with chromosome aberrations in free-living small rodents, chronically exposed to heavy metal pollution were used for detection the genome response to genotoxic agents in the environment. Yellow-necked mice (Apodemus flavicollis), common vole (Microtus arvalis) and East-Mediterranean (Macedonian) mice (Mus macedonicus) were collected in a polluted region near lead-zinc smelting factory – Asenovgrad (South Bulgaria, near Plovdiv) and in the background region of the Strandzha National Park (Southeastern Bulgaria). Mean frequencies of MN and aberrant cells in the individuals from the impact region were significantly higher compared to the mean frequencies from the same species in the background region. The comparative analysis of results confirmed that the species Apodemus flavicollis and Microtus arvalis may be suitable bioindicators for biomonitoring studies using MN test and chromosome aberrations. Obtained results demonstrated that the in vivo MN test may be a sensitive end-point for the detection of genotoxicity that may result from the simultaneous action of several metals and may be useful as a biomarker of environmental stress in situ.

Parveen and Shadab (2012) evaluated the genotoxic effect of heavy metal in Channa punctatus through the micronucleus test, chromosomal aberrations and sister chromatid exchange. The fish were kept separately in 0.5, 1.0, 2.0 and 5.0 ppm cadmium chloride for 3 days. Mean frequency of micronuclei in blood observed were 0.10, 0.15, 0.24, 0.34 and 0.39 in control, 0.5, 1.0, 2.0 and 5.0 ppm CdCl₂ respectively. In vivo chromosome preparation from kidney tissues was carried out. The mean frequency of cells with aberrations observed was 0.13, 0.20, 0.34, 0.60 and 0.95 in control, 0.5, 1.0, 2.0 and 5.0 ppm CdCl₂ respectively.
Likewise the mean frequency of SCE observed was 0.05, 0.16, 0.36, 0.44 and 0.52 in control, 0.5, 1.0, 2.0 and 5.0 ppm CdCl₂ respectively. It was revealed from the results of the study that cadmium produced genotoxic effects in fish.

Rudnicka et al., (2012) studied an in vitro culture of V79 cells of the Chinese hamster subjected to three tested PAHs: 5-amino-2,3-dihydro-1H-cyclopenta[c]phenanthrene (ACP[c]Ph), 5-amino-9-methoxy-2,3-dihydro-1H-cyclopenta[c]phenanthrene (AMCP[c]Ph) and cyclopenta[c]phenanthrene (CP[c]Ph). The in vitro micronucleus (MN) assay was applied in order to evaluate the genotoxic properties of the studied compounds. The highest genotoxic effect was observed for AMCP[c] Ph in a concentration of 0.0200μg·ml⁻¹. The genotoxic effect of the other two compounds was slightly lower.

Zhang et al., (2012) investigated the potential genotoxicity of Z. spinosi in vitro and in vivo. This was examined by the Bacterial reverse mutation (Ames) test using Salmonella typhimurium TA98, TA100, TA1535, TA1537 and Escherichia coli WP2uvrA. Chromosomal aberration was investigated using Chinese hamster lung cells and the micronucleus test using mice. Z. Spinosi did not induce mutagenicity in the Ames test and it did not produce chromosomal aberration in Chinese hamster lung cells with and without metabolic activation, nor in the micronucleated polychromatic erythrocytes in the bone marrow cells in mice concluding the result that Z. spinosi does not have mutagenic potential under the conditions examined in each study.

Rasgele et al., (2013) investigated the genotoxic effects of cobalt chloride and copper chloride in mouse bone marrow cells using the micronucleus (MN) assay. The three different concentrations of cobalt chloride (11.2, 22.5 and 45mg kg⁻¹) and copper chloride (1.17, 2.35 and 4.70mg kg⁻¹) were injected intraperitoneally to mice for 24 and 48 hours. It was observed that both of these heavy metals induced a significant increase in frequency of micronucleated polychromatic erythrocytes (MNPCE) at different concentrations in mice for 24 and 48 hours when compared with the control. Furthermore, the significant reduction for the polychromatic erythrocyte/ normochromatic erythrocyte (PCE/NCE) ratio which is indicative of bone marrow cytotoxicity was observed in bone marrow cells which were treated with copper chloride at all concentrations for 24 and 48 hours. No reduction of the PCE/NCE ratio was observed both 24 and 48 hours after all the doses of cobalt chloride tested as compared to the negative control. These results lead to the conclusion that copper chloride may have genotoxic and cytotoxic properties due to induction in the frequency of
MN and a reduction in PCE/NCE ratio in bone marrow cells of mice, whereas cobalt chloride induced only genotoxic effect in mice bone marrow.

Hayretdag et al., (2014) studied the mean micronucleated erythrocyte (MNE) frequencies and nuclear abnormalities in the peripheral blood erythrocytes of some Colubrid snakes from Turkey: Coronella austriaca, Dolicophis schmidti and two Natrix tessellata specimens collected from Vize (Kırklareli), Kızılcahamam (Ankara) and Digor (Kars). Different numbers of micronuclei were recorded in all snake specimens examined and in addition, three types of nuclear abnormalities were detected: nuclear bud, notched, and blebbed nuclei. The mean MNE frequency (%) was determined to be highest (0.30±0.08) in the N. tessellata specimen caught from Kızılcahamam, the lowest (0.13±0.07) in the D. schmidti specimen caught from Digor. Nuclear abnormalities were encountered in different numbers in the specimens under examination. As a result of this examination on snakes inhabiting three regions of Turkey with different demographical and physical geographical characteristics (Thrace, Central Anatolia, and Eastern Anatolia), they conclude that similar genotoxic effects reported in the erythrocytes might occur widely due to environmental pollutant impacts.

Iji and Adeogun (2014) evaluated the cytotoxic effect of effluents at inducing chromosomal aberrations, using this as a biomarker tool in wild Clarius pachynema for assessing and monitoring pollution of the aquatic environment. A total of 60 live fish (30 each downstream and upstream) were obtained and subjected to chromosomal analysis. Chromosomal aberration in the fish samples from downstream sector was recorded at a rate of 30%, while there were no aberrations in the samples collected upstream the effluent discharge point. Water sample analysis revealed a high concentration of ammonium and nitrates above permissible standards of Federal Environmental Protection Agency (FEPA) guidelines. Heavy metal analysis also revealed the presence of Cr, Cu, Pb, Zn and Fe above permissible standards from the downstream section of the river which revealed that the ever increasing discharge of effluents from the industry increased chromosomal damage in the aquatic components.

2.2 GENOTOXICITY IN AMPHIBIANS

Amphibians are generally believed to be sensitive to environmental perturbations, partly because of their central place in the food chain (being both prey and predator), because
they often utilize both terrestrial and aquatic habitats, and can have very different feeding ecologies at different stages of their life cycles. Frogs are the amphibians that have been shown to be sensitive to a range of environmental pollutants (Tyler, 1989) including agricultural pesticides such as Chlordane (Kaplan and Overpeck, 1964), DDT (Osborn et al., 1981), Dieldrin (Cooke, 1972; Brooks, 1981); herbicides such as Defenuron (Paulov, 1977 and heavy metals such as Mercury (Birge et al., 1977), Copper (Bando, 1976) and Zinc (Byrne et al., 1975). Rarely frogs have been used to monitor unspecified environmental pollutants. The potential to use frogs as environmental indicators was firstly done in Australia to monitor changes in water quality of local streams in the development area of the Ranger Uranium Mine in the Northern Territory (Tyler and Cappo, 1983).

Chang et al., (1974) observed the effects of methylmercury chloride on Rana pipiens tadpoles. Some of the tadpoles were allowed to be raised in water containing various concentrations of methylmercury chloride and also some were injected with 0.025ml of 0.1% aqueous solutions of methyl mercury chloride (0.025mg Hg), every other day for 10 days. All the tadpoles which were raised in water containing more than 0.05ppm mercury died within 48 hours. The distention of body cavities and eventual death of these tadpoles was believed to be the result of a disturbance of the osmotic regulator system by the mercury. Total arrest of further development and differentiation was observed in tadpoles which were raised in water containing 0.001-0.01 ppm mercury. Extensive deposition of blood pigment (hemosiderosis) was observed in the livers of mercury injected tadpoles. Such hemosiderotic condition was thought to be predisposed by hemolysis of the RBCs by mercury followed by severe peripheral edema and hemopoietic reactions in the kidneys of these tadpoles.

Terhivuo et al., (1984) studied the mercury content of common frogs (Rana temporaria) and common toads (Bufo bufo) collected in southern Finland. In southern Finnish rural municipality of Tuulos, which has a low environmental mercury load, the mean mercury contents of the common frog’s liver, kidney, muscle, lung and eggs ranged from 0.03 to 0.08mgHg/kg fresh weight. In two sample localities within the cities of Helsinki and Porvoo the range of the corresponding means was somewhat wider, viz. 0.03-0.19mgHg/kg fresh weight, but statistically significant differences were found only between the mean mercury content of the liver in the frogs from Tuulos and those of the frogs from both Helsinki and Porvoo. In Lake Kirkkojarvi in Hameenkyro, badly polluted by phenyl mercury compounds from a paper mill, only a minimal mercury load (less than 0.05mgHg/ fresh weight) were recorded in the few small specimens studied. In common toad (Bufo bufo) from
the unpolluted Tuullos area the mean mercury content was highest in liver (0.12 ppmHg/kg fresh weight) and lowest in muscle tissue (0.03 ppmHg/kg fresh weight). It was noted that the mean mercury content in the liver of southern Finnish frogs and toads was 3-10 times lower than in that of the same species from unpolluted areas of Yugoslavia and was as much as 110-420 times lower than in those from the mercury mining areas of Idrija in Yugoslavia. With regard to the other tissues (kidney, muscle and lung), the corresponding differences were somewhat smaller

Pathan and Nadkarni (1986) studied the effect of calcium rich environment on the ultimobranchial bodies of *Rana cyanophlyctis* treated with calciferol. The frog when injected with calciferol and placed in 0.8% solution of Calcium chloride showed an increase in the height of follicular epithelium, formation of secondary follicles and obliteration of the follicular lumen as compared with that of control and also there was heavy deposition of calcium carbonate in the paravertebral lime sacs. The observation indicated that hypercalcemic condition might have stimulated the ultimobranchial gland to secrete calcitonin which prevented excessive calcium mobilization.

Rao and Madhyastha (1987) conducted static bioassays to determine the relative acute toxicities of five heavy metals (mercury, cadmium, copper, manganese and zinc) to 1-week-old and 4-week-old tadpoles of the frog, *Microhyla ornata*. Toxic effects were calculated on the basis of LC$_{50}$ for 24h, 48h, 72h and 96h exposures at 25.5-26.0°c. Mercury was the most toxic and zinc was the least toxic of the heavy metals tested. The sensitivity of the tadpoles to the heavy metals increased with increased age.

Ruifang (1991) discussed approach to analysis of micronuclei (MN) and other nuclear anomalies (ONA) in erythrocytes of toad (*Bufo bufo andrewsi*) tadpoles, induced by exposure to polluted water of Daguan River. Tadpoles were exposed to polluted water of three different concentrations (33.3%, 50.0% and 100.0%) for 7 days. In the control, total frequency of nuclear anomalies (including micronuclei) was 4.1%. Total frequencies of MN and ONA in erythrocytes of tadpoles, showed distinct dose dependent increase. The result showed that MN and ONA in erythrocytes of toad tadpole is useful index for detecting genotoxic compounds in aquatic environment.

Kanamadi and Saidapur (1992) undertook a study on the effects of exposure to sublethal Mercuric chloride on the testis and fat body of frog *Rana cyanophlyctis*. Adult male frogs were kept in aquaria containing different concentration of mercuric chloride and the
experiments for LC$_{50}$ were carried out within the range (1-10mg/l). The result suggested that the sub lethal concentration of mercuric chloride inhibits spermatogenesis in *R. cyanophlyctis* as mercury causes severe damage to primordial germ cells in eggs prior to the first cleavage and results in reduced germ cell proliferation. Fat bodies in male *R. cyanophlyctis* are known to indicate nutritional status and the significant decrease in the percent mass of fat bodies in mercuric chloride-exposed frogs may be due to decreased intake of food.

Punzo (1993) studied the effect of mercuric chloride on fertilization and larval development in the river frog, *Rana heckscheri*. Exposure of gametes to 0.5mg/l mercuric chloride has no adverse effect on fertilization success. At 1.0mg/l, fertilization success (69%) decreased significantly. A further significant decrease was (27%) was observed at 2.5mg/l. At 5.0mg/l, fertilization was completely blocked in this species but exposure 5.0mg/l had no observable adverse effect on development as compared to control frogs. At conc.$\geq$ 1.0mg/l, significant development abnormalities were observed.

Yeliseeva *et al.*, (1995) conducted cytogenetic monitoring of amphibian and rodent populations and children from the radio contaminated regions of the Republic of Belarus as a follow up to Chernobyl nuclear accident. A statistically significant increase in the levels of cytogenetic damage in bone marrow cells of amphibians and rodents and in peripheral blood lymphocytes of children was found. The presence of chromosomal aberrations supports the conclusion that radiation is the causative agent. However, no direct relationship between the level of radionuclide contamination and the degree of the cytogenetic damage was found.

Ralph *et al.*, (1996) examined erythrocytes in two species of tadpoles, *Rana clamitans* and *Bufo americans*, using the alkaline single-cell gel (comet) assay. Time-dose experiment using methyl methanesulphonate (MMS) suggested that the peak level of DNA damage in *R. clamitans* tadpoles occurred 42 hr after exposure. *B. americans* tadpoles exposed to 6.25mg/l of MMS for 12 hours had a significant increase in DNA damage over that seen in the controls.

Ralph and Petras (1997) examined tadpole erythrocytes of two species, *Rana clamitans* and *Rana pipiens* using the alkaline single cell gel DNA electrophoresis (SCG) or comet assay to monitor genotoxicity in small bodies of water (e.g., creeks, ponds and drainage ditches). The higher levels of DNA damage in tadpoles collected from agricultural areas may be due to the pesticides used and the increased variation in DNA damage in the same areas is likely due to the impact of crop rotation, including leaving fields fallow, the
timing of rainfall and/or the application of pesticides. *R. clamitans* tadpoles, especially those collected from agricultural areas, also showed significant seasonal variation in DNA damage. This study indicated that both species are suitable for use in the alkaline SCG assay and as *in situ* sentinel organisms for environmental biomonitoring.

Calevro *et al.*, (1998) investigated the toxicity and teratogenicity in three different species of amphibians treated with chromium (Cr\(^{3+}\)), aluminium (Al\(^{3+}\)) and cadmium (Cd\(^{2+}\)) by examining their developmental abnormalities and mortality in embryos. Chromium and aluminium were lethal at 1.5mM concentration and seriously affect the differentiation of central nervous system, skeleton and eye and caused cephalic and trunk oedemas at lower concentrations, being aluminium significantly more harmful than chromium. Cadmium was found to be highly toxic as embryos exposed to concentrations ranging from 0.18 μm to 50 μm display malformations, delay and arrest of development in a dose dependent manner.

Ralph and Petras (1998) evaluated caged amphibian tadpoles and *in situ* genotoxicity monitoring of aquatic environments with the alkaline single cell gel electrophoresis (comet) assay. To determine it *Rana clamitans* (green frog) and *Bufo americanus* (American toad) tadpoles were housed in cages at 11 sites in southwestern Ontario (Canada). In a preliminary experiment, it was found that tadpoles caged at a polluted reference site (Tall grass Prairie Heritage Park in Windsor, Ontario) for either 7 or 14 days showed significant (*P*<0.05) increases in DNA damage, relative to tadpoles caged in the laboratory in dechlorinated water. As a result a 7 day exposure time was used. Significantly (*P*<0.05) increased levels of DNA damage, relative to their controls, were observed in tadpoles caged at three sites along two creeks draining a large petrochemical installation south of Sarnia, Ontario; at two sites in the Tall grass Prairie Heritage Park; and at a site along the Ecarte Channel which is part of the St. Clair River. The DNA damage levels of animals caged in Lake St. Clair, in the Trenton Channel of the Detroit River, at a landfill site, and in two creeks in the city of Windsor did not differ significantly (*P*>0.05) from their controls. This study demonstrates that caged tadpoles are suitable for monitoring most bodies of fresh water, particularly those aquatic habitats mentioned above where indigenous tadpoles are not present.

Schuytema and Nebeker (1999) studied the effects of ammonium nitrate, sodium nitrate and urea on red-legged frogs, pacific tree frogs and African clawed frogs. *Rana aurora* egg masses were collected from small ponds in the foothills of the Coast Range (Benton County). *Pseudacris regilla* tadpoles were raised from egg masses collected in Corvallis and
Xenopus laevis tadpoles were raised from eggs from an in-house breeding colony. All three species were maintained and tested under a 16:8 light: dark cycle. There was no mortality in the controls from the ammonium nitrate, sodium nitrate and urea tests. All R. aurora embryos died at concentrations of ≥ 105mg/L NH₄-N in the ammonium nitrate test and at 918mg/L NO₃-N in the sodium nitrate test. The 16-d LC50s (median lethal concentration) were 71.9mg/L NH₄-N and 636.3mg/L NO₃-N. The levels of NO₃-N present in solutions containing sufficient NH₄-N to cause adverse effects were insufficient to have an effect on survival or growth. This indicates the ammonium ion to be more toxic and to be the primary cause of mortality in ammonium nitrate exposures. Responses to urea were very similar between P. regilla and X. laevis. All died at a concentration of 15,000mg/L N, with 7% or less mortality at lower concentrations. The results indicated urea in its pure form to affect tadpoles only at extremely high concentrations. The ammonium ion appears to be the main contributor of toxicity of ammonium nitrate to amphibians while urea appeared to be relatively non-toxic to amphibians at normally used concentration.

Vinod and Naik (1999) analyzed the effect of lead acetate on the bone marrow chromosomes of frog, Haplobatracus tigerinus and found that significant increase in chromosome aberrations even after 48hrs with high dose and also after exposure of frogs to the higher dose of solution for 14 days.

Djomo et al., (2000) investigated amphibian micronuclei test in vivo (Jaylet test) to evaluate the genotoxicity of petrochemical waste waters which proceed from industrial processes after physicochemical and biological treatments into the Midouze River, southwest of France. The industry, whose waste waters were tested, refines crude oils and also produces sulphur and chlorine. The samples were collected at three sites: the waste from discharge point after treatment, the water without petrochemical effluent taken upstream of effluent outlet and effluent mixed to water, downstream of effluent outlet. The larvae of Pleurodeles waltl were exposed for 6 days to increasing concentrations of test waste water. At all concentrations tested, no toxic effect was observed with water collected upstream and downstream. Larvae reared in presence of the effluent taken at discharge point (at 1000ml/L) showed a light inability to catch and eat Daphnia. The results concerning the genotoxicity test revealed that the effluents from discharge point, positive response were obtained at 250 and 500ml/L with a dose effect. Compared to the negative control, the levels of micronuclei (MN) observed at 125, 250 and 500ml/L were, respectively, 1.5-fold, 3-fold and 6.5- fold
higher. The formation of MN was attributed to the action of many classes of aromatic and mineral compounds.

Kryukov (2000) studied the effects of lead on induction of micronuclei in anuran larvae. Groups of seven tadpoles were placed for 6, 12, 18, and 24 h in lead nitrate solutions with the final Pb$^{+2}$ concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0mg/L, which corresponded to 6.7, 13.3, 20.0, 26.7 and 33.3 MACs. Six-hour exposure was used in additional experiments with higher concentrations of lead: 1.4, 2.8, and 5.6mg/l (46.7, 93.3, and 186.7 MACs, respectively). Tadpoles not exposed to the effect of lead served as a control. The result revealed that the frequency of cells with micronuclei increased proportionally to the lead concentration and the duration of its action on the animals. Upon six-hour exposure, a statistically significant increase in the frequency of micronuclei was revealed under the effect of lead concentrations exceeding 1.0mg/l. Upon longer exposure (12, 18, and 24 h), this increase was observed at a concentration as low as 0.2mg/l. At a lead concentration of 1mg/ml and long periods of exposure (18 and 24 hrs), the clastogenic effect proved to decrease slightly. This was probably associated with an increase in the overall toxic effect of Pb$^{+2}$.

Lienesch et al., (2000) studied the effect of cadmium administration on oocyte development in Xenopus laevis. Adult female Xenopus were injected in the dorsal lymph sac with cadmium chloride (CdCl$_2$) at doses of 0.5, 0.75, 1.0, 3.0 or 0.5mg/kg every other day for 21 days. Significant adverse effects of Cd on oocyte development were observed. The percentage of oocytes at all stages of oogenesis was decreased while the population of atretic oocytes increased dramatically (p<0.0001). Numerous oocytes exhibited a speckled or mottled appearance. The observation indicated that Cd has the potential to significantly disrupt oogenesis and that examination of developing gametes may be a useful parameter for assessing the influence of environmental contaminants on reproductive capacity.

Vinod and Naik (2000) observed the occurrence of increase in dicentric chromosomes after nickel chloride (NiCl$_2$) treatment, breaks and hypodiploidy after mercuric chloride (MgCl$_2$) treatment and pulverization after cadmium chloride (CdCl$_2$) treatment in the Indian frog (Hoplobatractus tigerinus). The study reveals that HgCl$_2$ was most toxic followed by CdCl$_2$ and NiCl$_2$.

Ugurtas et al., (2001) reported a cytogenetic study on the Pelobates syriacus (Amphibia: Anura) in Bursa-turkey. In this study, 10 females and 6 males, a total of 16
specimens, were examined. The specimens were collected from near to Nilufer Stream, Bursa. 16 hours before sacrifice, 0.3% colchicine solution was injected under the skin into the ventrum. Mitotic chromosomes were prepared from the bone marrows of both the femur and humerus. The bone marrow was flushed out with 0.075 M KCl from the bones and incubated for 7 min at 37°C. Then this material was centrifuged at 1400 rpm for 10 min and fixed in methanol: acetic acid (3:1). The results showed that the chromosome complement of *Pelobates syriacus* consists of 26 chromosomes. The seventh pair was acrocentric, the thirteenth pair was metacentric and the remaining pairs were submetacentric.

Bulog et al., (2002) investigated the concentrations of some essential (Cu, Zn, Se) and some toxic elements (Hg, As) were determined in tissues (liver, kidneys, integument, and muscle) of the endemic cave salamander, *Proteus anguinus* and *Proteus anguinus parkelj* captured in the Planina Cave, Kompolje Cave, and Jelsevnik, all situated within the Dinaric Karst in Slovenia. The highest amounts of selenium and mercury were found in the liver of *Proteus anguinus* specimens from all three localities. Some of the animals from the Planina Cave showed higher concentrations of copper in the liver and muscle. Pigmented subspecies of *Proteus* from Jelsevnik showed increased values of arsenic in the integument and of zinc in the liver and integument. The liver of the animals contained the highest amounts of the metals analyzed and therefore considered as a target organ. Metal levels were also measured in the cave water and sediments. The waters were not polluted with metals. However, the metals were relatively high in the sediments, but not to levels considered contaminated.

Blaustein et al., (2003) reviewed the contribution of increasing UV radiation and environmental contamination to the global decline of amphibian populations and both affect amphibians at all life stages. Exposure to UV radiation and to certain contaminants can kill amphibians and induce sub lethal affects in embryos, larvae and adults. Moreover, UV radiation and contaminants interact with one another synergistically. Synergistic interactions of UV radiation with contaminants enhanced the detrimental effects of the contaminant and UV radiation.

Campana et al., (2003) evaluated pyrethroid lambda-cyhalothrin genotoxicity using the micronucleus test in *Rana catesbeiana* tadpoles. The effects of concentration and exposure time on the micronuclei frequency were studied in blood smears obtained from tadpoles exposed to four concentrations (0.02, 0.1, 0.2 and 0.4μg/L) of the compound for 24, 48, 72 and 96 h and 8, 15, 20 and 30 days. As a positive control, tadpoles were exposed to
cyclophosphamide (5mg/L). The micro nucleated cell frequency was expressed per 1,000 cells. *R. catesbeiana* tadpoles exposed to increasing concentrations of lambda-cyhalothrin showed an increase in the micronuclei frequency in peripheral blood. Tadpoles exposed to cyclophosphamide (CP) also showed a significant increase in micro nucleated erythrocytes which peaked after 15 days.

Selvi *et al.*, (2003) determined the 96-h LC_{50} value of cadmium chloride (CdCl\textsubscript{2} \cdot H\textsubscript{2}O), a metal salt widely used in industry, in the water frog (*Rana ridibunda*). The experiments were conducted in two series and a total of 140 frogs were used to determine acute toxicity. In addition, behavioral changes in the water frog were determined for each cadmium chloride concentration. Data obtained from the cadmium chloride investigation were evaluated by using the prohibit analysis statistical method and the 96 h LC\textsubscript{50} value for water frog was estimated to be 51.2mg/L. Frogs, subjected to different cadmium chloride concentrations, showed various behavioral disorders such as loss of balance, respiratory difficulty, slowness in motion, capsizing in water, sinking down to bottom and increased mucus secretion. These toxic effects increased, as the dose increased.

Feng *et al.*, (2004) conducted acute toxicity test, micronucleus (MN) test and comet assay of the two pesticides on amphibians. The values of LC\textsubscript{50}-48 h of imidacloprid were found to be 165mg /L for tadpoles of *Rana limnocharis* and 219mg /L for tadpoles of *Rana nigronaculata*. On the other hand, RH-5849 showed no acute toxicity to tadpoles during the 96 h exposure even it was saturated in the test solutions. There were significant differences in the MN frequencies between the negative controls and the treated groups at the dose of 8mg /L for imidacloprid (p < 0.05) and 40mg/L for RH-5849 (p< 0.01). Comet assay found significant differences (p< 0.01) in the distributions of DNA damage grades between the negative controls and groups treated in vitro with 0.05, 0.1, 0.2 and 0.5mg/L of imidacloprid and 5, 25, 50 and 100mg/L of RH-5849, respectively. DNA damage scores increased with the exposure levels of the two pesticides and dose–effect relationships were observed for both imidacloprid (r\textsuperscript{2}=0.92) and RH-5849 (r\textsuperscript{2} =0.98). The MN test and comet assay revealed potential adverse effects of the two pesticides on DNA in the erythrocytes of amphibians in aquatic and agricultural ecosystems.

Gauthier *et al.*, (2004) monitored the potential genotoxic effects and the detoxifying activities induced in organisms exposed to the water of river Dadou (Tarn, France). Two in vivo bioassays were performed in laboratory experiments, using larvae of the amphibian
Xenopus laevis. The first was the micronucleus test, using red blood cells and the second was the assay of ethoxyresorufin-o-deethylase (EROD) induction in the liver of exposed animals. Eight water samples were taken from the river and at outlet points from the two major industrial activities of the studied section of the water course: a spar-fluor mine and a water treatment plant. Genotoxic impact and EROD induction were measured in the larvae. The effluent of the filter-washing process from the water treatment plant was found to be particularly genotoxic, even after dilution in pure reconstituted water, but no particular genotoxicity was found, either in Dadou river water, or in the effluents from the mine. On the other hand, most of the water samples tested produced a clear induction of EROD activity compared to the level of enzymatic activity found in the liver of larvae reared in the river water sampled upstream of the industrial activities. These results were interpreted taking into account (i) the high concentrations of pollutants (fluorine and manganese) measured in the river water, (ii) the very low population levels inventoried in the downstream section of the river and (iii) the possible interactions between the substances present in the river water, particularly the classical EROD inducers PAHs and PCBs.

Haywood et al., (2004) assessed the toxicity of bio-available Zn, Cu, Pb, and Cd on the life stages of Xenopus laevis embryos and tadpoles. Cu and Cd were found to affect the hatching success of the embryos, with a strong negative relationship existing between the increase in Cu concentrations and the hatching of the embryos. Only concentrations above 0.6 ppm Cd affected the hatching of the embryos. All metals affected the survival of tadpoles over the seven days of exposure, with Zn and Cu showing a steady linear toxicity and Pb and Cd a threshold toxicity effect. Metals affected the growth of the tadpoles by reducing body length with increasing concentrations. An increase in the concentration of each metal resulted in an increase in the frequency and severity of malformations among the tadpoles. The percentage of malformed tadpoles was dependent on the metal and concentration to which they were exposed. Their data describe the relationships among hatching success, survival, growth rate and the frequency of malformations of tadpoles in relation to different concentrations of the four metals. Xenopus laevis embryos and tadpoles were used successfully in bioassays to biologically quantify the severity of Zn, Cu, Pb and Cd pollution.

James et al., (2004) evaluated the effects of multiple routes of cadmium exposure on juvenile American toads (Bufo americanus) using environmentally relevant concentrations. During or after exposure, toads were individually hibernated for 172 days at approximately 4°C. The following experiments were conducted: (1) dermal exposure (hibernation in soil
contaminated with up to 120μg Cd/g (dry weight)); (2) injection exposure (single injection with cadmium to achieve a maximum whole-body nominal concentration of 3μg Cd/g (wet weight) 12 days before hibernation in uncontaminated soil); and, (3) oral exposure (feeding with mealworms containing ≤16μg Cd/g (dry weight) for 50 days before hibernation in uncontaminated soil). No prehibernation mortality occurred in the injection and oral exposure studies. There was a significant treatment effect on whole-body cadmium concentration in toads orally or dermally exposed and on percent of cadmium retention in toads orally exposed. There was also a trend of increased time-to-burrowing and more toads partially buried with greater cadmium concentration in the dermal study, which indicated avoidance. In all 3 experiments, no significant differences were found among cadmium treatments in hibernation survival, percent of mass loss, or locomotors performance. However, toads fed mealworms averaging 4.7μg Cd/g (dry weight) had only 56% survival compared with 100% survival for controls.

Rollins-Smith et al., (2004) developed an amphibian model to test the effects of xenobiotic chemicals on development of the hematopoietic system. Diploid (2N) embryos (16–20hrs of age) of the South African clawed frog, *Xenopus laevis*, were exposed to 10μg/ml diazinon or 10⁻⁶ M lead acetate for 2 h. After 2 h, the ventral blood island (VBI) was transplanted from a chemically treated or untreated control embryo to an untreated triploid (3N) host embryo. After 55 days, the contribution of the donor VBI-derived stem cells to populations in the blood, thymus and spleen was assessed by flow cytometry. Diazinon, but not lead acetate, interfered with the ability of transplanted stem cells to contribute to hematopoiesis.

Bank et al., (2005) reported concentrations of methyl Hg (MeHg) and total Hg in larval northern two-lined salamanders (*Eurycea bislineata*) collected from streams in Acadia National Park (ANP), Maine, and Bear Brook Watershed, Maine (BBWM; a paired, gauged watershed treated with bimonthly applications (25kg/ha/yr) of ammonium sulfate [(NH₄)₂SO₄] since 1989), and Shenandoah National Park (SNP), Virginia. MeHg comprised 73–97% of total Hg in the larval salamander composite samples from ANP. At BBWM, significantly higher total Hg levels in larvae from the (NH₄)₂SO₄ treatment watershed were detected. At ANP total Hg concentrations in salamander larvae were significantly higher from streams in unburned watersheds in contrast with larval samples collected from streams located in watersheds burned by the 1947 Bar Harbor fire.
Additionally, total Hg levels were significantly higher in salamander larvae collected at ANP in contrast with SNP.

Ezemonye and Enuneku (2005) examined the potential for anthropogenic metal contamination to effect amphibian survival by evaluation of acute toxicity of heavy metals cadmium (Cd) and lead (Pb) to early larval stages of *Bufo maculatus* and *Ptychadena bibroni* at varying post-hatch maturation stages (3 days, 1, 2, 3 and 4 weeks) under laboratory conditions. The larval stages were exposed to nominal Cd and Pb concentrations of 0.10, 0.15, 0.20, 0.25 and 0.50μg/L. Derived 96-hour LC$_{50}$ values decreased with increase in maturation stages in both *B. maculatus* and *P. bibroni*. Mortality also increased with concentrations and exposure time for both species. Differential acute toxicity of Cd and Pb was observed in both species, with Cd showing much higher toxicity than Pb. Tadpoles of *B. maculatus* and *P. bibroni* showed species specific sensitivity to Pb and Cd respectively.

Mouchet *et al.*, (2005) evaluated the genotoxic potential of aqueous extracts of five sediments from French channels (draining water from dredged sediments), using larvae of the frog *Xenopus laevis*. Two genotoxic end points were analyzed in larvae: clastogenic and/or aneugenic effects (micronucleus induction after 12 d of exposure) and DNA-strand breaking potency (comet assay after 1 and 12 d of exposure) in the circulating blood. Additionally, *in vitro* bacterial assays (Microtox and Ames tests) were carried out and the results were compared with those obtained with larvae. Physico-chemical analyses were also taken into account. Analytical analyses highlighted in the five draining waters a heavy load of contaminants such as metals and hydrocarbons. The results obtained with the micronucleus test established the genotoxicity of three draining waters. The comet assay showed that all 5 draining waters were genotoxic after 1 d of exposure. Although 3 of them were still genotoxic after 12 d of exposure, DNA damage globally decreased between d 1 and 12. The comet assay can be considered as a genotoxicity-screening tool. Data indicate that both tests should be used in conjunction in *Xenopus*. Bacterial tests (Ames) revealed genotoxicity for only draining water. The results confirmed the relevance of the amphibian model and the need to resort to bioassays *in vivo* such as the *Xenopus* micronucleus and comet assays for evaluation of the ecotoxicological impact, an essential complement to the physicochemical data.

Chen *et al.*, (2006) evaluated sub lethal effects of lead on northern leopard frog (*Rana pipiens*) tadpoles. Northern leopard frogs (*Rana pipiens*) were exposed to environmentally relevant concentrations of lead in water (3, 10, and 100mg/L as lead nitrate) from the
embryonic stage to metamorphosis. Survival, growth, deformity, swimming ability, metamorphosis, and lead tissue concentrations were evaluated. Tadpole growth was significantly slower in the early stages (Gosner stages 25–30) in 100mg/L treatment. More than 90% of tadpoles in the 100 mg/L treatment developed lateral spinal curvature, whereas almost all the tadpoles in the other groups were morphologically normal. Spinal deformity was associated with abnormal swimming behavior. Maximum swimming speed of tadpoles in the 100mg/L treatment was significantly lower than that in the other groups. No significant effect of lead exposure was found on percentage metamorphosis, snout–vent length, mortality, and sex ratio of metamorphs. Time to metamorphosis was delayed in 100mg/L treatment. Lead tissue concentrations in the tadpoles ranged from 0.1 to 224.5mg/kg dry mass, were positively related to lead concentrations in the water.

Mouchet et al., (2006) evaluated the toxic potential of Cd in larvae of the frog Xenopus laevis after 12 days of exposure to environmentally relevant contamination levels, close to those measured in the river Lot (France). Several genotoxic and detoxification mechanisms were analyzed in the larvae: clastogenic and/or aneugenic effects in the circulating blood by micronucleus (MN) induction, metallothionein (MT) production in whole larvae, gene analyses and Cd content in the liver and also in the whole larvae. The results show: (i) micronucleus induction at environmental levels of Cd contamination (2, 10, 30μgL−1); (ii) an increased and concentration dependent quantity of MT in the whole organism after contamination with 10 and 30 μgCdL−1 (a three- and six-fold increase, respectively) although no significant difference was observed after contamination with 2 μgCdL−1; (iii) Cd uptake by the whole organism and by the liver as a response to Cd exposure conditions; (4) up-regulation of the genes involved in detoxification processes and response to oxidative stress, while genes involved in DNA repair and apoptosis were repressed. The results confirmed the relevance of the amphibian model and highlight the complementarily between a marker of genotoxicity, MT production, bioaccumulation and genetic analysis in the evaluation of the ecotoxicological impact.

Murphy et al., (2006) conducted a study to correlate the effect of atrazine concentration on the morphology and histology of gonads of native ranid frogs collected from sites in agriculture and non-agricultural areas in Machigan. Juvenile and adult green frogs (Rana clamitans), bullfrogs (R. catesbeiana) and leopard frogs (R. pipiens) were collected in the summers of 2002 and 2003. Atrazine concentrations were below the limit of quantification at non-agricultural sites, and concentrations did not exceed 2μg/L at most
agricultural sites. One concentration that was greater than 200μg atrazine/L was measured once at one site in 2002. Hermaphroditic individuals with both male and female gonad tissue in either one or both gonads were found at a low incidence at both non-agricultural and agricultural sites, and in both adults and juveniles. Testicular oocytes (TO), were found in male frogs at most of the sites, with the greatest incidence occurring in juvenile leopard frogs. Testicular oocytes incidence was not significantly different between agricultural and non-agricultural sites with the exception of juveniles collected in 2003. Atrazine concentrations were not significantly correlated with the incidence of hermaphroditism, but maximum atrazine concentrations were correlated with Testicular oocytes incidence in juvenile frogs in 2003.

Natale et al., (2006) analyzed the acute and chronic effect of Cr(VI) assessed short-(96 h, ‘acute’) and long-term (1272 h, ‘chronic’) exposure to Cr(VI) at lethal (3 to 90mg l\(^{-1}\)) and sub lethal concentrations (0.001 to 12mg l\(^{-1}\)) on Hypsiboas pulchellus tadpoles (Fam. Hylidae) from central eastern Argentina. Fertilized eggs collected from a clean pond near La Plata (Buenos Aires Province) were used for acute and chronic toxicity testing. Assays were done under controlled laboratory conditions. Results of chronic exposure were used to assess the effect of factors such as toxicant concentration and age of organisms at the beginning of exposure on the response variables (growth, development and survival until metamorphosis). Results indicated a higher sensitivity to Cr (VI) of individuals first exposed as tadpoles than those first exposed as embryos during acute and chronic exposure. Exposure to the highest sub lethal concentrations (6 to 12mg l\(^{-1}\)) of the toxicant showed early inhibitory effects on growth of all treated organisms compensated at longer exposure periods with an increase in the growth rate compared to the control groups.

Punzo and Law (2006) conducted studies to assess the effects of nitrate-related compounds on survival, growth, and hematological responses in tadpoles of the Cuban tree frog, Osteopilus septentrionalis . Stage-25 tadpoles were exposed to a nitrate dilution series and exposed to distilled water (controls), 40 ppm nitrate, or 100 ppm nitrate. Survivorship was significantly higher for control animals as compared to those exposed to 40 and 100 ppm nitrate. Total blood Hb concentrations were not significantly altered by exposure to sodium nitrite, and a significant positive correlation was found between methemoglobinemia (MHb) and nitrite concentration over the test range of 1.0 to 50.0 mg/l. Percentage Hb was significantly correlated with nitrite concentration. Percentage MHb for all treatment groups was significantly higher (18.4 to 45.3 %) than that of controls (5.4 %).
Sparling et al., (2006) studied the effect of lead contaminated sediment on *Rana sphenocephala* tadpoles, which were laboratory-raised from early free-swimming stage through metamorphosis at lead concentrations of 45, 75, 180, 540, 2360, 3940, 5520, and 7580 mg/kg dry weight in sediment. Corresponding pore water lead concentrations were 123, 227, 589, 1833, 8121, 13,579, 19,038, and 24,427 μg/L. Tadpoles exposed to lead concentrations in sediment of 3940 mg/kg or higher died within 2 to 5 days of exposure. At lower concentrations, mortality through metamorphosis ranged from 3.5% at 45 mg/kg lead to 37% at 2360 mg/kg lead in sediment. The LC50 value for lead in sediment was 3728 mg/kg (95% CI=1315 to 72,847 mg/kg), which corresponded to 12,539 μg/L lead in pore water (95% CI= 4000 to 35,200 μg/L). Early growth and development were depressed at 2,360 mg/kg lead in sediment (8100 μg/L in pore water) but differences were not evident by the time of metamorphosis. The most obvious effect of lead was its pronounced influence on skeletal development. Whereas tadpoles at 45 mg/kg lead in sediment did not display permanent abnormalities, skeletal malformations increased in frequency and severity at all higher lead concentrations. By 2360 mg/kg, 100% of surviving metamorphs displayed severe spinal problems, reduced femur and humerus lengths, deformed digits, and other bone malformations. Lead concentrations in tissues correlated positively with sediment and pore water concentrations.

Bank et al., (2007) reported total Hg and methyl Hg (MeHg) concentrations for water, sediment, and green frog (*Rana clamitans*) and bullfrog (*Rana catesbeiana*) tadpoles (age, approximately one year) from Acadia National Park (ANP; ME, USA). Total Hg concentrations (mean ± standard error) in green frog and bullfrog tadpoles were 25.1 ± 1.5 and 19.1 ± 0.8 ng/g wet weights, respectively. Mean total Hg was highest for green frog tadpoles sampled from the Schooner Head site (ANP, Bar Harbor, ME, USA), a small, and semi permanent beaver pond. Methyl Hg comprised 7.6 to 40% of the total Hg in tadpole tissue (wet-wt basis), and mean total Hg levels in tadpoles were significantly different among pond sites (n = 9). Total Hg in pond water was a significant predictor of tadpole total Hg levels. Dissolved organic carbon was a significant predictor of both total Hg and MeHg in water, and total Hg in water also was strongly correlated with MeHg in water. Of the nine pond ecosystems sampled at ANP, 44% had a methylation efficiency (water MeHg to total Hg ratio) of greater than 10%, and 33% had total Hg levels in sediment that were approximately equal to or greater than the established threshold level effect concentration for freshwater sediments (0.174 mg/kg dry wt.). The data indicated that wetland food webs in ANP likely are susceptible to high levels of total Hg bioaccumulation and that methylation...
dynamics appear to be influenced by local abiotic and biotic factors, including disturbances by beavers and In situ water chemistry patterns.

Krishnappa and Venkateshwarlu (2007) tested the genotoxic effect of a carbamate pesticide on Indian cricket frog, *Limnonectes limnocharis*. The frequency of chromosomal aberrations are not significant even at higher doses, micronucleus and sperm abnormalities were significantly more in higher doses of pesticide exposure during 72 and 96 hrs treated groups compared to controls. There is evidence that environmental metal levels affect the immune function. In the particular case of the impact of heavy metals, information available suggests that the immune system is a target for low-dose Pb exposure. Among vertebrates it was shown that amphibians are capable of forming antibodies against a variety of antigens, causing several responses such as anaphylactic response and rejecting grafts.

Rosenberg et al., (2007) assessed the production of antibodies against sheep red blood cells (SRBC) in the anuran *Bufo arenarum* after six weekly injections of sub lethal doses of lead (50mgkg\(^{-1}\), as lead acetate). Natural antibodies (natural heteroagglutinins) were also quantified against SRBC. Both assessments were carried out employing an ELISA method developed to this end, measuring absorbance (A). For natural anti-SRBC antibodies in both control (C) and Pb treated (T) toads, there was a non significant tendency to increase the initial absorbances (C initial: 0.69±0.39 A; T initial: 0.54±0.30 A), relative to those registered at the end of the experiments (C final: 0.89±0.49 A; T final: 0.76±0.31A); the T/C ratios also did not show changes. The only significant difference was found between initial and final samples from lead-treated toads (p<0.014). The immune anti-SRBC antibody levels of toads immunized with SRBC showed a significant lower increase (p<0.05) in lead-treated animals (T final: 0.66±0.36 A), as compared to control toads (C final: 0.91±0.50 A) at the end of the experiment. It was thus concluded that the changes due to the assayed doses of Pb in the levels of antibodies cannot be explained on the basis of only one single action mechanism of the metal, but as the result of a conjunction of effects over different immunocompetent cell subpopulations. These different responses suggested that factors affecting animals exposed to a foreign stimulus are different from those influencing the response of wild animals.

Bouhafs et al., (2009) conducted a study on micronucleus induction in erythrocytes of tadpole *Rana saharica* (green frog of North Africa) exposed to Artea 330EC, a systemic fungicide widely used to struggle against cereals parasites, was evaluated. Tadpoles, *R. saharica* (species widely exist in northern Africa) were exposed to different concentrations
(50, 75, 100, 150ml/L) of Artea 330EC and MMS (methyl methane sulfonate) as a positive control in a concentration of 1.56mg/L. The Test procedure in this study was described in French Standard (AFNOR). The toxic conditions mitotic indices showed in erythrocytes were high, in few erythrocytes nuclear morphological aberrations, like several binucleated cells were presented. About MN frequencies, there were significant differences between the positive control (MMS) and the groups treated with Artea 330EC concentrations whatever the time of exposure. There was a dose-effect relationship from 08 days of exposure on erythrocytes of R. saharica. The results revealed a genotoxic effects of Artea 330EC on R. saharica tadpoles only at the highest concentrations (100 and 150μl/L) with the longest time of exposure (12 days).

Brodeur et al., (2009) evaluated acute and subchronic toxicity of arsenite (As\(^{3+}\)) and zinc (Zn\(^{2+}\)) to stage 25 tadpoles of Rhinella arenarum in both single and joint laboratory exposures. LC50 values obtained for As\(^{3+}\) were elevated and remained within the range of 46 to 50 mg/L of As\(^{3+}\) between 4 and 17 d of exposure. Growth of tadpoles was completely inhibited with 30 mg/L of As\(^{3+}\), demonstrating the presence of ecologically relevant sublethal effects at concentrations lower than those resulting in lethality. With respect to Zn\(^{2+}\), a 96-h LC50 value of 2.49 mg/L was calculated in soft water. Contrary to results obtained for As\(^{3+}\), LC50 values of Zn\(^{2+}\) gradually decreased with increasing exposure duration, from 2.49mg/L at 96 h to 1.30mg/L after 21 d. In joint exposures to both metals, the type of interaction observed between As\(^{3+}\) and Zn\(^{2+}\) was concentration dependent. Lethal effects of As\(^{3+}\) were mitigated, unaffected, or potentiated by 0.01, 0.1, and 1–2mg/L of Zn\(^{2+}\), respectively. However, although 0.01mg/L of Zn\(^{2+}\) significantly reduced lethality of As\(^{3+}\) exposed tadpoles, the same concentration of Zn\(^{2+}\) did not help to reverse the stunt growth of these animals.

Ahmad and Saleh (2010) conducted clastogenic studies on Tandaha Dam water in Asser. Clastogenic effects of home wastes and agricultural contaminates of Tandaha dam located at Alahad municipality in the southwestern part of Saudi Arabia were investigated in peripheral erythrocytes of Rana ridibunda. Examination of blood smears showed that the formation of micronuclei significantly increased during one year (f=9.89, df =3) with p value < 0.05 and were more abundant compared to the control group. This increase in the formation of micronucleus indicates that agricultural pollution increases the risk of clastogenic effects on peripheral erythrocytes of Rana ridibunda and may have similar effects on the human population located around the dam.
Bommarito et al., (2010) invested that Polycyclic aromatic hydrocarbons (PAHs) can affect amphibians in lethal and many sub lethal ways. There are many natural and anthropogenic sources of PAHs in aquatic environments. One potentially significant source is runoff from surfaces of parking lots and roads that are protected with coal tar sealants. Coal tar is 50% or more PAH by wet weight and is used in emulsions to treat these surfaces. Breakdown of sealants can result in contamination of nearby waters. The toxicity of PAHs can be greatly altered by simultaneous exposure to ultraviolet radiation. The study exposed larvae of the spotted salamander (Ambystoma maculatum) to determine if coal tar sealant can have negative effects on aquatic amphibians and if coal tar toxicity is influenced by ultraviolet radiation. Spotted salamanders were exposed to 0, 60, 280 and 1500 mg coal tar sealant/kg sediment for 28 days. Half of the animals were exposed to conventional fluorescent lighting only and half were exposed to fluorescent lighting plus ultraviolet radiation. No significant mortality occurred during the experiment. Exposure to sealants resulted in slower rates of growth, and diminished ability to swim in a dose-dependent fashion. Exposure to ultraviolet radiation affected the frequencies of leukocytes and increased the incidence of micronucleated erythrocytes. There was an interactive effect of sealant and radiation on swimming behavior. They conclude that coal-tar sealant and ultraviolet radiation increased sub lethal effects in salamanders, and may be a risk to salamanders under field conditions.

Hothem et al., (2010) studied mercury contamination in three species of anuran amphibians from the cache creek watershed, California, USA. In this study, adult and juvenile American bullfrogs (Lithobates catesbeianus) and foothill yellow-legged frogs (Rana boylii), adult Northern Pacific tree frogs (Pseudacris regilla), and larval bullfrogs were collected and analyzed for total Hg. One or more species of amphibians from 40% of the 35 sites had mean Hg concentrations greater than the US Environmental Protection Agency’s tissue residue criterion for fish (0.3μg/g). Of the bullfrog tissues analyzed, the liver had the highest concentrations of both total Hg and methyl mercury. Total Hg in carcasses of bullfrogs was highly correlated with total Hg in leg muscle, the tissue most often consumed by humans.

Lawrence and Isioma (2010) evaluated the acute toxicity of Endosulfan (organochlorine) and Diazinon (organophosphate) pesticides to adult amphibians, Bufo regularis to determine uptake and effect of environmentally relevant concentrations on survival, morphology and behavior. Toxicity characterizations were also assessed using standard indices. Toads were exposed for 96 hrs to varying concentrations of the pesticides;
0.25, 0.50, 0.75 and 1mg/l. Mean percentage mortality increased significantly (p<0.05) with concentrations and exposure duration for Endosulfan and Diazinon pesticides and was significantly (p<0.05) different from the control, indicating that pesticide induced lethality. The results showed that Diazinon (LC50=0.44mg/l) was more toxic than Endosulfan (LC50 = 0.73mg/l). Derived safe concentrations were 0.07 and 0.04mg/l for Endosulfan and Diazinon, respectively. Estimated Toxicity index values (TIV) and Hazard Quotients (HQ) for all the concentrations were above one (1) indicating potential risk of the pesticides to the toad. Bioconcentration of the pesticides after 96 h increased with increasing concentrations indicating that uptake was concentration dependent. There was a significant positive correlation between tissue concentration and mortality (p< 0.01) for both pesticides. The pesticides also caused dose-dependent deformities and behavioral abnormalities. More pronounced poisoning symptoms were observed in Diazinon and at higher concentrations.

Paunescu et al., (2010) investigated histological and histochemical alterations in the liver of the frog Rana ridibunda induced by the action of the insecticide Reldan 40EC (chlorpyrifos-methyl). The animals used in the experiment were treated with 0.01ml/g body weight Reldan 40EC and kept at 4-6ºC, respectively at 22-24ºC. The toxic was administrated by intraperitoneal shots (one shot every two days, in a scheme of three weeks). At the end of the experiment an increase in the area occupied by the Kuppfer cells as well as an increase in their color intensity was observed. Mild karyomegalia and polyploidy together with accumulation of infiltrates was evident. A fibrosis around the blood vessels and between hepatocytes was also observed.

Bosch et al., (2011) determined the genotoxic effect of cyclophosphamide and glyphosate in a commercial formulation using the micronucleus test in peripheral blood erythrocytes of Odontophrynus cordobae and Rhinella arenarum, amphibians widely distributed in the Province of Córdoba, Argentina. For this, the basal frequency of the micronucleated erythrocytes (MNE) was determined by: 0.40±0.18 MNE/1000 erythrocytes in Odontophrynus cordobae and 0.30±0.09 MNE/1000 erythrocytes in Rhinella arenarum. The frequency of MNE in Odontophrynus cordobae increased after 5 days of exposure to glyphosate (100 mg ai/L) and cyclophosphamide. After 2 and 5 days of exposure to glyphosate (200, 400 and 800 mg ai/L), the MNE frequency in Rhinella arenarum was higher than the basal frequency, as it occurred in the group exposed to cyclophosphamide. Regarding acute toxicity and genotoxicity, the results show that Odontophrynus cordobae was more sensitive to cyclophosphamide and glyphosate exposure than Rhinella arenarum. A
correlation was detected between exposure concentration and MNE frequency in *Rhinella arenarum*.

Ezemonye and Enuneku (2011a) studied hematological changes in *Bufo maculates* treated with sub lethal concentrations of Cadmium. Adult *Bufo maculatus* was exposed to sub lethal cadmium concentrations of 0.25, 0.50, 1.00 and 2.00mg/L. The toxicant from which the cadmium concentrations were prepared was cadmium chloride (CdCl$_2$.H$_2$O). There were three replicate tanks per treatment and three individuals per tank including control groups. The hematologic alterations based on the examination of blood indices during the 28 days of exposure showed that total erythrocyte count (TEC), hematocrit (Hct) and hemoglobin (Hb) concentration decreased (P<0.05) relative to controls. The decline was concentration-dependent as concentration of cadmium increased. The decline in hemoglobin and hematocrit in the experimental organism could be due to a decrease in the synthesis or release of erythrocytes into the circulation or an increase in the rate of erythrocyte destruction inflicted by cadmium toxicity. There was significant (P<0.05) elevation in total leukocyte count (TLC) with increase in the concentration of cadmium. The increase in total leukocyte count observed could be attributed to a stimulation of the immune system in response to tissue damage caused by cadmium toxicity. The study has shown that the exposure of the *Bufo maculates* toad to cadmium can inflict alterations in the hematologic indices, which could induce unfavorable physiological changes in the amphibian, which may lead to death.

Ezemonye and Enuneku (2011b) investigated biochemical alterations in *Hoplobatrachus occipitalis* exposed to sub lethal concentrations of cadmium. The adult crowned bullfrog, *Hoplobatrachus occipitalis* was exposed to 0.25, 0.50, 1.00 and 2.00mg/L cadmium for 28 days. The effect of cadmium on selected biochemical parameters- superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) in the liver were tested. Biochemical observations revealed significant (P<0.05) dose-dependent increase in the specific activity of superoxide dismutase (SOD) and catalase (CAT) relative to controls. This could be due to increased production of these antioxidants to counteract oxidative stress and lipid peroxidation induced by cadmium. Glutathione (GSH) level decreased with increase in the concentration of heavy metal. Thiobarbituric acid reactive substances (TBARS) which is an index of lipid peroxidation increased as concentration of cadmium increased. The increased level of TBARS in the liver of cadmium exposed frogs is an indication of increased membrane lipid peroxidation which could lead to cell damage.
Ezemonye and Enuneku (2012) assessed the hepatic bioaccumulation of cadmium in *Hoplobatrachus occipitalis* and *Bufo maculatus* for 14 and 28 days respectively. The amphibians were exposed to sub lethal cadmium concentrations of 0.25, 0.50, 1.00 and 2.00mg/l. Bioaccumulation in *H. occipitalis* and *B. maculatus* increased significantly (p<0.05) in both periods of exposures. There was no significant difference in bioaccumulation between the 14 and 28 days exposures. There was also no significant difference in the bioaccumulation of cadmium between two amphibian species. The study suggested that the release of cadmium into the environment could possibly affect the well-being of amphibians as well as resulting in further decline of these very sensitive organisms that contribute significantly to the food web.

Todd *et al.*, (2012) tested the efficacy of using amputated toes (‘‘toe clips’’), a common byproduct when marking amphibians in population and genetic studies, to determine mercury (Hg) concentrations in amphibians. They examined total mercury (THg) concentrations in American toads (*Bufo americanus*) collected along a contamination gradient at a Hg-contaminated field site. Significant positive correlations between toe THg and blood THg concentrations in adult males and females collected in two different years was found that blood and toe clips could be used to predict maternal transfer of Hg, an important mechanism of reproductive toxicity in wildlife. Maternal toe THg concentrations were more highly correlated with egg THg concentrations than were maternal blood THg concentrations. Results indicate that amputated toes are effective for identifying Hg concentrations in amphibians.

Wast *et al.*, (2012) described the effect of lead nitrate, lead acetate and mercuric acetate on tadpole of fresh water *Rana tigrina* at the five different concentrations (50μg/l, 100μg/l, 200μg/l, 400μg/l and 800μg/l) for 120 hrs exposure. Mercury acetate caused 100% mortality within six hours in three different concentration (200μg/l, 400μg/l and 800μg/l) used while in 50μg/l concentration 100% tadpoles were survived up to 24 hrs. However gradual decrease in survival percentage was noted in further duration of exposure i.e. 80 % survived up to 48 hrs, 60% survived up to 72 hrs and 20 % survived up to 96 hrs. In 120 hrs exposure of same concentration all the tadpole were observed died. In both lead acetate and lead nitrate, in 50μg/l concentration all the tadpoles survived up to 12 hrs. However, in 96 hr and 120hrs exposure of the lead nitrate and lead acetate 80% and 60% tadpole survived respectively.
Ilizaliturri-Hernandez et al., (2013) measured the exposure to lead and evaluated hematological and biochemical effects in specimens of giant toads (*Rhinella marina*) taken from three areas surrounding an industrial complex in the Coatzacoalcos River downstream. Lead levels in toads' blood are between 10.8 and 70.6μg/dl and are significantly higher in industrial sites. They found a significant decrease in the delta-aminolevulinic acid dehydratase (δ-ALAD) activity in blood from 35.3 to 78 % for the urban–industrial and industrial sites, respectively. In addition, we have identified a strong inverse relationship between the δ-ALAD activity and the blood lead levels (r0−0.84, p<0.001). Hemoglobin and mean corpuscular hemoglobin levels, as well as the condition factor, were found to be lower at industrial sites compared with the reference sites. Results suggest that the *R. marina* can be considered a good biomonitor of the δ-ALAD activity inhibition and hematological alterations at low lead concentrations.

Jayawardena et al., (2013) evaluated heavy metals (Cd, Cu, Zn and Pb) induced toxicity to the Indian green frog, *Euphlyctis hexadactylus* in the polluted wetland, Bellanwila-Attidiya sanctuary in the western province. Naturally exposed frogs, from this site were compared with their healthy counterparts from the reference site at the catchment of Labugama reservoir. Standard methodology assessed toxicity based on frog erythrocyte morphometry, histopathology of major organs and serum biomarkers of hepatic and of nephro toxicity. Erythrocyte morphometry measured mean cell volume (MCV), mean nuclear volume (MNV), aspect ratio and nucleo-cytoplasmic ratio. The exposed versus healthy *E. hexadactylus* reported discrepancies in all these tested parameters; particularly, MCV of exposed animals (179.0 ± 32.58 μm3) was significantly lower (P=0.0001) than that of reference animals (200.79 ± 35.96 μm3). Histopathological assessment showed pronounced disturbances in tissue development; severe bile secretion, hemorrhages and sinusoidal dilations of liver, distortion of alveolar sacs in the lungs, damaged Bowman’s capsules in the kidney and damaged glandular cells of the skin epidermis. Liver injuries observed were clearly explained by increased activity of serum hepatic marker enzymes, aspartate transaminase, alanine transaminase and gamma glutamyl transferase, indicating cellular leakage and loss of functional integrity of frog hepatocyte membrane in the polluted site. Reduced total protein and albumin levels suggested hepatic dysfunction. Significantly elevated levels of urea (P=0.033) and higher creatinine in blood indicated renal dysfunction.

Taiwo et al., (2014) analyzed the effect of heavy metals on Wild African Tiger frogs (*Hoplobatrachus occipitalis*) and the resulting production of oxidative stress enzymes, the
concentrations of the heavy metals, cadmium (Cd), copper (Cu), iron (Fe), zinc (Zn), lead (Pb) and nickel (Ni) were also investigated in the tissues of *H. occipitalis* as well as in water and sediment samples collected from five different locations in Lagos State, Nigeria. The activities of superoxide dismutase (SOD), reduced glutathione (GSH) and level of lipid peroxidation product, malondialdehyde (MDA) were analyzed in the liver of the sampled frogs. Most measured physicochemical characteristics of the water varied significantly across the sampling locations (P<0.05). The levels of metals (mg/kg dry weight) in muscle tissues also varied significantly. Zn was the most accumulated metal, followed by Fe, Cu and Ni, while Pb was the least. The mean of SOD and reduced GSH in the frogs indicate some responses to oxidative stress which varied significantly among sampling areas (P<0.05). MDA values however did not consistently correlate with either oxidative stress or heavy metal concentrations in the frogs. The water-sediment-tissue analysis for heavy metals demonstrated that the sediment concentrated more heavy metals than water, while the frog tissues accumulated these metals particularly in more polluted areas.

Zocche et al., (2014) studied the impact of mineral coal on amphibians. The chemical elemental contents and oxidative stress indexes in *Hypsiboas faber* from coal-mining areas and in an unpolluted area in the Catarinense Coal Basin, Brazil, were assessed. The highest contents of sulfur, chlorine, iron, zinc, and bromine were registered in specimens from the coal-mining area, whereas the highest contents of potassium calcium, and silicon were registered in specimens from the control area. It was found that there was a significant increase (p<0.05) in the activity of super oxide dismutase (SOD) and glutathione peroxidase (GPx) in the animals from the coal-mining area, whereas the level of catalase showed no differences between the animal groups. The levels of TBARS showed no differences between the tested groups. However, carbonylation decreased significantly (p<0.05) in animals from the coal-mining area, and there was a significant increase (p<0.05) in the formation of total thiols in animals from the coal-mining area, thus concluded that the antioxidant system of *H. faber* is sensitive to pollutants present in coal-mining wastes and may be a potential biomarker for monitoring the level of contaminants in the environment.
Chapter 3

Material and Methods
General methods to biomonitor (use of organisms to monitor contaminants and to imply possible effects to biota or sources of toxicants to humans (Goldberg, 1986) and apply biomarkers (cellular, tissue, body fluid, physiological or biochemical changes in individuals that are used quantitatively during biomonitoring to imply presence of significant pollutants or as early warning systems for imminent effects) are also important technologies developed. One of the major problems in biomonitoring genotoxic pollutants is the choice of test organisms. Unequal sensitivity among species caused by metabolic rates, physiological conditions and target organs can yield misleading results. Conventional test animals used in ecotoxicology include alga, earthworm, fish, avian species and mouse.

The present investigation was aimed to bio-monitor the genotoxic effects of different heavy metals salts using frog, *Euphlyctis cyanophlyctis* as ‘test animal’ by employing chromosomal aberration test (CAT) and micronucleus test (MNT) as the most conventional biomarker tools of *In vivo* techniques for genotoxicity testing and determining the effect on the mitotic index (MI). In order to evaluate the genotoxic potential of different heavy metal salts, *In vivo* toxicity tests were conducted for different sets of exposure period under sublethal concentrations of various heavy metal salts.
Various methods and techniques used during present work are as follow:

3.1 MATERIAL

3.1.1 Selection of model animal:

Amphibians have many compelling features that make them ideal as animal models (Hopkins, 2007). Frogs and toads are about 90% of all amphibians (McDiarmid and Mitchell 2000). Therefore, they are an important link between human and ecosystem health (Hayes et al., 2002) and main components of aquatic and terrestrial ecosystems (Unrine et al., 2007).

Out of the three frogs species found in and around Jammu region, *E. cyanophlyctis* has been selected as a model for genotoxicity experiments. The following practical aspects were taken into consideration during the selection of the test organism (Butler et al., 1971; Rand and Petrocelli, 1985; Rosenberg et al., 1971; Hellawell, 1986; USEPA 1976, 1979):

- Well known taxonomically.
- Living in water and have low dispersal rate.
- Holds ecologically important position in the food chain.
- Wide range of habitats and distribution.
- Available in good number in fresh water ponds, ditches etc.
- Straight forward maintenance.
- Collection of organism does not affect the survival of the species in nature.
- Widely available for most of the time of year except for extreme winter, easily obtained.
- Temperature and oxygen tolerances.
- High sensitivity to environmental pollutants.
- Effortless testing without the use of extensive instruments and high manpower.
- IUCN status- least concern (very common)

3.1.2 Systematic position of the test frog species:

<table>
<thead>
<tr>
<th>Class</th>
<th>Amphibia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order</td>
<td>Anura</td>
</tr>
<tr>
<td>Suborder</td>
<td>Procela</td>
</tr>
<tr>
<td>Family</td>
<td>Dicroglossidae</td>
</tr>
<tr>
<td>Subfamily</td>
<td>Dicroglossinae</td>
</tr>
</tbody>
</table>
Genus : *Euphlyctis*
Specimen : *cyanophlyctis*

3.1.3 Collection and maintenance of the frog specimens

Active, healthy and live specimens of *Euphlyctis cyanophlyctis* weighing, (Fig. 1a,1b,2a and 2b) 50-70gms were collected from the unpolluted lentic water bodies (ponds and ditches) from Jammu District (Altitude 300-4200), using hand nets to prevent injury to the animal during capture and transported to the laboratory in a covered bucket. Frogs were acclimatized in plastic containers of 20 L capacity containing 3 L of dechlorinated tap water, for 5 days prior to the experiments. Containers were placed on a slant to provide the option of both aqueous and dry environment. Water was changed every two days and the container cleaned thoroughly. Frogs were fed with earthworms twice weekly. Uneaten earthworms and faecal wastes were removed and water replenished regularly. Feeding were stopped 24hrs prior to commencement of toxicity tests and frogs were not fed during experimental period.

3.1.4 Test Chemicals used

Various heavy metal salts used during the experiment were:

- **a)** Cadmium in the form of Cadmium Chloride (CdCl$_2$.1/2H$_2$O).
- **b)** Chromium in the form of Potassium Dichromate (K$_2$Cr$_2$O$_7$).
- **c)** Copper in the form of Copper Sulphate (CuSO$_4$.5H$_2$O).
- **d)** Lead in the form of Lead Acetate (Pb (COOH)$_2$).
- **e)** Manganese in the form of Manganese chloride (MnCl$_2$).
- **f)** Mercury in the form of Mercuric Chloride (HgCl$_2$).
- **g)** Nickel in the form of Nickel Chloride (NiCl$_2$).
- **h)** Zinc in the form of Zinc Chloride (ZnCl$_2$).

3.2 METHODOLOGY

3.2.1 Preparation of Chemicals

**A) Colchicine (0.01%)**: It was prepared by mixing 100 mg colchicine powder in 10ml of distilled water.
Fig. 1a. *E. cyanophlyctis* in water

Fig. 1b. *E. cyanophlyctis* in moist habitat on land

Fig. 2a. Male and female frogs in captivity

Fig. 2b. Frogs in captivity
B) Hypotonic solution (0.48% NaCl): It was prepared by adding 480 mg of NaCl in 100ml of distilled water.

C) Carnoys fixative: It was prepared by mixing methanol and glacial acetic acid in the ratio of 3:1.

D) Preparation of Giemsa stain:

1) Stock solution: It was prepared by dissolving 2g of Giemsa powder in 66 ml of glycerine. This solution was incubated for 2 hrs at 60°C. After cooling the contents, 66 ml of methanol was added. This stock solution was placed in fridge in dark air-tight bottles for further use.

2) Working solution of Giemsa: It was made by diluting the stock solution with distilled water or phosphate buffer.

E) 0.2 N HCL solution: Prepared by adding 1 ml of concentrated HCl in 49ml of distilled water.

F) Barium hydroxide (BaOH) solution (5%): Fresh solution of barium hydroxide was prepared by mixing 5 gm of barium hydroxide in 100 ml of distilled water.

G) Standard saline citrate (2xSSC): It was prepared by mixing 2.2gm of NaCl per 125ml of distilled water and 1.2gm of sodium citrate in 125ml of distilled water.

H) Colloidal developer: Prepared by mixing gelatin powder (2gm) and formic acid (1ml) in 100 ml of distilled water.

I) Silver nitrate solution: Prepared by dissolving 4 gm of AgNO₃ in 3ml of distilled water.

J) Phosphate buffer (pH 7): Prepared by mixing Na₂HPO₄ (anhydrous; 1.42gm) and NaH₂PO₄.2H₂O (1.56gm) in 500ml of distilled water.

3.2.2 Method for Cytological preparation:

The methodology involved arresting the chromosomes at metaphase stage through colchicine treatment, hypotonic treatment, fixation and slide preparation, followed by staining and microscopy. Mitotic chromosomes from frog were made from the rapidly dividing cells. Therefore, anterior kidney, intestine and bone marrow were selected for chromosomal preparations.
3.2.2.1 Procedure:

Cytogenetic analysis of male and female specimens of model species was carried out following the conventional colchicine-hypotonic-acetic-alcohol air-drying Giemsa staining technique (modified after Tijo and Whang, 1965). *In-vivo* injection of colchicine treatment was used. An intramuscular and intraperitoneal injection of 0.1% colchicine solution was given to the frog at the rate of 1ml/100gm body weight. After 4 hour, frogs were dissected from the ventral side so as to remove the kidneys, intestine and bone marrow (Haertel *et al*., 1974; MacGregor and Varley, 1986). The tissue was chopped into very fine pieces and subjected to hypotonic treatment at room temperature for an hour. Tissue was fixed in freshly prepared Carnoys fixative for 45 minutes and after every 15 minutes fixative was changed. Air-drying dabbing method was used for preparing slides. Tissue was dabbed on clean and dry slides and eventually air-dried. Slides were stained in 2% Giemsa stain for about 40-50 minutes and differentiated in distilled water and then again air-dried.

3.2.2.2 Chromosome Banding Methods

Some slides were subjected to differential staining or banding. Two banding methods were used to study the heterochromatin distribution and location of NORs in chromosomes. The procedure for which is as under:

a. **C-banding:**

The slides were processed for C-banding following the technique of Sumner (1972) with slight modification. 5-7 days old slides were incubated in 0.2N HCl at room temperature for 40 minutes at room temperature. These were then rinsed in distilled water and treated in a saturated solution of 5% Barium hydroxide for 10 minutes. After the treatment, slides were again rinsed in distilled water and incubated in standard saline citrate (2xSSC) at 60°C for an hour, rinsed finally in distilled water and stained for 35-45 minutes in 2% Giemsa (pH 7).

a. **NOR-banding:**

Some slides were also processed for NOR-banding following the technique of Howell and Black (1980). Two drops of fresh colloidal developer and four drops of silver nitrate were put on air dried slide with a dropper. After mixing the solution well; a cover slip was put on the slide and incubated at 70°C for 8-10 minutes until the
mixture turned golden brown. The slide was washed and coverslip was removed under running water. Slide was rinsed with distilled water and air dried.

3.2.3 Scanning and Photomicrography:

The stained slides were scanned thoroughly for well spread metaphase plates which were then observed under 10X eyepiece and 100X objective using oil immersion and the best complements were selected for photomicrography. Selected suitable metaphases conventionally stained with Giemsa and those showing NOR and C-banding were photomicrographed at 1000X magnification showing clear images by using Nikon YS100 binocular research microscope and Samsung SDC-313 camera.

3.2.4 Photokaryotype Preparation

The photographic prints were prepared and individual chromosomes were cut out from them. Pairing of homologous chromosomes was done on the basis of their centromere position, length, arm length and gross morphology. Chromosomes were arranged in their decreasing order of length and finally after morphometric measurement arranged in a karyogram. The chromosomes were categorized and identified according to the morphological classification of chromosomes proposed by Levan et al., (1964), as metacentric, submetacentric, subtelocentric and telocentric (Table 1).

Table 1: Nomenclature for classification of chromosomes (Levan et al., 1964).

<table>
<thead>
<tr>
<th>Centromeric position</th>
<th>Arm ratio (p/q)</th>
<th>Chromosome type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>1.00-1.70</td>
<td>Metacentric(M)</td>
</tr>
<tr>
<td>Submedian</td>
<td>1.71-3.00</td>
<td>Submetacentric(SM)</td>
</tr>
<tr>
<td>Subterminal</td>
<td>3.01-7.00</td>
<td>Submetacentric(ST)</td>
</tr>
<tr>
<td>Terminal</td>
<td>7.01 and above</td>
<td>Telocentric(T)</td>
</tr>
</tbody>
</table>

3.2.5 Morphometric Analysis

The length of chromosome, long arm, and short arm was measured using stage micrometer and oculometer. Centromeric index, relative length percentage and arm ratio were calculated from the short arm and long arm measurements of the chromosomes. Centromeric index and arm ratio depicts the morphology of the chromosomes whereas relative length percentage tells us about the size of the chromosome in relation to
chromosomes in the karyogram. Following morphometric criteria were taken into account in the present investigation.

(a) Actual length of chromosomes: It was determined in micrometers (μm).
(b) Mean Length of chromosomes: It was calculated by taking the average of the length of homologous chromosomes.
(c) Mean total haploid length: The actual lengths of all the chromosomes in a haploid set were added to determine the mean total haploid length.
(d) Total diploid length: It was calculated by adding the actual length of all the chromosomes in a diploid set and it was expressed in micrometers. This value is also known as total complement length (TCL).
(e) Total complement length percentage and relative length percentage: the TCL% and RL% of each pair of chromosomes were worked out from the total complement length of diploid set by the following formula.
   TCL% = Absolute length of chromosomes/TCL×100
   RL% = Absolute length of chromosomes/Absolute length of the largest chromosome of the complement ×100
(f) Centromeric index of the chromosomes:
   CI% = length of short arm/Total length of chromosome× 100
(g) Arm ratio = Length of long arm (q)/ Length of short arm (p)

3.2.6 Preparation of Histogram and Idiogram

Relative length percentage was taken as criterion for preparation of Histogram. Chromosome pair number was represented on X-axis and corresponding relative length percentage on Y-axis. Chromosome pairs were arranged in decreasing order of their length.

Idiogram of chromosome pairs was constructed to represent the shape and size of the chromosomes. These were prepared according to the centromeric position, length of chromosomes and in order of decreasing lengths by using haploid formula as the basic parameter. Each chromosome pair was represented as thick vertical bar having length in accordance with the chromosomes. The length of each vertical bar above the X-axis represent the length of short arm and its portion below the X-axis represent the length of long arm. The vertical bars were arranged in descending order of their length.

3.2.7 Estimation of median-lethal concentration (LC₅₀) of test chemicals
The frogs were given heavy metal treatment through intraperitoneal injection. In this injection method, the known amount of heavy metal salt was administered into the animal body. In animals, IP injection is used predominantly in veterinary medicine and animal testing for the administration of systemic drugs and fluids due to the ease of administration compared with other parenteral methods. Thus, this method yield more consistent results and is used more extensively. It is a statistically derived single concentration of a toxicant that can be expected to cause 50% death of a given population of organisms under a defined set of experimental conditions. For all the salts, the LC$_{50}$ values at 96hrs for *Euphlyctis cyanoophyctis* were calculated using standard method of Finney (1980) and are given in Table 2. All heavy metal salts were dissolved in distilled water and the four sublethal doses were arbitrarily chosen for each heavy metal salt treatment. The exposure time was also specified as 24hrs, 48hrs, 72hrs and 96hrs.

**Table 2: LC$_{50}$ values and sublethal concentrations of heavy metal salts used during present work.**

<table>
<thead>
<tr>
<th>Salt</th>
<th>LC$_{50}$ value</th>
<th>Sublethal concentration used</th>
</tr>
</thead>
<tbody>
<tr>
<td>CdCl$_2$.1/2H$_2$O</td>
<td>15mg/kg</td>
<td>1.5mg/kg, 3.0mg/kg, 6.0mg/kg, 12.0mg/kg</td>
</tr>
<tr>
<td>K$_2$Cr$_2$O$_7$</td>
<td>20mg/kg</td>
<td>2.0mg/kg, 4.0mg/kg, 8.0mg/kg, 16.0mg/kg</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>10mg/kg</td>
<td>3.5mg/kg, 5.0mg/kg, 6.5mg/kg, 8.0mg/kg</td>
</tr>
<tr>
<td>[Pb(COOH)$_2$]</td>
<td>42mg/kg</td>
<td>4.5mg/kg, 9.0mg/kg, 18.0mg/kg, 36.0mg/kg</td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>35mg/kg</td>
<td>4.0mg/kg, 8.0mg/kg, 16.0mg/kg, 32.0mg/kg</td>
</tr>
<tr>
<td>HgCl$_2$</td>
<td>18µg/kg</td>
<td>1.5µg/kg, 3.0µg/kg, 6.0µg/kg, 12.0µg/kg</td>
</tr>
<tr>
<td>NiCl$_2$</td>
<td>25mg/kg</td>
<td>2.5mg/kg, 5.0mg/kg, 10.0mg/kg, 20.0mg/kg</td>
</tr>
<tr>
<td>ZnSO$_4$</td>
<td>29mg/kg</td>
<td>3.0mg/kg, 6.0mg/kg, 12.0mg/kg, 24.0mg/kg</td>
</tr>
</tbody>
</table>

*3.2.8 Experimental Design*

Toxicity tests were conducted in accordance with standard methods (Brodie, 1968). Acclimatized, live, active and apparently healthy frog specimens were treated through IP injection having a precise measurement of the amount of heavy metal given once in the treatment period. The groups of frogs, treated with four sub-lethal concentrations of each heavy metal salt mentioned above for 24, 48, 72 and 96hrs of duration frogs were not fed during experimental periods and the frogs of the control group (without the heavy metal...
treatment) were maintained on the normal food i.e. earthworms in similar experimental conditions.

3.2.9 Genotoxicity Assays

Bioassays are used in monitoring scheme in toxicology studies (Weber, 1993). Effective monitoring procedures, including those for genotoxicity, must involve assays that are highly sensitive, relatively straightforward to apply and inexpensive. Such assays are often used to monitor the acute toxicity of aquatic effluents including heavy metals according to the guideline set by the regulatory authority. Of the numerous short term tests for detecting genotoxicity, the following tests were selected at the beginning of the study because of their sensitivity and ease of application in the frog as model organism:

3.2.9.1 Chromosome Aberration Test (CAT)

The chromosome aberration test has been considered as an authentic one in assessing the mutagenic potentiality of physical and chemical agents (Manna, 1989). Chromosomal mutation is a macro damage of chromosomes. Chromosome aberration include structural aberrations such as fragments or intercalations and numerical aberrations (unequal segregation of homologous chromosomes during cell divisions, which leads to a loss or surplus of chromosomes (aneuploidy and polyplody). Cytogenetic effects can be studied either in whole animals (“in vivo”) or in cells grown in culture (“in vitro”). Usually, the cell culture is exposed to the test substance and afterwards treated with a metaphase-arresting substance (Colchicine). Following suitable staining the cells in metaphase are analyzed microscopically for the presence of chromosomal aberrations.

In the present study, the chromosome aberration test was performed on four specimens of frogs in each experimental group. The frogs were exposed to four sub lethal concentrations of each heavy metal for 24, 48, 72 and 96hrs. After particular duration and concentration, chromosomal preparations were made from bone marrow and intestinal tissues as described earlier. Well spread metaphase chromosomes were selected and scoring of aberrations was done under oil immersion objective. A total of 400 metaphases were selected for detection of chromosome aberrations after every 24hrs of treatment. The data for each animal was pooled together in respective groups and expressed as mean frequency (mean±SD) per 100 cells. Slides from untreated frog (four specimens for every 24hrs of
exposure duration) served as control and treated slides were photomicrographed at 1000x magnification.

### 3.2.9.2 Micronucleus test (MNT)

The micronucleus test, developed by (Schmid, 1975) and (Heddle, 1973), is an *in vivo* and *in vitro* short-time screening method, widely used to detect genotoxic effects (Villarini *et al.*, 1998). It is one of the simplest, reliable, least expensive and rapid screening system for both clastogenic (chromosome breakage and formation of acentric fragments) and aneugenic (chromosome lagging and effects on spindle) effects (Heddle *et al.*, 1983, Orhan *et al.*, 1993).

Micronuclei (MN) are cytoplasmic chromatin-containing bodies that appear in the cell like a small, satellite nucleus around the cell nucleus that are formed from acentric chromosomal fragments or whole chromosomes that are not incorporated in either of the daughter nucleus after cell division. At telophase nuclear envelope forms around the lagging chromosomes and fragments which uncoil and gradually assume morphology of an interphase nucleus with the exception that they are smaller than the main nuclei (1/5 to 1/20) in the cell hence the term micronuclei (Heddle *et al.*, 1991). The MNT is a simple, reliable, less expensive and sensitive assay for *in vivo* evaluation of genotoxic potential of various agents. Jaylet *et al.* (1986) first adapted the MN test to amphibians. Many MN tests on amphibians have been proven to be suitable for evaluating mutagens and genotoxic agents (Fernandez *et al.*, 1993; Godet *et al.*, 1996; Djomo *et al.*, 2000). Chen and Xia (1993) used MN test in erythrocytes of tadpoles, *Rana nigronaculata*, to evaluate mutagens and genotoxic agents in aquatic environment. Presently, four frog specimens were given treatment with each heavy metal salt concentration for 24, 48, 72 and 96hrs. Separate batch of same number of frog was kept as control for each duration of treatment i.e. 24, 48, 72 and 96hrs, and peripheral erythrocytes (RBCs), intestine cells, kidney cells were used for performing MNT. Procedure for preparation of micronuclei slides from kidney and intestine was same as that used for the chromosomes aberration test except that the colchicine pre-treatment was not given.

1) **Collection of blood from frog:**

The only reliable method of blood collection is by cardiac puncture. In frog the ventricle is located near the xiphisternum. Rinsed syringe with heparin solution (75U/ml) and left approximately 2mm gap at top of syringe column. After feeling the pulsating heart on the
ventral side of thoracic region, pricked the needle in the middle of the heart. As blood started flowing into syringe, the dispenser was very gently pulled outwards to collect the blood.

2) Preparation of micronuclei slides:

A blood films were made from a drop of blood according to the method of blood cell smear (Carmena, 1971a,b).

- Taken a heparinized syringe (1ml) and drew 0.05-0.1ml blood by heart puncture, made a thin smear on pre-cleaned slide with the help of blood smear slide.
- Air dried the slides over night at room temperature.
- Fixed the slides by dipping them into absolute methanol for 5-10 minutes.
- Air dried the slides for at least 1hr.
- Stained the slides in 2% Giemsa stain in phosphate buffer for 30 minutes.
- Washed the slides with distilled water to remove all the stain particles carefully. Air dried the slides over night.
- Observed the slide under microscope using 40/10x objective lenses and scored the micronucleated cells.

3) Scoring of MN slides:

Only micronuclei not exceeding 1/3rd of the main nucleus diameter, clearly separable from the main nucleus and with distinct borders and of the same colour as the nucleus were scored. The frequency of micronuclei in each tissue was established by estimating the number of micronuclei in at least 1500 interphase cells/specimen (total of 6000 interphase cells from four specimens used per concentration and duration) and was expressed as mean±standard deviation (SD) per 1000 cells. The micronucleated interphase cells from different tissues were photomicrographed at 1000x magnification.

3.2.9.3 Mitotic index (MI)

The mitotic index was established by estimating the number of metaphase per 1000 cells counted (total of 4000 cells counted from four specimens for each concentration and duration) which was expressed as mean±SD. For this purpose bone marrow and intestinal tissue were used.
3.2.10 Statistical analysis

Data from the chromosome aberration test, mitotic index and micronucleus test were expressed as mean±SD and the Data were evaluated using the non-parametric Kruskal-Wallis test. The computer software called ‘PRIMERS-4.0’ was used to perform statistical analysis of the data. A value of p<0.05 was considered to indicate the level of significance. Statistical significance in the frequencies of chromosome aberrations, mitotic index and micronuclei between experimental and control groups after each dose and duration of exposure, were evaluated.
Chapter 4

Observations
In the present course of investigation on *Euphlyctis cyanophlyctis*, the following parameters were taken into consideration:

4.1 Cytogenetic analysis

4.2 Genotoxicity analysis

4.1.1 **Chromosomal observation from male specimen of *Euphlyctis cyanophlyctis***

a). Somatic metaphase obtained from intestine, bone marrow and kidney (Fig. 3a-3c)

Diploid chromosome number recorded 2n=26

Thirteen pairs of chromosomes were placed into two groups comprising:

Group A: five pairs of large chromosomes.

Group B: eight pairs of small chromosomes.

The chromosomes in both the groups were of two types: metacentric and submetacentric

b). Karyotype prepared from metaphase of intestine cells (Fig. 4a-4b)

Number of metacentric chromosomes=7 pairs (biarmed)

Number of submetacentric chromosomes=6 pairs (biarmed)

c). Chromosomal formula, n=7M+6SM

d). Sex chromosome=Not distinguishable

e). Morphometric data of somatic karyotype (Table 3)
4.1.2 **Chromosomal observation from female specimen of *Euphlyctis cyanophlyctis***

**a).** Somatic metaphase obtained from intestine, bone marrow and kidney (Fig. 6a-6c)

Diploid chromosome number recorded 2n=26

Thirteen pairs of chromosomes were placed into two groups comprising:

- Group A: five pairs of large chromosomes.
- Group B: eight pairs of small chromosomes.

The chromosomes in both the groups were of two types: metacentric and submetacentric

**b).** Karyotype prepared from metaphase of intestine cells (Fig. 7a-7b)

- Number of metacentric chromosomes=7 pairs (biarmed)
- Number of submetacentric chromosomes=6 pairs (biarmed)

**c).** Chromosomal formula, n=7M+6SM

**d).** Sex chromosome=Not distinguishable

**e).** Morphometric data of somatic karyotype (Table 4)

   i.  Actual mean length of the largest chromosome = 1.20
   ii. Actual mean length of the smallest chromosome =0.60
   iii. Relative length percentage of the largest chromosome =100.0
   iv.  Relative length percentage of the smallest chromosome =50.0
   v.   Ratio of the largest to the smallest chromosome =2.0
   vi.  Mean total haploid length =9.8
   vii. Total diploid length =19.76

**f).** Histogram of the chromosomes (Fig. 8a)

**g).** Idiogram of the chromosomes (Fig. 8b)
Fig. 3a-3c Metaphase complements from intestine (3a), bone marrow (3b) and kidney (3c) of male specimen of *E. cyanophlyctis*
Fig. 4a A conventional stained somatic metaphase complement of male
*Euphlyctis cyanophlyctis* (2n=26)

Fig.4b Karyotype of male *Euphlyctis cyanophlyctis* (2n=26)
Chromosomal formula=7M+6SM
Fig. 5a Histogram of male specimen of *E. cyanophlyctis*.

Fig. 5b Idiogram of male specimen of *E. cyanophlyctis*
Fig. 6a-6c Metaphase complements from intestine (3a), bone marrow (3b) and kidney (3c) of female specimen of *E. cyanophlyctis*
Fig. 7a A conventional stained somatic metaphase complement of female *Euphlyctis cyanophlyctis* (2n=26)

Fig. 7b Karyotype of female *Euphlyctis cyanophlyctis* (2n=26)
Chromosomal formula=7M+6SM
Fig. 8a Histogram of female specimen of *E. cyanophlyctis*.

Fig. 8b Idiogram of female specimen of *E. cyanophlyctis*
4.1.3 Observations of differential banding in *Euphlyctis cyanophlyctis*

1. C-banding:

C-banding of the somatic metaphase complement revealed the presence of centromeric C-bands in all the chromosomes showing the presence of heterochromatin. Some telomeric C-bands were also seen in some chromosomes but the bands were very light (Fig. 9a-9b).

2. NOR-banding:

The Ag-NOR staining showed a densely stained, well defined and conspicuous pair of nuclear regions on the long arm of the chromosome pair no. 10 (10q) (Fig. 10a-10b).

The above cytogenetic observations and morphometric analysis of the test species, *Euphlyctis cyanophlyctis*, revealed that this frog processes all the characteristic of a best model organism for the genotoxic studies in addition to their abundance in the natural environment, where collection of organisms does not affect the survival of the species in nature. Extremely favourable cytological features required for the genotoxic study were found in the karyotype of *E. cyanophlyctis*. Chromosomal analysis revealed its diploid chromosome number as 26 with comparatively large-sized chromosomes, which were easily recognizable as metacentric and submetacentric. No telocentric chromosome and also no sex chromosome could be designated even on the basis of differential staining (Fig. 9a-9b and 10a-10b).

4.2 GENOTOXICITY STUDIES

4.2.1 Chromosomal aberrations test (CAT) and mitotic index (MI)

a) Chromosome aberrations test (CAT):

In *Euphlyctis cyanophlyctis*, which was selected as a model animal, the diploid number was 26 (2n=26). The karyotype observed comprised of all biarmed chromosomes with NF=52. The chromosomes were having clear morphology and can be easily recognizable as five pair of large chromosomes and eight pairs of smaller chromosomes and were of two types (metacentric and submetacentric). Six pairs of chromosomes were submetacentric type i.e. pair 2, 3, 4, 9, 10 and 11 was found to be submetacentric while other chromosomes were found to be metacentric type. Haploid formula for the complement was calculated as n=7M+6SM. However, sex chromosome was not distinguishable. The chromosomes of the somatic metaphases obtained from intestine, bone marrow
Fig. 9a C-stained metaphase complement of *Euphlyctis cyanophlyctis*

Fig. 9b C-banded karyotype of *Euphlyctis cyanophlyctis* showing the presence of centromeric C-bands in all the chromosomes
Fig. 10a NOR-stained metaphase complement of *Euphlyctis cyanophlyctis*

Fig. 10b NOR-stained karyotype of *Euphlyctis cyanophlyctis* showing the location of NORs on the 10\textsuperscript{th} pair of submetacentric chromosomes
and kidney of control specimens showed clear morphology without any chromosome aberrations. The various chromosomal abnormalities observed during present investigations on frog, *E. cyanophlyctis*, after treatment with different heavy metal salts were:

- Chromosome fragmentation (Cf),
- Ring chromosomes (Rc),
- Terminal chromatid deletion (Tcd),
- Minutes (M),
- Centromeric gaps (Cg),
- Terminal association of chromosomes (Tac),
- Stickiness (Stk),
- Clumping (C)
- Pycnosis (Py) and
- Stretching (Sth)

In the present investigation, chromosomal aberrations recorded could be categorized as clastogenic and physiological type. The clastogenic effects on the chromosomes obtained after treatment with heavy metals included chromosomal fragmentation, ring chromosomes, terminal chromatid deletion, minutes and centromeric gaps. The physiological type of aberrations included terminal association of chromosomes, stickiness, clumping, pycnosis and stretching. These aberrations have been described as:

a) Chromosome fragmentation (Cf): Cf results from chromosomal breaks. In the present investigations, Cf also included chromatid or chromosome breaks, thinning of chromatids and centric as well as acentric fragments. The break gives an acentric fragment from the telomeric region which may be either aligned with the main chromosomes. Sister chromatid separation at the centromere was found to give rise to centric fragments. This process was believed to occur due to longitudinal centromeric split. The lightly stained regions on the chromosomes, generally near the centromere, were regarded as chromosome gap (if present at the same locus on both sister chromatids) and were believed to be fragile enough to give rise to acentric fragments after becoming totally destained. Theses destained loci on the chromatid were termed as chromatid breaks (or chromosome breaks if the break is present at the same locus on both sister chromatids). Such breaks have been observed in the chromosomes where the broken acentric fragment is lying close to the parent chromosome.
Chromatin extrusion or disorganization was also found to give rise to fragments. These have no definite chromosome outline. The size of the fragments was also found to vary enormously.

b) Ring chromosomes (Rc): This aberration included both big and small or dot shaped rings. These appear ring shaped due to the deletion of telomeric regions followed by joining of the ends of chromosome to give rise the formation of centric ring. The joining of the ends of acentric fragment could form the acentric ring. Therefore both centric and acentric rings were observed in the present investigations. Narrowing of the centromeric region from the sides accompanied by the joining of telomeric ends of sister chromatids was also observed to give rise to rings.

c) Terminal chromatid deletion (Tcd): Chromosomes whose chromatids were of unequal size due to deletion of some of the terminal portion show this abnormality. When only a part of the chromatid was deleted, it was termed as terminal chromatid deletion in contrast to the deletion of whole chromatid from the centric region. Later case was termed as whole chromatid deletion, however, it was considered under terminal chromatid deletion.

d) Minutes (M): In most of the cases, severe fragmentation of chromosomes seemed to result in the formation of minute chromosomes which were smaller than normal chromosome fragments and usually occur in large number in a complement. The presence of a few minutes in a complement was also a common observation. However, when whole of the metaphase complement consisted of minute chromosomes, it might be stated that such an effect could be due to extreme condensation of the chromosomes after heavy metal treatment rather than fragmentation. Thus, the cause of formation of minute chromosomes needs to be ascertained from further studies.

e) Centromeric gaps (Cg): A centromeric gap represents a condition in which centromeric region of a chromosome elongates/stretches, becomes narrow or thin and gets lightly stained or even destained. The longitudinal split in the centromeric region was also considered in this aberration.

f) Terminal association of chromosomes (Tac): a specific pattern of joining of ends of chromosomes so as to form an array was considered as terminal association of chromosomes. This may be due to the erosion of telomeric regions of chromosomes thus making the ends sticky. This association was observed to involve two to as many
as all the chromosomes of the complement. Sometimes the chromosomal fragments were also seen joined with the telomeric ends of the chromosomes.

g) Stickiness (Stk): Stickiness arises due to DNA depolymerisation. The joining of few chromosomes at positions other than their ends was considered as stickiness. This association was also observed to involve two to as all the chromosomes of the complement. The morphology of the associated chromosomes remained intact and the individual chromosomes could be easily distinguished in case of stickiness.

h) Clumping (C): The severe effect of stickiness was observed to result into clumping of a few to all the chromosomes of a complement. Clumping was usually considered as a case of coming together of all or most of the chromosomes of a complement. In the case of clumping, the morphology of the associated chromosomes did not remain intact so that the individual chromosomes could not be distinguished. Clumping was observed to result from DNA depolymerisation or chromatin disintegration/extrusion. Certain chromosomes were observed to clump in a flower shaped arrangement as if their centromeres were associated.

i) Pycnosis (Py): It was characterized by differential staining of chromosomal parts. This is due to more condensation in some regions and lesser in other regions. The condensed regions show positive pycnosis and stain more darkly with Giemsa stain and vice versa. In some instances, whole chromosomes were seen to show positive pycnosis compared to the rest of the chromosomes of the same complement.

j) Stretching (Sth): Stretching of a few to as all the chromosomes of the complement was also observed in the present investigation after treatment with various heavy metal salts. In this case, the affected chromosomes become unusually long and thin compared to the chromosomes of the control. After treatment with different heavy metal salts for different durations and concentrations, the results of chromosome aberration test obtained have shown a wide range of differences in the frequency and percentage of abnormalities with respect to the type of heavy metal used. Frequencies of different chromosome aberrations induced by individual heavy metal salt are described below in detail. In the present text, abbreviated forms of different chromosome aberrations as depicted above will be used.

b) Mitotic index (MI):
The mitotic index depicts the number of dividing cells among total cells observed. It was expressed as mean±SD. After treatment with different heavy metals, the results of MI obtained as per the dose and duration of exposure are described below in detail.

The results of chromosomal aberration test and mitotic index observed after different heavy metal treatment have been revealed in details as follows:

**CADMIUM TREATMENT**

1. Intestine

**CAT:** Frequencies of different chromosome aberrations induced in intestine after exposure to sublethal concentrations of cadmium have been summarized in Fig. 11 and Table 5. Frequency of total aberrations was found to be 5.25±0.95 (at 24hrs), 10.0±0.57 (at 48hrs), 15.25±1.89 (at 72hrs) and 25.0±1.0 (at 96hrs) in treatment with 1.5mg/kg of cadmium. Some abnormalities showed an increase, some decreased whereas others showed no change in their frequency value with an increase in duration of exposure. Maximum frequency was recorded Sth followed by C and Stk at 96 hrs of exposure and the minimum frequency was recorded for Cf and Cg. In frog treated with 6.0mg/kg of the test chemical, frequencies of total aberrations was found to be 3.75±3.30, 19.0±0.5, 25.25±1.0 and 36.75±1.29 at 24, 48, 72 and 96hrs of duration respectively. In this case, except for Rc, M, Cg, Tac and Stk, all other individual aberrations were found to increase with the increase in duration of exposure; however the percentage of Tcd was found to be same after 48hrs of duration. Sth and Py followed by Rc and C were recorded in maximum percentage after 96hrs of exposure and Stk was found to have least frequency. At a sublethal concentration of 6.0mg/kg, frequencies of total chromosomal aberrations were found to be 11.75±0.5 (at 24hrs), 21.25±1.15 (at 48hrs), 26.5±1.25 (at 72hrs) and 41.5±0.81 (at 96hrs). In this case, Rc and Cf followed by Tcd, Stk and Py were recorded in higher percentage at 96hrs whereas C was recorded in minimum percentage at 96hrs. Frog treated 12.0 mg/kg of test chemical also showed an elevation in the frequencies of total chromosomal aberrations from 24 to 96hrs i.e. 18.75±0.95, 34.0±1.89, 44.75±0.57 and 68.0±1.91 respectively. Rc and Sth were found to have maximum frequency and Stk was found to have minimum frequency at 96hrs of exposure. Some of the aberrations showed an increase whereas other decreased with an increase in the durations of exposure after different treatments. The data was significantly different from the respective controls at different exposure periods (at 24, 48, 72 and 96hrs, p<0.01) as shown in Table 5 and Fig. 12.
MI: Table 5 and Fig. 13 represent the frequencies of MI in the intestine of frog exposed to four sublethal concentrations of cadmium. The values showed a decrease from 24 to 96hrs in all treatment groups which were statistically significant from respective controls (at 24, 48 and 72hrs, p<0.01; at 96hrs, p<0.001) in all treatment doses. Minimum (14.47±0.67) and maximum (12.8±1.33) decrease of MI was recorded after 24 and 96hrs of exposure respectively in treatment with 1.5mg/kg. Frog treated with 3.0mg/kg of test compound showed 11.7±0.08, 9.95±0.65, 9.12±0.09 and 8.92±0.62 values of MI at 24, 48, 72 and 96hrs of duration respectively. Minimum (10.95±0.15) and maximum (6.12±0.17) values of MI after treatment with 6.0mg/kg were recorded at 24 and 96hrs of exposure respectively. Similar trend of decrease of MI was revealed after treatment with 12.0mg/kg of test chemical. The values recorded were 7.9±0.54, 7.3±0.96, 6.2±0.14 and 5.65±0.12 after 24, 48, 72 and 96hrs of exposure respectively.

2. Bone marrow:

CAT: Results of chromosome aberration test obtained in bone marrow showed a steady increase in percentage of aberrations with the increase of concentrations and durations of exposure. The frequencies of total chromosomal aberrations recorded after treatment with 1.5mg/kg were found to be 3.0±0.57, 4.25±0.5, 6.0±1.0 and 8.5±0.95 at 24, 48, 72 and 96hrs of exposure respectively as shown in Fig. 11 and Table 6. There was a two fold increase in frequencies of total aberrations from 24 to 72hrs and from 48 to 96hrs. Individually, only few abnormalities increased with increase in exposure periods, among which Py followed by Rc and Tcd were found in abundance at 96hrs compared to other abnormalities particularly Stk and Sth which were shown to have least percentage. Values of Stk and C remained same during all the exposure periods at treatment dose of 1.5mg/kg excepting for 24hrs where no C was found. Also not a single M, Tac, Py were recorded at 24hrs, similarly not a single Cg, Py and Sth recorded at 48hrs and not a single Cf was found at 72hrs of exposure duration. The values of total chromosomal abnormalities found in case of 3.0mg/kg treatment were 2.0±0.81, 6.75±0.81, 8.0±0.0 and 12.0±0.5 at 24, 48, 72 and 96 hrs of exposure periods respectively. Maximum incidence was recorded for Rc and Sth followed by Tcd while minimum incidence was recorded for C compared to other abnormalities at 96hrs of duration. At 24hrs of duration, except for Stk, Py and Sth, not a single chromosomal aberration was observed. In treatment with 6.0mg/kg, the total aberrations increased from 4.5±0.81 at 24hrs to 26.0±0.81 through 10.0±0.5at 48hrs and 14.75±0.57 at 72hrs. Maximum frequency of Rc and Sth followed by M and Stk were observed after 96hrs of exposure period while minimum
frequency was observed for Tac and Py at 96hrs. M and Stk were not recorded at 24 hrs of
duration. When treated with 12.0mg/kg of test chemical, the values of total chromosomal
aberrations were found to increase almost four times from 24 hrs (20.0±0.57) to 96hrs
(79.5±2.63) of exposure. Some of the aberrations showed an increase whereas others
decreased with an increase in the duration of exposure. Tac followed by C were found to be
maximum while Tcd was found to be minimum at 96hrs among various abnormalities.
Results obtained were significantly different from the controls in all the exposure periods (at
24, 48, 72hrs, p<0.01; at 96hrs, p<0.001) as shown in Table 6 and Fig. 14.

MI: Table 6 and Fig. 15 represent the frequencies of MI in the bone marrow of frog
exposed to four sub-lethal concentrations of cadmium. The values showed a decrease from
24hrs to 96hrs in all the treatment groups which were statistically significant from respective
controls except after 24hrs of exposure (at 48 and 72hrs, p<0.05; at 96hrs, p<0.01). Minimum
(8.72±1.097) and maximum (8.57±1.1) decrease of MI was recorded after 24 and 96hrs of
exposure in treatment with 1.5mg/kg. Frog treated with 3.0mg/kg of test chemical showed
8.72±1.11, 8.55±0.71, 8.27±1.02 and 8.1±0.60 values of MI at 24, 48, 72 and 96hrs of
duration respectively. Minimum (8.42±0.72) and maximum (7.07±0.61) values of MI after
treatment with 6.0mg/kg were recorded at 24 and 96hrs of exposure. Similar trend of
decrease of MI was revealed after treatment with 12.0mg/kg of test chemical. The values
recorded were 6.72±0.330, 6.57±0.34, 5.42±0.67 and 5.35±0.34 after 24, 48, 72 and 96hrs of
exposure respectively.

The results of CAT revealed higher percentage at 72 and 96hrs of exposure compared
to 24 and 48hrs of exposure in various tissues of exposed specimens. Predominant aberrations
recorded in intestine were Rc and Stk followed by Tcd and Cg whereas C, Tac, Sth were
recorded in least frequencies. In case of bone marrow, Tac, C, Sth and Py were found to be
predominant aberrations whereas Tcd followed by Cg were recorded in least percentage i.e in
bone marrow physiological aberrations were more prominent than clastogenic aberrations.
Total percentage of chromosomal aberrations was found to be highest in bone marrow
followed by intestine. Analysis of MI after treatment with various sublethal concentration of
cadmium revealed mitodepressive effect of the heavy metal studied. The effect was
predominant in intestine followed by bone marrow which is reverse as in case of CAT.
a= Chromosome fragmentation, b= Ring chromosomes, c=Terminal chromatid deletion, d=Minutes, e= Centromeric gaps, 
f= Terminal association of chromosomes, g= Stickiness, h= Clumping, i= Pycnosis, j= Stretching

Fig.11 Chromosome aberrations in intestine (A,B,C,D) and bone marrow (E,F,G,H) after cadmium treatment
Table 5: Frequencies of chromosomal aberrations (%) and mitotic index (%) in intestine of Euphlyctis cyanophlyctis after treatment with Cadmium

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<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
<th>Mitotic index (Mean ± SD)</th>
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a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4)
For mitotic index, 1000cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed= 400
Cf= Chromosome fragmentation, Rc= Ring chromosomes, Tcd=Terminal chromatid deletion, M=Minutes, Cg= Centromeric gaps, Tac= Terminal association of chromosomes, Stk= Stickiness, C= Clumping, Py= Pycnosis, Sth= Stretching, Contr.= Control
Fig. 12 Frequencies of total chromosomal aberrations in intestine after treatment with cadmium

Fig. 13 Mitotic index of intestinal cells after treatment with cadmium
Table 6: Frequencies of chromosomal aberrations (%) and mitotic index (%) in bone marrow of *Euphlyctis cyanophlyctis* after treatment with Cadmium

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<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
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<th>Total aberrations (Mean ± SD)</th>
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*a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4) For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed= 400
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Fig. 14 Frequencies of total chromosomal aberrations in bone marrow after treatment with cadmium

Fig. 15 Mitotic index of bone marrow cells after treatment with cadmium
**CHROMIUM TREATMENT**

1. **Intestine**

   **CAT:** The types and frequencies of chromosomal aberrations recorded in treatment with chromium have been shown in the Fig. 16 and Table 7. Data was found to be statistically significant from respective controls at different exposure periods (at 24hrs, p<0.05; at 48, 72 and 96hrs, p<0.01 versus control values for all treatment doses). At a sublethal dose of 2.0mg/kg, percentage of total chromosomal aberrations recorded were 10.0±0.0, 11.25±0.95, 17.75±0.95 and 28.75±1.25 after 24, 48, 72 and 96hrs of exposure periods respectively. Tac followed by Rc and Tcd was recorded in highest percentage after 96hrs of exposure while Stk was recorded the least whereas not a single M was found at 24hrs of exposure. Frequencies of total aberrations were found to be 4.0±0.81 (at 24hrs), 12.5±1.9 (at48hrs), 28.25±1.25 (at72hrs) and 38.5±0.5 (at 96hrs) in frog after treatment with 4.0mg/kg. In this case, Cf and Sth were found to be predominant aberrations at 96hrs of exposure. Tcd and Cg were the least recorded aberrations. Frog treated with 8.0mg/kg of test chemical also showed an elevation in the frequencies of total chromosomal aberrations from 24 to 96hrs i.e. 4.5±0.5, 11.75±0.5, 19.0±0.95 and 37.25±2.6 respectively. Cf, Rc, M and Sth were found to have maximum frequency at 96hrs of exposure and minimum frequency at 96hrs was recorded for Stk. Not a single Cf, Tcd, Cg, Stk and Py were found at 24hrs and not a single Tac and C were found at 48hrs of exposure. At a sublethal concentration of 16.0mg/kg, frequencies of total chromosomal aberrations were found to be 11.0±1.41, 21.0±2.16, 38.5±0.81 and 65.0±1.82 after 24, 48, 72 and 96hrs of exposure periods respectively. The frequencies of Re and Sth were found elevated at 96hrs of treatment whereas C was having least percentage compared to other abnormalities. Total number of chromosomal aberrations recorded after different treatments showed a dose dependent increase from 24 to 96hrs of exposure periods compared to respective controls (Table 7 and Fig. 17).

   **MI:** Frequencies of MI in intestinal cells of frog treated with different sublethal concentrations of chromium is given in Table 7 and Fig.18. The data was found to be statistically significant from respective controls at different exposure periods (at 24, 48 and 72hrs, p<0.01; at 96hrs, p<0.001 versus control values for all treatment doses). Mitotic index, at a dose of 2.0mg/kg were found to remain same after 24 and 48hrs (12.25±0.71 and 12.2±0.62 respectively)
but decrease afterwards from 72 to 96hrs (11.9±0.55 and 9.85±0.12 respectively). The mitodepressive effect of chromium became more pronounced after treatment with 4.0mg/kg as the values of MI decreased from 24hrs (12.5±0.29) to 96hrs (9.62±0.41). Minimum (7.67±0.49) and maximum (4.0±0.98) frequency of MI in treatment with 8.0mg/kg of chromium were recorded after 24 and 96hrs of exposure. Mean values of MI observed after treatment with 16.0mg/kg were 6.05±0.46, 4.65±1.13, 3.55±0.35 and 2.8±0.29 of exposure respectively.

2. Bone marrow

**CAT:** Effect of various concentrations of chromium (2.0, 4.0, 8.0 and 16.0mg/kg) on the chromosomes of bone marrow cells is shown in Fig. 16 and Table 8. The most significant effects (at 24, 48, 72 and 96hrs, p<0.01) were observed in all treatment groups in terms of induction of various types of chromosome which showed an increase with an increase in the duration of exposure. Total aberrations recorded in the treatment with 2.0mg/kg were 2.75±0.95 at 24hrs, 9.0±0.81 at 48hrs, 14.75±0.70 at 72hrs and 23.25±2.21 at 96hrs of duration. The frequency of individual aberration had increased with increase in duration of exposure except Cf, M, C, Py and Sth. Cg, C, Py and Cg were the most predominant effects and these were observed in considerable percentage only at 96hrs of exposure whereas Tac, Stk, Py and Sth were the only effects found at 24hrs of exposure and M and Cg were noted to be absent at 48hrs of treatment. Frequencies of total aberrations observed after treatment with 4.0mg/kg at different exposure periods were 4.25±0.5 (at 24hrs), 17.0±1.82 (48hrs), 24.0±2.06 (72hrs) and 34.75±1.5 (96hrs). Some of the aberrations showed an increase whereas other decreased with the increase in the durations of exposure. Sth and Py were the predominant effects and these were observed in considerable percentage after 96hrs of exposure. 9.25±1.63, 20.25±1.82, 26.5±1.41 and 39.5±1.21 were the values of the total aberrations observed at 24, 48, 72 and 96hrs of exposure periods respectively in treatment with 8.0mg/kg of test chemical. Tcd followed by Cf, Rc, M and Py were much elevated compared with other types of aberrations after all the durations of exposure. The incidences of total chromosomal aberrations were found to be 16.75±1.70 (at 24hrs), 32.0±0.95 (at 48hrs), 42.75±0.5 (72hrs) and 70.0±0.81 (at 96hrs) in treatment with 16.0mg/kg of test chemical. Except for M, Tad, Py and Sth, all the aberrations showed time dependent increase in the percentage. Cf and Stk were the predominant effects, observed in considerable percentage after 96hrs of exposure whereas not a single Cf, Cg and C were found at 24hrs of exposure (Table 8 and Fig. 19).
**MI:** Table 8 and Fig. 20 represent the frequencies of MI in the bone marrow cells of frog exposed to four sublethal concentrations of chromium. The values showed a decrease from 24 to 96hrs in all treatment groups which were statistically significant from respective controls (at 24, 48, 72 and 96hrs, p<0.01). Decrease in the mitotic index at lower dose (2.0mg/kg) and at high dose (16.0mg/kg) was less predominant than the decrease at other doses (4.0 and 8.0mg/kg) even though it was significantly different from the respective controls at all doses and time intervals. Minimum and maximum decrease recorded after treatment with 2.0mg/kg was 8.95±0.68 at 24hrs and 7.85±0.20 at 96hrs of exposure. Values of mitotic index obtained in bone marrow after treatment with 4.0mg/kg were found to be 7.5±1.19, 7.02±0.68, 6.92±0.51 and 6.92±0.65 at 24, 48, 72 and 96hrs of exposure periods respectively, the value remain same at 72 and 96hrs of durations. Frog treated with 8.0mg/kg of test chemical showed a maximum (5.75±1.43) and minimum (4.47±0.95) decrease of mitotic index at 24 and 96hrs of exposure period respectively. Maximum decrease in the mitotic index was recorded after treatment with highest dose of chromium (16.0mg/kg) at all the exposure periods compared to controls. The values recorded were 6.77±0.39, 6.47±1.47, 6.0±1.32 and 5.12±1.06 after 24, 48, 72 and 96hrs of exposure respectively.

Present results of chromosome aberrations in intestine and bone marrow after using chromium as test chemical have revealed that the frequencies of total aberrations were found to be predominant in bone marrow compared to intestine. In case of intestine, the predominant aberrations recorded were Rc and Stk while in bone marrow, the maximum frequency was found for Cf and Stk, all having the same percentage value. The maximum frequency of chromosome aberrations was recorded only after treatment with maximum sublethal concentration of test chemical at 96hrs of exposure period which was regarded as the effective sublethal concentration at 96hrs of exposure period. The frequency C was found to be minimum in both intestine as well as bone marrow. Maximum decrease of mitotic index was observed in intestine compared to bone marrow.
CHROMIUM TREATMENT

INTESTINE

BONE MARROW

a= Chromosome fragmentation, b= Ring chromosomes, c= Terminal chromatid deletion, d= Minutes, e= Centromeric gaps, f= Terminal association of chromosomes, g= Stickiness, h= Clumping, i= Pycnosis, j= Stretching

Fig. 16 Chromosome aberrations in intestine (A,B,C,D) and bone marrow (E,F,G,H) after chromium treatment
Table 7: Frequencies of chromosomal aberrations (%) and mitotic index (%) in intestine of *Euphlyctis cyanophlyctis* after treatment with Chromium

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
<th>Mitotic index (Mean ± SD)</th>
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<td>Cf</td>
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</table>

Note: a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4)

For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed = 400

Cf= Chromosome fragmentation, Rc= Ring chromosomes, Tcd=Terminal chromatid deletion, M=Minutes, Cg= Centromeric gaps, Tac= Terminal association of chromosomes, Stk= Stickiness, C= Clumping, Py= Pycnosis, Sth= Stretching, Contr.= Control
Fig. 17 Frequencies of total chromosomal aberrations in intestine after treatment with chromium

Fig. 18 Mitotic index of intestinal cells after treatment with chromium
Table 8: Frequencies of chromosomal aberrations (%) and mitotic index (%) in bone marrow of *Euphycys cyanophyctis* after treatment with Chromium

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
<th>Mitotic index (Mean ± SD)</th>
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<td>Cf  Rc Tcd M Cg Tac Stk C Py Sth</td>
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<td>6.92±0.65^b</td>
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<td>20.25±1.82^b</td>
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<td>6.77±0.39^b</td>
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<td>32.0±0.95^b</td>
<td>6.47±1.47^b</td>
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<td>42.75±0.5^b</td>
<td>6.0±1.32^b</td>
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<td>70.0±0.81^b</td>
<td>5.12±1.06^b</td>
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*a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4) For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed = 400*

Cf= Chromosome fragmentation, Rc= Ring chromosomes, Tcd=Terminal chromatid deletion, M=Minutes, Cg= Centromeric gaps, Tac= Terminal association of chromosomes, Stk= Stickiness, C= Clumping, Py= Pycnosis, Sth= Stretching, Contr.= Control
Fig. 19 Frequencies of total chromosomal aberrations in bone marrow after treatment with chromium

Fig. 20 Mitotic index of bone marrow cells after treatment with chromium
COPPER TREATMENT

1. Intestine

CAT: Treatment In vivo with four sublethal concentrations (3.5mg/kg, 5.0mg/kg, 6.5mg/kg and 8.0mg/kg) of coppersulphate resulted in induction of various chromosomal aberrations in the intestinal cells which increased with the increase in doses and time of exposure as shown in Fig. 21 and Table 9. The values of total aberrations recorded in treatment with 3.5mg/kg after 24, 48, 72 and 96hrs of duration were 4.0±0.81, 7.0±0.0, 14.0±1.41 and 26.0±1.82 respectively. Maximum frequency was observed for M and Cg whereas minimum frequency was observed for Tcd, Stk and Sth. The only aberrations observed at 24hrs were Cg, Stk, C and Sth. Not a single Rc, Tcd, Cg, Tac and C was observed at 48hrs and also not a single Stk, C and Py was observed at 72hrs of exposure. Frog treated with 5.0mg/kg showed clear time dependent increase of total aberrations from 7.0±0.81 at 24hrs to 30.0±1.25 at 96hrs of exposure. Excepting for Cf, Stk, Py and Sth, similar trend of time dependent increase was observed in the frequencies of individual aberrations. Highest number of Cf and Sth were recorded after 96hrs and minimum number was found to be Stk compared to other aberrations. Not a single Tcd, M, Tac, C and Sth (at 24hrs), Tcd, Stk and Py (at 48hrs) and C and Sth (at 96hrs) were observed. Minimum (13.0±1.15) and maximum (37.0±0.81) value of total aberrations were found at 24hrs and 96hrs respectively in the treatment with 6.5mg/kg. In this case, Tcd have same frequency for all the duration of exposure and not a single C was found at 24, 48 and 96hrs of exposure. Some of the aberrations showed an increase whereas other decreased with an increase in duration of exposure. The frequency of Sth followed by Rc was highest among various aberrations and the number of Tcd and Stk were found to be lowest at the same duration of exposure. The trend of time dependent increase continued even at the highest dose tested (8.0mg/kg) for which the minimum (24.0±1.25) and maximum (76.0±2.06) value of total aberrations were recorded at 24hrs and 96hrs. Except for Cg and Sth, individual aberrations increased with an increase in the duration of exposure wherein M followed by Cf had the highest frequency (at 96hrs). However, minimum frequency was obtained for Cg, C and Py at 96hrs of exposure. Further, the frequencies of abnormalities scored in all the treatments were significantly higher (at 24, 48, 72 and 96hrs, p<0.001) compared to the respective controls (Table 9 and Fig. 22).

MI: A significant decrease (at 24, 48 and 72hrs, p<0.01; at 96hrs, p<0.001 versus respective controls in all the treatment groups) in the mitotic index was evident in intestine of frog treated with four sublethal concentrations of test chemical and evaluated after 24, 48, 72 and 96hrs of exposure.
durations (Table 9 and Fig. 23). The values obtained after treatment with 3.5mg/kg showed decrease from 10.67±0.70 to 8.75±1.13 after 24 to 96hrs of exposure. Similar trend of decrease in the mitotic index was observed after treatment with 5.0mg/kg where the minimum and maximum values were 9.47±0.27 and 8.17±0.45 obtained after 24 and 96hrs respectively. Mitotic index was found decreased after all the exposure periods during treatments with 6.5mg/kg and 8.0mg/kg. Values recorded for 6.5mg/kg were 7.42±0.51 (24hrs), 6.80±0.21 (48hrs), 5.95±0.33 (72hrs) and 4.92±0.57 (96hrs) whereas the values of mitotic index for 8.0mg/kg were 4.02±0.99, 3.95±0.71, 3.65±0.40 and 3.52±1.03 at 24, 48, 72 and 96hrs of exposure respectively.

2. Bone marrow

CAT: Results of chromosome aberration test (Fig. 21 and Table 10) obtained in bone marrow showed a steady increase in percentage of aberrations with the increase in concentrations and durations of exposure. Values in the treated groups were found to be significantly higher (at 24, 48 and 72hrs, p<0.01; at 96hrs, p<0.01) versus respective controls in all the treatment groups. The highest and lowest percentage of abnormalities observed after treatment with 3.5mg/kg was 21.75±0.95 (after 96hrs of exposure period) and 4.25±0.5 (after 24hrs of exposure period) respectively. A time dependent increase was observed in the frequencies of individual aberrations except for Tac and also not a single Cg (at24hrs) and Stk (at 48hrs) was observed. However, the number of Rc followed by number of M was much elevated compared with other type of aberrations after all the durations of exposure. The frequencies of total aberrations found in treatment with 5.0mg/kg of copper were 12.75±1.5, 19.5±1.29, 24.5±1.29 and 36.75±1.25 at 24, 48, 72 and 96hrs of exposure respectively. Some of the aberrations showed an increase whereas other decreased with the increase in the durations of exposure. Among various aberrations, Rc followed by M and Tac were found in maximum frequency whereas minimum frequency was observed for Stk at 96hrs. Similarly, total aberrations observed after treatment with 6.5mg/kg of test chemical were 20.5±1.70 (at 24hrs), 26.25±2.5 (at 48hrs), 30.0±0.81 (at 72hrs) and 50.75±0.95 (at 96hrs). Maximum percentage at 96hrs was recorded for Cf while minimum percentage was obtained for Stk. The frequency of some individual aberrations increased while in some, it decreased with the duration of exposure except for Stk, the value recorded is same for Stk after 24hrs of duration. The frog treated with 8.0mg/kg of test compound revealed the frequencies of total aberrations as 24.75±1.82, 36.75±2.21, 56.00±1.5 and 80.25±1.25 after 24, 48, 72 and 96hrs of exposure respectively. Among various abnormalities
recorded, maximum frequency was observed for M followed by Cf and Tac at 96hrs of exposure whereas Stk was observed with least frequency after 96hrs of exposure. The frequency of individual aberration had increased with increase in duration of exposure except for Rc, Tcd, Stk and Py (Table 10 and Fig. 24).

**MI:** In the present investigation, the cytotoxicity of copper sulphate after treatment with four sublethal concentrations was evident as the mitotic index was found to decrease significantly from respective controls except after 24hrs of duration in each concentration where be non-significant (at 48hrs, p<0.05; at 72 and 96hrs, p<0.01) (Table 10 and Fig. 25). Frog treated with 3.5mg/kg of test chemical showed a slight decrease of mitotic index in relation to durations of exposure and compared to respective controls. The values recorded were 7.25±0.86 (24hrs), 7.85±0.05 (48hrs), 6.32±1.20 (72hrs) and 6.4±0.29 (96hrs). The mitotic index at 24hrs was not significantly different from the control value of 7.8±0.18 whereas all the values observed after 24hrs were significant compared to respective controls. In the treatment with 5.0mg/kg, minimum (6.85±0.44) and maximum (5.87±1.01) decrease was recorded just after 24hrs and 96hrs of exposure periods respectively. The value of mitotic index at 24hrs was not significant compared to control value. With treatment of 6.5mg/kg, the mitotic index decreased from 7.75±0.20 (at 24hrs) to 6.52±1.03 (at 96hrs) through 7.52±0.30 (at 48hrs) and 7.47±0.59 (72hrs) and the value was not significantly different from the control. Similar decreasing trend in the value of mitotic index was observed after treatment with the highest sublethal concentration of 8.0mg/kg. Value of mitotic index (8.95±0.62) at 24hrs was not significant compared to control. The minimum (8.95±0.62) and maximum (5.5±0.98) decrease was observed at 24hrs and 96hrs of duration respectively.

Results of CAT that highest percentage of different chromosomal aberrations were induced only after treatment with maximum dose of 8.0mg/kg of test chemical in both the tissues of the exposed specimens. Different aberrations which were found to be predominant at different doses of exposure were M followed by Cf and Tac (in bone marrow); Cf and Tcd (in intestine) while Stk (in bone marrow) and Cg, C and Py (in intestine) were found to have least frequency. In both the tissues, clastogenic effects were found to be more predominant than physiological type of aberrations. Total percentage of chromosomal aberrations was found to be highest in bone marrow followed by intestine. Analysis of MI after treatment with various sublethal concentrations of copper revealed mitodepressive effect of heavy metal studied. The effect was predominant in intestine than in bone marrow.
COPPER TREATMENT

INTESTINE

BONE MARROW

a= Chromosome fragmentation, b= Ring chromosomes, c=Terminal chromatid deletion, d=Minutes, e= Centromeric gaps, f= Terminal association of chromosomes, g= Stickiness, h= Clumping, i= Pycnosis, j= Stretching

Fig. 21 Chromosome aberrations in intestine (A,B,C,D) and bone marrow (E,F,G,H) after copper treatment
Table 9: Frequencies of chromosomal aberrations (%) and mitotic index (%) in intestine of Euphlyctis cyanophlyctis after treatment with Copper

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
<th>Mitotic index (Mean ± SD)</th>
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<tr>
<td></td>
<td></td>
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<td>Cf</td>
<td>Rc</td>
<td>Tcd</td>
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\(^a=p<0.05\), \(^b=p<0.01\), \(^c=p<0.001\), \(n.s=\) non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; \(df=4\)) For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed = 400

\(\text{Cf}=\) Chromosome fragmentation, \(\text{Rc}=\) Ring chromosomes, \(\text{Tcd}=\) Terminal chromatid deletion, \(\text{M}=\) Minutes, \(\text{Cg}=\) Centromeric gaps, \(\text{Tac}=\) Terminal association of chromosomes, \(\text{Stk}=\) Stickiness, \(\text{C}=\) Clumping, \(\text{Py}=\) Pycnosis, \(\text{Sth}=\) Stretching, \(\text{Contr.}=\) Control
Fig. 22 Frequencies of total chromosomal aberrations in intestine after treatment with Copper

Fig. 23 Frequencies of mitotic index in intestinal cells after treatment with Copper
Table 10: Frequencies of chromosomal aberrations (%) and mitotic index (%) in bone marrow of *Euphlyctis cyanophlyctis* after treatment with Copper

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<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
<th>Mitotic index (Mean ± SD)</th>
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a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4). For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed= 400.

Cf= Chromosome fragmentation, Rc= Ring chromosomes, Tcd=Terminal chromatid deletion, M=Minutes, Cg= Centromeric gaps, Tac= Terminal association of chromosomes, Stk= Stickiness, C= Clumping, Py= Pycnosis, Sth= Stretching, Contr.= Control
Fig. 24 Frequencies of total chromosomal aberrations in bone marrow after treatment with Copper

Fig. 25 Mitotic index of bone marrow cells after treatment with Copper
LEAD TREATMENT

1. Intestine

CAT: Treatment in vivo with 4.5mg/kg, 9.0mg/kg, 18.0mg/kg and 36.0mg/kg of lead acetate resulted in a significant increase in the frequencies of chromosomal aberrations compared to respective controls (at 24, 48, 72 and 96hrs, p<0.01) after all the exposure periods (Fig. 26 and Table 11). After treatment with 4.5mg/kg, the percentage of total chromosomal aberrations was found to increase with the duration of exposure. The highest percentage of chromosome aberrations (18.0±0.816) was recorded after 96hrs and the least percentage (11.5±1.29) was obtained after 24hrs of treatment. Cf, Cg, Stk and Sth were not observed at 48hrs whereas M and Py were absent after 96hrs of exposure. Rc followed by Tcd and Cg were observed in highest percentage while other types of aberrations had almost equal percentage after 96hrs of exposure. After treatment with 9.0mg/kg, percentage of aberrations increased from 20.5±1.914 at 24hrs to 34.5±1.41 at 96hrs through 24.0±2.16 at 48hrs and 31.0±1.82 at 96hrs. Some of the aberrations increased whereas other decreased with an increase in duration of exposure with no C found at 96hrs of exposure. However among all the abnormalities, highest frequency was scored by Rc followed by Cf and Tcd at 96hrs of duration. Total number of chromosomal aberrations recorded after treatment with 18.0mg/kg also showed a time dependent increase from 24 to 96hrs of exposure period which were significant at all the exposure periods compared to controls. There was a total of 30.0±0.81, 42.0±1.63, 47.0±1.41 and 55.0±1.5 percentage of total aberrations after 24, 48, 72 and 96hrs of exposure periods respectively. The percentage of Rc followed by Cf and Stk was much elevated at 96hrs of treatment whereas C showed highest frequency at 72hrs of exposure. Least frequency was observed of Cg, C and Py at 96hrs of exposure as compared to other abnormalities. The percentages of total aberrations observed after treatment with 36.0mg/kg were 63.5±1.29, 74.0±2.70, 87.5±2.08 and 99.5±1.41 after 24, 48, 72 and 96hrs respectively. Some of the individual aberrations showed an increase, others decreased and some remain constant in the duration of exposure. However at 96hrs of exposure, C followed by Cf and Py were highest and Stk was the least observed chromosome abnormality (Table 11 and Fig. 27).

MI: A significant decrease of mitotic index was observed in various treatments and at all the exposure periods compared to respective controls (at 24 and 48hrs, p<0.01; at 72 and 96hrs, p<0.001) (Table 11 and Fig. 28). Lowest (12.05±0.44) and highest (10.57±0.49) decrease in the value of mitotic index was obtained at 24 and 96hrs respectively in treatment with 4.5mg/kg of test chemical.
Similarly, minimum value of mitotic index obtained in the treatment with 9.0, 18.0, 36.0mg/kg of test chemical was 13.0±0.57, 10.25±0.50 and 6.75±0.64 respectively obtained at 24hrs of exposure. However, maximum decrease in the mitotic index was found to be 11.72±0.37, 6.92±0.69 and 5.34±0.43 recorded after 96hrs in treatment with 9.0, 18.0, 36.0mg/kg respectively.

2. Bone marrow

CAT: A significantly higher (at 24, 48, 72 and 96hrs, p<0.001 for all the treatment doses versus controls) dose and time dependent increase in the frequencies of chromosome aberrations was observed in the intestinal tissue treated with four sublethal concentrations of lead acetate (4.5, 9.0, 18.0 and 36.0mg/kg) (Fig. 26 and Table 12). At a sublethal concentration of 4.5mg/kg, minimum and maximum percentage of chromosome aberrations recorded was 3.0±0.81 (after 24hrs of exposure) and 20.0±0.81 (after 96hrs of exposure). Except for Cf, Rc and Py, all the aberrations showed a time dependent increase in the percentage. The aberrations which showed higher percentage were Sth followed by Rc. Other types of aberrations had almost equal percentage at each of the exposure durations. The highest and lowest percentage of total chromosomal abnormalities observed after treatment with 9.0mg/kg of test compound was 30.0±0.81 (after 96hrs of exposure) and 6.75±1.5 (after 24hrs of exposure). The chromosomal aberrations which showed higher percentage were Rc followed by Sth and the effect with lower percentage was Cg. During treatment with 18.0mg/kg, lowest and highest frequency of chromosomal aberrations scored was 14.5±1.29 and 46.75±0.95 after 24hrs and 96hrs of exposure periods. Among various chromosomal abnormalities scored, highest frequency was recorded for Stk, Rc and Sth after 96hrs of exposure period. Least frequency was observed for Cf and Py after 96hrs of duration. Some abnormalities showed an increase, some decreased whereas others showed no change in their frequency value with an increase in duration of exposure (Table 12 and Fig. 29). Lowest and highest frequency of chromosome aberrations recorded after treatment with 36.0mg/kg was 37.75±1.25 (after 24hrs of exposure) and 81.5±0.57 (after 96hrs of exposure). Among various aberrations recorded, Py followed by Rc and Sth were found in higher frequencies compared to other abnormalities and C had lowest frequency at 96hrs. Except Cg, M, Stk and C, all the aberrations showed a time dependent increase in the percentage.

MI: Mitodepressive effect of lead acetate treatment on intestinal cells was observed due to the decreased mitotic index. The effect was significantly different from the respective controls (at 24 and 48hrs, p<0.01; at 72 and 96hrs, p<0.001) versus respective controls in all treatment groups) and
showed dose and time dependent decrease (Table 12 and Fig. 30). After treatment with low doses of 4.5mg/kg and 9.0mg/kg of lead acetate, the maximum and minimum values 10.77±1.82 (4.5mg/kg; 24hrs) and 8.97±0.17 (4.5mg/kg; 96hrs) and 9.42±1.05 (9.0mg/kg; 24hrs) and 8.10± 0.42 (9.0mg/kg; 96hrs). Similarly, the values obtained after treatment with 18.0mg/kg were 7.47±0.40 (after 24hrs), 6.70±2.16 (after 48hrs), 7.17±0.27 (after 72hrs) and 5.975±1.75 (after 97hrs) which showed a decrease with increase in the duration of exposure. The maximum and minimum values of mitotic index in treatment with 36.0mg/kg were 5.92±0.20 (after 24hrs) and 3.92±0.92 (after 96hrs).

Results of chromosomal aberration test showed that lead acetate treatment in both the tissues induced a whole spectrum of chromosome aberrations which could be broadly classified into clastogenic and physiological type. The former aberrations include chromosomal fragmentation, ring chromosomes, terminal chromatid deletion, minutes and centromeric gap and the later include terminal association of chromosomes, stickiness, clumping, pycnosis and stretching. Among the various chromosome abnormalities scored in intestine, C followed by Cf and Py showed the highest frequency and the frequency of Stk was found to be least. In case of bone marrow, predominant aberrations were found to be Py followed by Rc and Sth while C and Stk were the least observed aberrations. Therefore, the highest percentage of individual aberrations has been recorded among the physiological type of aberrations i.e. C and Py and also the least percentage was also observed in physiological type of aberrations showing that this type of aberrations had a wider range of the values recorded. The maximum percentage of chromosomal aberrations was observed in case of intestine than in bone marrow. The results of mitotic index showed a general trend of decrease in both the treated tissues versus respective control values. Maximum decrease in the mitotic index was evident in case of intestine compared to bone marrow.
LEAD TREATMENT

INTESTINE

a= Chromosome fragmentation, b= Ring chromosomes, c=Terminal chromatid deletion, d=Minutes, e= Centromeric gaps, f= Terminal association of chromosomes, g= Stickiness, h= Clumping, i= Pycnosis, j= Stretching

Fig. 26 Chromosome aberrations in intestine (A,B,C,D) and bone marrow (E,F,G,H) after lead treatment
Table 11: Frequencies of chromosomal aberrations (%) and mitotic index (%) in intestine of *Euplectis cyanophlyctis* after treatment with Lead

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<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
<th>Mitotic index (Mean ± SD)</th>
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*a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4)*

For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed= 400

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Fig. 27 Frequencies of total chromosomal aberrations in intestine after treatment with Lead

Fig. 28 Mitotic index of intestinal cells after treatment with Lead
Table 12: Frequencies of chromosomal aberrations (%) and mitotic index (%) in bone marrow of *Euphlyctis cyanophlyctis* after treatment with Lead

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<th>Duration (Hrs)</th>
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a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4)

For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed= 400

Cf= Chromosome fragmentation, Rc= Ring chromosomes, Tcd=Terminal chromatid deletion, M=Minutes, Cg= Centromeric gaps, Tac= Terminal association of chromosomes, Stk= Stickiness, C= Clumping, Py= Pycnosis, Sth= Stretching, Contr.= Control
Fig. 29 Frequencies of total chromosomal aberrations in bone marrow after treatment with Lead

Fig. 30 Mitotic index of bone marrow cells after treatment with Lead
MANGANESE TREATMENT

1. Intestine

CAT: treatment in vivo with four sublethal concentrations (4.0, 8.0, 16.0 and 32.0mg/kg) of manganese chloride resulted in the induction of various chromosomal aberrations in the intestine which increased with the increase of doses and time of exposure as shown in Fig. 31 and Table 13. The values of total aberrations recorded in treatment with 4.0mg/kg after 24, 48, 72 and 96hrs of duration were 2.75±0.57, 6.0±0.5, 7.75±0.95 and 13.25±0.57 respectively. Maximum frequency was observed for Py whereas Cf had minimum frequency among all the aberrations recorded; however Tcd was only found at 48hrs of duration and not a single Cg, Tac, Stk, C, Py and Sth was observed at 24hrs of exposure. Frog treated with 8.0mg/kg showed clear time dependent increase of total aberrations 4.0±0.57 at 24hrs to 28.5±1.5 at 96hrs of exposure. Highest number of Stk and Py were recorded after 96hrs and minimum number was found to be of M. as compared to other aberrations. Except for Cf, Rc, Cg and C, all other aberrations were not found at 24hrs. Minimum (7.25±0.95) and maximum (29.25±1.91) value of total aberrations were observed at 24hrs and 96hrs respectively in treatment with 16.0mg/kg of test chemical. Individual aberrations except for Cf, C and Py also resulted to have maximum percentage at 96hrs. The frequency of Sth was found to be highest among various abnormalities at 96hrs and the number of Cf and C were found to be lowest at the same duration of exposure. The trend of time dependent increase continued even at the highest dose tested (32.0mg/kg) for which the minimum (10.0±0.5) and maximum (56.5±1.0) value of total aberrations were recorded at 24 and 96hrs. Individual aberrations, except Cf, Rc, Tcd and Py increased with an increase in the duration of exposure wherein Py and Sth had the highest frequency (at 96hrs). However, minimum frequency was obtained for Tcd at 96hrs of exposure. Further, the frequencies of abnormalities scored in all the treatments were significantly higher (at 24, 48, 72 and 96hrs, p< 0.01) compared to the respective controls (Table 13 and Fig. 32).

MI: The values of mitotic index in intestinal tissue recorded after treatment with different sublethal concentrations of manganese have been shown in Table 13 and Fig. 33. The values of MI were only slightly decreased at 24, 48, 72 and 96hrs with respect to controls in all treatment groups. In all the sublethal concentrations studied, the MI after 24 and 48hrs of duration was found to be non significant compared to the respective control but was significant after 72 and 96hrs of exposure (p<0.05) versus respective control. Frog treated in vivo with 4.0mg/kg of test chemical showed a decrease trend of MI with respect to the increase in duration of exposure. Values recorded were
11.95±0.79, 11.87±0.80, 11.52±1.93 and 10.72±1.19 after 24, 48, 72 and 96hrs of exposure respectively. Minimum (10.37±1.06) and maximum (7.85±0.67) values of MI after treatment with 8.0mg/kg were recorded at 24 and 48hrs of exposure. At a sublethal concentration of 16.0mg/kg, the value of MI decreased from 7.8±0.64 (at 24 hrs) to 6.25±0.56 (at 96hrs) through 7.1±0.29 (at 48hrs) and 6.4±0.29 (at 72hrs). Similar trend of decrease of MI was revealed after treatment with 32.0mg/kg of test chemical. The values recorded were 5.7±0.58, 4.77±0.37, 4.75±1.31 and 4.45±0.68 after 24, 48, 72 and 96hrs of exposure respectively.

2. Bone marrow

CAT: The frequencies of different types of chromosomal aberrations induced in bone marrow cells after treatment with four sublethal concentrations of manganese have been illustrated in Fig. 31 and Table 14. At a sublethal concentration of 4.0mg/kg, percentage of total chromosomal aberrations increased from 1.0±0.57 (at 24hrs) to 15.0±1.0 (at 96hrs) through 3.5±0.0 (at 72hrs) and 6.75±1.25 (at 96hrs). Among various individual abnormalities, most of them have maximum frequency at 96hrs. Some aberrations were totally absent at different durations of exposure; for example except for Cg and Py; Cf, Rc and M, all other aberrations were not observed at 24hrs and 48hrs respectively, M, Cg, Tac and Stk were absent at 72hrs. The values of total chromosomal abnormalities found in case of 8.0mg/kg treatment were 2.5±0.57, 11.75±0.57, 9.5±1.5 and 20.5±1.25 at 24, 48, 72 and 96hrs respectively. Maximum incidence was recorded for Sth followed by Stk and C minimum incidence was recorded for Cg compared to other aberrations at 96hrs of duration. At 24hrs of exposure, not a single Cf, Tcd, M, Cg, Stk and C was observed and not a single Tcd was found at 72hrs of exposure. Frog exposed to 16.0mg/kg of test chemical revealed the percentage of total chromosomal aberrations as 4.5±0.0 at 24hrs, 14.5±0.57 at 48hrs, 20.5±0.81 at 72hrs and 31.25±1.5 at 96hrs of durations. Maximum frequency was recorded for Rc and M while minimum frequency was observed for Py at 96hrs of exposure. Exposure to 32.0mg/kg of test chemical induced minimum (9.25± 0.5) and maximum (48.75±1.29) frequencies of total aberrations at 24 and 96hrs of exposure periods respectively. Individual aberrations except for Rc, M, C and Py, increased with increase in the exposure periods being highest at 96hrs. Rc followed by Sth were the predominant aberrations observed at 96hrs. Data obtained was statistically significant in all the exposure groups with respect to controls at different exposure periods (at 24, 48, 72 and 96hrs, p<0.01) as shown in Table 14 and Fig. 34.
**MI:** In the present investigation, the values of mitotic index were recorded as $9.72 \pm 0.26$, $9.75 \pm 0.36$, $9.62 \pm 0.20$ and $9.45 \pm 0.23$ in the control group after 24, 48, 72 and 96hrs of exposure (Table 14 and Fig. 35). Cytotoxicity of manganese chloride was evident in the bone marrow cells as the mitotic index was found to decrease in all the exposure periods after treatment with 4.0mg/kg ($9.50 \pm 0.35$ at 24hrs, $8.82 \pm 0.62$ at 48hrs, $8.85 \pm 0.35$ at 72hrs and $6.97 \pm 0.17$ at 96hrs), 8.0mg/kg ($8.40 \pm 0.37$ at 24hrs, $7.85 \pm 0.45$ at 48hrs, $7.37 \pm 0.29$ at 72hrs and $7.30 \pm 0.25$ at 96hrs), 16.0mg/kg ($8.30 \pm 0.34$ at 24hrs, $7.92 \pm 0.30$ at 48hrs, $7.25 \pm 0.47$ at 72hrs and $7.05 \pm 0.54$ at 96hrs) and 32.0mg/kg ($5.80 \pm 0.29$ at 24hrs, $5.40 \pm 0.53$ at 48hrs, $5.02 \pm 0.28$ at 72hrs and $4.7 \pm 0.31$ at 96hrs) of manganese chloride. The values were significantly different from the respective controls except for 24 hrs of exposure (at 48hrs, $p<0.05$; at 72 and 96hrs, $p<0.01$) in all the treatment groups.

The chromosomal aberrations recorded in different tissues revealed a dose and time dependent increase in their frequencies which were significantly different from their respective controls at all exposure periods. Most frequent aberrations recorded in intestine after treatment with manganese were Sth, Py and C whereas in bone marrow, Rc and Sth were the most frequent aberrations recorded. Sth was observed to be frequent abnormality recorded in both the tissues however, maximum percentage of chromosomal aberrations was found in intestine followed by bone marrow. Maximum decrease of MI was found in intestine and the minimum effect of manganese treatment on MI was revealed in bone marrow.
MANGANESE TREATMENT

INTESTINE

BONE MARROW

a= Chromosome fragmentation, b= Ring chromosomes, c=Terminal chromatid deletion, d=Minutes, e= Centromeric gaps,
f= Terminal association of chromosomes, g= Stickiness, h= Clumping, i= Pycnosis, j= Stretching

Fig. 31 Chromosome aberrations in intestine (A,B,C,D) and bone marrow (E,F,G,H) after manganese treatment
Table 13: Frequencies of chromosomal aberrations (%) and mitotic index (%) in intestine of *Euphlyctis cyanophlyctis* after treatment with Manganese

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<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
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a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4)  
For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed= 400. Cf= Chromosome fragmentation, Rc= Ring chromosomes, Tcd=Terminal chromatid deletion, M=Minutes, Cg= Centromeric gaps, Tac= Terminal association of chromosomes, Stk= Stickiness, C= Clumping, Py= Pycnosis, Sth= Stretching, Contr.= Control
Fig. 32 Frequencies of total chromosomal aberrations in intestine after treatment with Manganese

Fig. 33 Mitotic index of intestinal cells after treatment with Manganese
Table 14: Frequencies of chromosomal aberrations (%) and mitotic index (%) in bone marrow of *Euphlyctis cyanophlyctis* after treatment with Manganese

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<th>Conc. (mg/kg)</th>
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a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4)

For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed= 400. Cf= Chromosome fragmentation, Rc= Ring chromosomes, Tcd=Terminal chromatid deletion, M=Minutes, Cg= Centromeric gaps, Tac= Terminal association of chromosomes, Stk= Stickiness, C= Clumping, Py= Pycnosis, Sth= Stretching, Contr.= Control
Fig. 34 Frequencies of total chromosomal aberrations in bone marrow after treatment with Manganese

Fig. 35 Mitotic index of bone marrow cells after treatment with Manganese
1. Intestine

CAT: Highly significant frequencies of total chromosomal aberrations were observed in the intestine after various treatments of mercury (at 24, 48, 72 and 96 hrs, \( p < 0.001 \)) which is shown in Fig. 36 and Table 15. The incidence of chromosomal aberrations in treatment with mercury at a concentration of 1.5µg/kg was found to be 5.25±0.5, 7.0±0.95, 15.5±1.55 and 20.75±0.57 after 24, 48, 72 and 96hrs of exposure respectively. Percentage of Sth was found to be highest among various types of aberrations scored at 96hrs. In treatment with 3.0µg/kg, minimum and maximum value was 4.5±0.0 and 21.0±1.5 at 24 and 96hrs of exposure. Maximum frequency was observed for Tac and Sth at 96hrs of exposure while Py was the least observed. Minimum and maximum frequency of chromosomal aberrations induced after treatment with 6.0µg/kg was 6.0±0.5 (at 24hrs) and 25.0±0.5 (at 96hrs). Rc followed by Stk was the most frequent abnormality in this treatment while Py was found in minimum frequency. At the highest sublethal concentration tested (12.0µg/kg), pattern of total chromosomal aberrations showed a time dependent increase; being minimum at 24hrs with a frequency of 13.5±1.29 and maximum at 96hrs with a frequency of 80.5±2.63. Rc followed by Py showed highest incidence at 96hrs while Tcd and M had minimum frequency (Table 15 and Fig. 37).

MI: The mitodepressive effect of treatment with sublethal concentrations of mercury was evident on the intestinal cells as its mitotic index decreased with an increase in the dose and exposure periods as shown in Table 15 and Fig. 38. The values were found to be statistically significant and different compared to respective controls after different durations of exposure in all treatment groups (at 24, 48, 72 and 96hrs, \( p < 0.01 \)). At low dose (1.5µg/kg and 3.0µg/kg), the value of mitotic index decreased considerably from 24hrs to 96hrs of dose treatment, the values observed at low dose (1.5µg/kg) decrease from 10.25±0.45 to 8.47±0.43 and the mitotic index recorded at 3.0 µg/kg also show a similar trend in decrease from 24 to 96hrs of duration i.e. 10.05±0.91 to 8.37±1.11 respectively. In the frog exposed to 6.0µg/kg of the test chemical, the observed divisional frequency were 8.7±0.60, 7.65±0.97, 7.5±1.17 and 6.45±7.17 at 24, 48, 72 and 96hrs respectively. Similar trend of decrease in the mitotic activity of intestinal cells was observed in treatment with 12.0µg/kg, the values recorded were found to be 7.9±0.63, 6.12±0.90, 5.4±0.73 and 4.5±0.35 at 24, 48, 72 and 96hrs of exposure periods respectively.

2. Bone marrow:
**CAT:** Frequencies of different chromosome aberrations induced in bone marrow after exposure to sublethal concentrations of mercury have been summarized in Fig. 36 and Table 16. Frequencies of total aberrations were found to be 6.25±1.25 (at 24hrs), 23.75±1.25 (at 48hrs), 26.5±0.57 (at 48hrs) and 37.25±1.0 (at 96hrs) in treatment with 1.5µg/kg of mercury. Maximum frequency was recorded for Rc followed by C and Sth and then by Cg and Cf at 96hrs of exposure while minimum frequency was recorded for Tac and Py. Some of the aberrations showed an increase whereas others decreased with an increase in the duration of exposure. Frequencies of total aberrations were found to be 12.75±1.25 (at 24hrs), 26.75±0.95 (at 48hrs), 35.75±3.69 (at 72 hrs) and 49.25±0.95 (at 96hrs) in frog after treatment with 3.0µg/kg of the test metal. In this case, Rc, Tac and C were recorded in maximum percentage followed by Sth, the least effect observed was Cg after 96hrs of exposure. At sublethal concentration of 6.0µg/kg, frequencies of total chromosomal aberrations were found to be 17.75±0.5, 38.75±0.95, 50.5±0.81 and 70.25±1.73 after 24, 48, 72 and 96hrs of exposure periods respectively. In this case, Stk and Tac were recorded in higher percentage followed by Rc, C and Py while least percentage aberrations were recorded for Cf at 96hrs of exposure period. Frog treated with 12µg/kg of test chemical showed an elevation in the frequencies of total chromosomal aberrations from 24 to 96hrs i.e. 25.25±2.64, 45.5±2.21, 70.0±0.57 and 98.5±1.0 respectively. Rc, Tcd, Cf and Sth were found to have maximum frequency while Py was recorded in least percentage at 96hrs. The data was significantly different from the respective controls at different exposure periods (at 48hrs, p<0.05; at 24, 72 and 96hrs, p<0.01) as shown in Table 16 and Fig. 39.

**MI:** Table 16 and Fig. 40 represent the frequencies of MI in the bone marrow of frog exposed to four sublethal concentrations of mercury. The values showed a decrease from 24 to 96hrs in all the treatment groups which were statistically significant from respective controls (at 24 and 48hrs, p<0.05; at 72 and 96hrs, p<0.01). Minimum (14.08±1.03) and maximum (11.7±0.08) decrease of MI was recorded after 24 and 96hrs of exposure in treatment with 1.5µg/kg. Frog treated with 3.0µg/kg of test compound showed 13.6±1.82, 12.8±1.33, 12.07±1.17 and 9.2±0.89 values of MI at 24, 48, 72 and 96hrs of duration respectively. Minimum (13.3±2.85) and maximum (8.62±1.40) values of MI after treatment with 6.0µg/kg were recorded at 24, 48, 72 and 96hrs of exposure. Similar trend of decrease of MI was revealed after treatment with 12.0µg/kg of test chemical. The values recorded were 13.7±0.49, 10.52±2.27, 8.67±1.38 and 7.3±0.96 after 24, 48, 72 and 96hrs of exposure.

Results of chromosomal aberration test showed that higher percentage of different chromosomal aberrations at 72 and 96hrs of exposure compared to 24 and 48hrs of exposure in
intestine as well as bone marrow of exposed specimens. In case of intestine, the aberrations which
were found to be predominant were Rc, and Py while Tcd and M were recorded to have the least
percentage. Predominant aberrations recorded in bone marrow were Rc, Tcd and Cf while Stk and C
were found to have minimum frequency. Total percentage of chromosome aberrations was found to
be highest in bone marrow and than in intestine. The result mitotic index showed a general trend of
decrease in both the treated tissues versus respective control values. Maximum decrease in the mitotic
index was evident in case of bone marrow followed by intestine.
a= Chromosome fragmentation, b= Ring chromosomes, c=Terminal chromatid deletion, d=Minutes, e= Centromeric gaps, f= Terminal association of chromosomes, g= Stickiness, h= Clumping, i= Pycnosis, j= Stretching

Fig. 36 Chromosome aberrations in intestine (A,B,C,D) and bone marrow (E,F,G,H) after mercury treatment
Table 15: Frequencies of chromosomal aberrations (%) and mitotic index (%) in intestine of *Euphycis cyanophyle*itis after treatment with Mercury

<table>
<thead>
<tr>
<th>Conc. (µg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
<th>Mitotic index (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contr.</td>
<td>24</td>
<td>4</td>
<td>Cf 0 0 0 0 Rc 0 0.25 0.75 0.75 0.25 0.25 0.25</td>
<td>0.5±0.5</td>
<td>10.4±0.96</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>0 0 0 0 Rc 0.5 0.25 0 0.75 0.75 0.25 0.25</td>
<td>0.75±0.57</td>
<td>10.4±0.64</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>96</td>
<td>4</td>
<td>0 0 0 0 Rc 0.75 0 0 0 0.25 0.25</td>
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<td>10.5±1.11</td>
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<tr>
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<td>5.25±0.5^c</td>
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</tr>
<tr>
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<td>13.0±0.57^c</td>
<td>9.57±1.23^c</td>
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<td>14.5±1.25^c</td>
<td>8.9±0.74^c</td>
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<tr>
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<td>80.5±2.63^c</td>
<td>4.5±0.35^c</td>
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a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4) For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed = 400

Cf= Chromosome fragmentation, Rc= Ring chromosomes, Tcd= Terminal chromatid deletion, M= Minutes, Cg= Centromeric gaps, Tac= Terminal association of chromosomes, Stk= Stickiness, C= Clumping, Py= Pycnosis, Sth= Stretching, Contr.= Control
Fig. 37 Frequencies of total chromosomal aberrations in intestine after treatment with Mercury

Fig. 38 Mitotic index of intestinal cells after treatment with mercury
Table 16: Frequencies of chromosomal aberrations (%) and mitotic index (%) in bone marrow of *Euphylys cyanophyllystis* after treatment with Mercury

<table>
<thead>
<tr>
<th>Conc. (µg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
<th>Mitotic index (Mean ± SD)</th>
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<td>Tcd</td>
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</table>

a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4)

For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed= 400

Cf= Chromosome fragmentation, Rc= Ring chromosomes, Tcd=Terminal chromatid deletion, M=Minutes, Cg= Centromeric gaps, Tac= Terminal association of chromosomes, Stk= Stickiness, C= Clumping, Py= Pycnosis, Sth= Stretching, Contr.= Control
Fig. 39 Frequencies of total chromosomal aberrations in bone marrow after treatment with Mercury

Fig. 40 Mitotic index of bone marrow cells after treatment with Mercury
NICKEL TREATMENT

1. Intestine

CAT: The frequency of different types of chromosomal aberrations induced in intestine are summarized in Fig. 41 and Table 17. Mean frequencies of total aberrations reached 2.5±1.29, 8.25±0.95, 14.0±1.15 and 26.5±1.73 after 24, 48, 72 and 96hrs of exposure periods during treatment with 2.5mg/kg of nickel. Except for Cf and Tcd, time dependent increase was observed in the frequency of individual aberrations. Maximum frequency at 96hrs was recorded for Rc and Stk and minimum was observed for Tcd while other aberrations showed almost same frequencies of aberrations. After treatment with 5.0mg/kg of test chemical, frequencies of total chromosomal aberrations showed an increase from 6.25±0.5 (at 24hrs) to 26.75±1.5 (at 96hrs) through 13.0±2.16 (at 48hrs) and 21.25±1.25 (at 72hrs). Py followed by Rc and Sth were found in maximum frequency after 96hrs while least frequency was recorded for Tac. After treatment with 10.0mg/kg, mean frequencies of total chromosomal aberrations reached 13.0±1.41 at 24hrs, 16.75±1.5 at 48hrs, 27.75±2.06 at 72hrs and 31.75±0.95 at 96hrs of exposure periods. Among various abnormalities, Py followed by Rc and Sth were observed in maximum frequency at 96hrs and Cf was observed in minimum percentage. Nickel treatment induced highest chromosomal aberrations which increased from 27.75±1.70 (at 24hrs), to 60.5±1.29 (at 96hrs) through 38.0±2.82 (at 48hrs) and 44.25±2.21 (at 72hrs) after treatment of frog with 20.0mg/kg of test chemical. Frequency of Rc, Py followed by Cf and Tcd were maximum among various abnormalities at 96hrs while that of Sth were minimum at 96hrs of exposure duration. Data obtained was found to be statistically significant compared to respective controls in all the treatment groups (at 24, 48, 72 and 96hrs, p<0.01 versus controls in all treatment doses) as shown in Table 17 and Fig. 42.

MI: Table 17 and Fig. 43 represent the frequencies of MI in the intestinal cells of frog exposed to four sublethal concentrations of nickel. The values showed a decrease from 24 to 96hrs in all the treatment groups which were statistically significant from respective controls (at 24, 48 and 72hrs, p<0.01; at 96hrs, p<0.001) in all treatment doses. Minimum (14.17±0.70) and maximum (13.32±1.08) decrease of MI was recorded after 24 and 96hrs of exposure respectively in treatment with 2.5mg/kg. Frog treated with 5.0mg/kg of test compound showed 12.12±1.44, 11.87±0.45, 11.67±1.35 and 10.7±0.65 values of MI at 24, 48, 72 and 96hrs of duration respectively. Minimum (9.55±0.79) and maximum (7.55±0.54) values of MI after treatment with 10.0mg/kg were recorded at 24 and 96hrs of exposure respectively. Similar trend of decrease of MI was revealed after treatment
20.0mg/kg of test chemical. The values recorded 8.12±0.98, 6.25±0.96, 6.2±1.14 and 4.95±0.95 after 24, 48, 72 and 96hrs of exposure respectively.

2. Bone marrow

**CAT:** Frequencies of different chromosomal aberrations induced after treatment with four sublethal concentrations of zinc have been shown in Fig. 41 and Table 18. At a sublethal concentration of 2.5mg/kg, the percentage of total aberrations increased from 4.75±1.25 (at 24hrs) to 14.25±1.5 (at 96hrs) through 5.0±0.81 (at 48hrs) and 8.0±1 (at 72hrs). Tcd was only recorded at 96hrs of exposure whereas Tac was not found at 24 and 48hrs of exposure, also not a single Cf was recorded at 48hrs of exposure. Highest frequency among individual aberrations was recorded for Rc and Stk and least was observed for Cg and Py after 96hrs of exposure. Total chromosomal aberrations observed after treatment with 5.0mg/kg were also found to increase with the duration of exposure and the values recorded were 5.75±1.5 at 24hrs, 13.5±1.29 at 48hrs, 14.75±1.73 at 72hrs and 25.25±0.57 at 96hrs. Maximum percentage was recorded for C and minimum was observed for Stk, Py and Sth. Tac was not found at 24hrs of exposure. Frog exposed to 10.0mg/kg of test chemical revealed the percentage of total aberrations as 12.0±0.81 at 24hrs, 15.75±1.70 at 48hrs, 26.25±2.87 at 72hrs and 41.5±1.29 at 96hrs of duration. Some of the aberrations increase whereas other decreased with an increase in duration of exposure. Maximum frequency was recorded for Py followed by Cf and minimum frequency was recorded for Sth at 96hrs of exposure. Exposure to 20.0mg/kg of test chemical induced minimum (26.25±1.70) and maximum (69.5±1.73) frequency of total aberrations at 24 and 96hrs of exposure respectively. All the aberrations except Cf, C and Py showed time dependent increase in the percentage. Maximum frequency at 96hrs was obtained for Cf while minimum frequency at 96hrs was recorded for C. Data obtained was statistically significant in all the exposure groups with respect to controls at different exposure periods (at 24 and 48hrs, p<0.01; at 72 and 96hrs, p<0.001 versus control values for all treatment doses) as shown in Table 18 and Fig. 44.

**MI:** Cytotoxicity of nickel after treatment with four sublethal concentrations was evident as the mitotic index was found to decrease significantly from respective controls except after 24hrs of exposure where it was found to be non significant (at 48hrs, p<0.05; at 72 and 96hrs, p<0.01). Minimum (15.2±0.72) and maximum (14.4±0.54) decrease of MI was recorded after 24 and 96hrs of exposure respectively in treatment with 2.5mg/kg. Frog treated with 5.0mg/kg of test chemical showed 13.9±1.67, 13.82±1.03, 13.32±1.08 and 12.35±0.73 values of MI at 24, 48, 72 and 96hrs of duration respectively. Minimum (12.3±0.21) and maximum (12.2±1.20) values of MI after treatment
with 10.0mg/kg were recorded at 24 and 96hrs of exposure respectively. Similar trend of decrease of MI was revealed after treatment with 20.0mg/kg. The values recorded were 10.65±0.54, 9.87±0.71, 8.27±0.66 and 7.55±0.54 after 24, 48, 72 and 96hrs of exposure respectively as evident in Table 18 and Fig. 45.

The results of CAT revealed higher percentage of different chromosomal aberrations at 72 and 96hrs of exposure compared to 24 and 48hrs of exposure in both the tissues of exposed specimens. Predominant aberrations recorded in intestine were Rc along with Py followed by Cf and Tcd while in bone marrow the percentage of Cf was found to be maximum. In case of intestine, the least observed frequency was found to be for Sth whereas C followed by Py was the least recorded aberrations in case bone marrow. Total percentage of chromosome aberrations was found to be highest in bone marrow followed by intestine. After treatment with various sublethal concentrations of nickel, analysis of MI revealed mitodepressive effect of the heavy metal studied except for 24hrs of exposure in bone marrow where the results obtained were not statistically significant from the respective controls. However the overall effect was predominant in intestine compared to bone marrow.
Fig. 41 Chromosome aberrations in intestine (A,B,C,D) and bone marrow (E,F,G,H) after nickel treatment
Table 17: Frequencies of chromosomal aberrations (%) and mitotic index (%) in intestine of *Euphlyctis cyanophlyctis* after treatment with Nickel

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
<th>Mitotic index (Mean ± SD)</th>
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<td></td>
<td></td>
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<td>Rc</td>
<td>Tcd</td>
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a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4)

For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed= 400

Cf= Chromosome fragmentation, Rc= Ring chromosomes, Tcd= Terminal chromatid deletion, M= Minutes, Cg= Centromeric gaps, Tac= Terminal association of chromosomes, Stk= Stickiness, C= Clumping, Py= Pycnosis, Sth= Stretching, Contr.= Control
Fig. 42. Frequencies of total chromosomal aberrations in intestine after treatment with Nickel

Fig. 43 Mitotic index of intestinal cells after treatment with Nickel
Table 18: Frequencies of chromosomal aberrations (%) and mitotic index (%) in bone marrow of *Euphlyctis cyanophlyctis* after treatment with Nickel

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
<th>Mitotic index (Mean ± SD)</th>
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Fig. 44 Frequencies of total chromosomal aberrations in bone marrow after treatment with Nickel.

Fig. 45 Mitotic index of bone marrow cells after treatment with Nickel.
ZINC TREATMENT

1. Intestine

CAT: Frequencies of different chromosomal aberrations induced in intestine after exposure to sublethal concentrations of zinc have been summarized in Fig. 46 and Table 19. The data was significant compared to the respective controls in all treatment groups (at 24, 48 and 72hrs, p<0.01; at 96hrs, p<0.001). Frequencies of total aberrations were found to be 0.5±0.5 (at 24hrs), 2.0±0.0 (at 48hrs), 2.5±0.57 (at 72hrs) and 6.0±0.81 (96hrs) in treatment with 3.0mg/kg of zinc. Maximum frequency was recorded for Rc, M and C and minimum was observed for Stk, Py and Sth at 96hrs compared to other individual aberrations. In frog treated with 6.0mg/kg of test chemical, frequencies of total aberrations were found to increase from 1.75±0.5 (at 24hrs) to 8.25±1.15 (at 96hrs) through 3.0±0.81 (at 48hrs) and 5.5±1.0 (at 72hrs). In this case, highest percentage of individual aberrations recorded were Tcd, M, Py and Sth, lowest percentage was recorded for Stk while other aberrations had the same percentage after 96hrs of exposure. At a sublethal concentration of 12.0mg/kg, frequencies of total chromosomal aberrations were found to be 4.5±0.57, 8.0±0.0, 12.5±0.5 and 16.25±0.81 after 24, 48, 72 and 96hrs of exposure periods respectively. Some of the aberrations showed an increase while others decreased with increase in duration of exposure. Rc and Py were found to be maximum while Tac was found to be minimum at 96hrs among various abnormalities. Similar trend of increase of total chromosome abnormalities was observed after treatment with 24.0mg/kg of test chemical. Values observed from 24 to 96hrs were 11.5±0.5, 16.25±1.0, 26.5±1.73 and 39.25±0.5 respectively. Py followed by Rc and Tcd were found to have maximum frequency at 96hrs of exposure while that of Tac had minimum frequency (Table 19 and Fig. 47).

MI: Table 19 and Fig. 48 showed the frequencies of MI in intestine of frog exposed to four sublethal concentrations of zinc. Values showed a non significant decrease from 24hrs to 96hrs in all the treatment groups compared to respective controls. Minimum (8.55±0.37) and maximum (5.97±0.17) decrease of MI was recorded after 24 and 96hrs of exposure respectively in treatment with 3.0mg/kg. Frog exposed to 6.0mg/kg of test chemical showed 8.02±0.35, 7.6±0.29, 7.0±0.53 and 6.97±0.49 values of MI at 24, 48, 72 and 96hrs of duration respectively. Minimum (5.62±0.28) and maximum (4.87±0.29) decrease of MI values after treatment with 12.0mg/kg were recorded at 24hrs and 96hrs of exposure respectively. After treatment with 24.0mg/kg of the test chemical, the values recorded were 8.1±0.77, 8.05±0.3, 7.27±40 and 7.15±0.50 after 24, 48, 72 and 96hrs of exposure respectively.
2. Bone marrow

**CAT:** The types and frequencies of chromosomal aberrations recorded in treatment with zinc have been illustrated in the Fig. 46 and Table 20. Frequency of total aberrations was found to be 1.0±0.57 (at 24hrs), 2.0±0.57 (at 48hrs), 2.5±0.57 (at 72hrs) and 4.5±0.81 (at 96hrs) after treatment with 3.0mg/kg of zinc. Maximum frequency was recorded for Tcd and Sth at 96hrs of exposure while least frequency was observed in Cg and C; however Stk was not observed at 96hrs of exposure. Some of the aberrations showed an increase whereas others decreased with the increase in the duration of exposure. Frequencies of total aberrations were found to be 2.0±0.0 (at 24hrs), 3.25±0.5 (at 48hrs), 3.5±0.95 (at 72hrs) and 6.0±0.5 (at96hrs) in frog after treatment with 6.0mg/kg of test chemical. In this case, Tcd and Tac were found in maximum percentage at 96hrs of duration while other types of aberrations had almost same percentage after 96hrs exposure. At a sublethal concentration of 12.0mg/kg, frequencies of total chromosomal aberrations were found to be 2.75±0.57, 5.0±0.0, 8.0±1.55 and 15.75±0.57 after 24, 48, 72 and 96hrs of exposure periods respectively. In this case, Sth followed by Cg was recorded in highest percentage at 96hrs whereas lowest percentage was found for Tac. Frog treated with 24.0mg/kg of zinc showed an elevation in frequencies of total chromosomal from 24 to 96hrs i.e. 7.0±0.81, 18.0±0.95, 20.0±1.5 and 31.25±1.73 respectively. Tcd followed by Rc was found to have maximum frequency at 96hrs of exposure and Stk was recorded in minimum percentage. Data was significantly different from the respectively controls at different exposure periods except after 24hrs of duration (at 48hrs, p<0.05; at 72 and 96hrs, p<0.01) as shown in Table 20 and Fig. 49.

**MI:** Table 20 and Fig. 50 represent the frequencies of MI in the bone marrow cells of frog exposed to four sublethal concentrations of zinc. The values of MI were only slightly decreased from 24, 48, 72 and 96hrs with respect to controls in all the treatment groups. In all the sublethal concentrations studied, the MI after 24, 48, 72 and 96hrs of duration was found to be non significant compared to respective controls. Minimum (16.1±0.18) and maximum (12.77±0.17) decrease of MI was recorded after 24 and 96hrs of exposure respectively in treatment with 3.0mg/kg .Frog treated with 6.0mg/kg of test compound showed 15.15±0.17, 14.97±0.20,13.9±0.21and 9.67±0.17 values of MI at 24, 48, 72 and 96hrs of duration respectively. Minimum (12.67±0.22) and maximum (6.85±0.17) values of MI after treatment 12.0mg/kg were recorded at 24 and 96hrs of exposure.
respectively. After treatment with 24.0mg/kg of test chemical, the values recorded were 11.97±0.12, 9.25±0.12, 6.92±0.18 and 5.95±0.23 after 24, 48, 72 and 96hrs of exposure respectively.

The results of CAT revealed higher percentage of different chromosomal aberrations at 96hrs of exposure compared to 24, 48 and 96hrs of exposure in both the tissues of exposed specimens. In case of intestine, aberrations which were found to be predominant were Py followed by Rc and Tcd while Tac had minimum incidence but in case of bone marrow, the percentage of Tac followed by Rc was found to be maximum and the frequency of Stk was recorded in least percentage. Since maximum frequency of total chromosomal aberrations was induced only after treatment with 24mg/kg of zinc at 96hrs of exposure period, so it could be regarded as the effective sublethal dose and period of exposure of zinc for induction of chromosome type aberrations in intestine and bone marrow. Total percentage of chromosome aberrations was found to be highest in intestine in comparison to bone marrow. Analysis of MI treatment with various sublethal concentrations of zinc revealed that zinc did not cause significant mitodepressive effect on the tissues studied. This is because the effect of zinc in treated series was not significantly different from the respective controls in all the exposure groups in both intestine and bone marrow.

Based on the results obtained for CAT after the exposure of frog (E. Cyanophlyctis) to different sublethal concentrations of different heavy metals, all the heavy metals tested had proved to be potentially genotoxic except zinc. The genotoxic potential of heavy metal toxicant was evident as Pb >Hg>Cu>Cd>Cr> Ni >Mn> Zn.
Fig. 46 Chromosome aberrations in intestine (A,B,C,D) and bone marrow (E,F,G,H) after zinc treatment
Table 19: Frequencies of chromosomal aberrations (%) and mitotic index (%) in intestine of *Euphyctis cyanophyllis* after treatment with Zinc

<table>
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<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
<th>Mitotic index (Mean ± SD)</th>
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a=p<0.05, b=p<0.01, c=p<0.001, ns = non-significant (represent values significantly different from the respective controls; Kruskal-Wallis test; df = 4)

For mitotic index, 1000 cells were analyzed for each fish, total cell analyzed = 4000; For chromosome aberration test, 100 cells were analyzed for each frog, total cells analyzed = 400

Cf= Chromosome fragmentation, Rc= Ring chromosomes, Tcd=Terminal chromatid deletion, M=Minutes, Cg= Centromeric gaps, Tac= Terminal association of chromosomes, Stk= Stickiness, C= Clumping, Py= Pycnosis, Sth= Stretching, Contr.= Control
Fig. 47 Frequencies of total chromosomal aberrations in intestine after treatment with Zinc.

Fig. 48 Mitotic index of intestinal cells after treatment with Zinc.
Table 20: Frequencies of chromosomal aberrations (%) and mitotic index (%) in bone marrow of *Euphyctis cyanophlyctis* after treatment with Zinc

<table>
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<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
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<th>Chromosome aberrations scored (in percentage)</th>
<th>Total aberrations (Mean ± SD)</th>
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Fig. 49 Frequencies of total chromosomal aberrations in bone marrow after treatment with Zinc

Fig. 50 Mitotic index of bone marrow cells after treatment with Zinc
4.2.2 Micronucleus test (MNT)

Micronucleus test was performed in the intestinal cells, red blood cells and kidney cells of exposed and control frog specimens. Micronuclei (MN) are cytoplasmic chromatin-containing bodies that appear in the cell like a small, satellite nucleus around the cell nucleus that are formed from acentric chromosomal fragments or whole chromosomes that are not incorporated in either of the daughter nucleus after cell division. Only cytoplasmic particles not exceeding 1/3rd of the main nucleus diameter, non-retractile structures clearly distinguishable from main nucleus and with distinct borders and resembling main nucleus with respect to staining properties were considered as micronuclei.

A normal intestinal cell of *Euphlyctus cyanophlyctis* contained a conspicuous round nucleus although the position may vary in the cytoplasm from cell to cell which may be central or peripheral. The cells obtained from the control specimens were having the similar characteristics (Fig. 51A, 55A, 59A, 63A, 67A, 71A, 75A, 79A). MN of an intestinal cells was small non-retractile circular (Fig. 51C, 59D, 79B) or ovoid (Fig. 51B, 59C) or even rod shaped (Fig. 55B, 79C) particles lying in the cytoplasm and resembling a nucleus with respect to staining properties. Size of MN in intestinal cells were fairly of larger size compared to MN of RBCs but did show variation from cell to cell. Position of MN also varied from one cell to another, some located very near to the nucleus (Fig. 51D, 63B, 67C, D, 75B) or some located very far even at the periphery of the cell (Fig. 51F, 59H, 63F, 79G). A single micronucleus per affected cell has been observed to be of a common occurrence whereas presence of more than one nucleus in a cell was also observed (Fig. 63G, 71G).

Normal mature erythrocytes of *Euphlyctus cyanophlyctis* are fairly large structures having centrally placed elliptical or rounded nuclei in the cytoplasm. It has a well defined boundary and clear cytoplasm which facilitates the identification of fragments in the cytoplasm. Such normal erythrocytes were the characteristics of control specimens (Fig. 51E, 55E, 59E, 63E, 67E, 71E, 75E, 79E). In majority of cases, MN has a dot like or circular appearance. Size as well as location of MN within the cytoplasm varied from cell to cell, the shape was round in most of the RBCs but small dot shaped MN were also observed (Fig. 55G, 67F, 79H). Position of MN in the cytoplasm also varied, some located very near to the nucleus (Fig. 51G-H, 59G, 71F) or some located very far even at the periphery of the cell (Fig. 51F, 59H, 63F, 79G). A single micronucleus per affected cell has been observed to be of a common occurrence whereas presence of more than one nucleus in a cell was also observed (Fig. 63G, 71G).
A normal kidney cell of *Euplyctus cyanophlyctis* like that of intestinal cell contained a conspicuous round nucleus although the position may vary in the cytoplasm from cell to cell which may be central or peripheral. The cells obtained from the control specimens were having the similar characteristics (Fig. 51I, 55I, 59I, 63I, 67I, 71I, 75I, 79I). MN of normal kidney was small non-retractile circular (Fig. 51K, 63J-L, 67J, 71K) or ovoid (Fig.55L, 71J, 79L) or even rod shaped (Fig. 55J, 59K) particles lying in the cytoplasm and resembling a nucleus with respect to staining properties. Size of MN in intestinal cells were fairly of larger size compared to MN of RBCs but did show variation from cell to cell. Position of MN also varied from one cell to another, some located very near to the nucleus (Fig.55J, 59J, 67K, 75J) or some located very far even at the periphery of the cell (Fig. 51K-L, 55K, 63K, 67J-L,71L).

The results of micronucleus test observed after different heavy metal treatment have been revealed in details as follows:

**CADMIUM TREATMENT**

1. **Intestine:**

   Frequencies of MN recorded in intestinal tissue after different treatments are shown in Fig. 51(B-D) and Table 21. The data after treatment with different doses was found to be statistically significant compared to respective controls (at 24hrs, p<0.05; at 48hrs and 72hrs, p<0.01 and at 96hrs, p<0.001). In frog treated with 1.5mg/kg, 1.12±0.670, 1.47±0.35, 1.97±1.419 and 2.47±0.618 MN were observed at 24, 48, 72 and 96hrs of exposure respectively. At a concentration of 3.0mg/kg, frequency of MN was found to be 2.95±1.776, 3.47±1.765, 4.15±1.276 and 4.97±0.865 after 24, 48, 72 and 95hrs of exposure respectively. An increasing trend in the MN frequency was also observed after treatment with 6.0mg/kg of test chemical viz. 3.62±2.053 (at24hrs), 4.32±1.757 (at48hrs), 5.3±0.571 (at72hrs) and 6.3±0.852 (at96hrs). A time dependent increase in MN frequency was observed after treatment with 12.0mg/kg of cadmium i.e. 4.97±0.865, 6.62±0.531, 9.8±2.046 and 10.8±1.458 after 24, 48, 72 and 96hrs of duration respectively as shown in the Table 21 and Fig. 52.

2. **RBCs:**

   Frequencies of MN observed in RBC are shown in Fig. 51(F-H) and Table 21. Dose and time dependent increase in the induction of MN was observed in this tissue after various treatments. Values of MN were found to be statistically significant from the respective controls (at 24hrs, p<0.05; at 48hrs, p<0.01, at 72 and 96hrs, p<0.001). At a sublethal concentration of 1.5mg/kg, MN
frequencies were 1.12±0.670, 1.8±0.627, 2.47±0.618 and 2.97±1.244 after 24, 48, 72 and 96hrs respectively. Frog treated with 3.0mg/kg revealed the MN frequencies to be 3.47±0.670, 4.8±1.232, 5.3±1.435 and 6.3±0.852 recorded at 24, 48, 72 and 96hrs respectively. Frequencies of MN recorded after treatment with 6.0mg/kg were found to increase from 3.3±1.205 (at 24hrs) to 7.15±0.848 (at 96hrs) through 4.62±0.942 (at 48hrs) and 5.8±0.848 (at 72hrs). Values of MN were also found to increase in a time dependent manner i.e. 4.12±0.994, 6.47±1.147, 8.95±1.377 and 10.82±0.994 at 24, 48, 72 and 96hrs respectively in treatment with 12.0mg/kg of test chemical as shown in the Table 21 and Fig. 53.

3. **Kidney:**

Frequencies of MN observed in kidney after different treatments have been tabulated in Fig. 51(J-L) and Table 21). The data was found to be statistically significant compared to the respective controls except at 24hrs of duration (at 48hrs, p<0.05; at 72 and 96hrs, p<0.01). In frog treated with 1.5mg/kg, 0.62±0.531, 1.47±0.35, 1.8±0.62 and 2.47±0.618 MN were observed at 24, 48, 72 and 96hrs of exposure respectively. After 24, 48, 72 and 96hrs of exposure in treatment with 3.0mg/kg, frequency of MN were recorded as 1.3±0.571, 2.5±1.0, 3.3±0.571 and 3.3±1.205 respectively. Frequencies of MN recorded after treatment with 6.0mg/kg were found to increase from 1.8±0.627 (at 24hrs) to 4.65±1.22 (at 96hrs) through 2.97±0.865 (at 48hrs) and 4.47±1.499 (at 72hrs). In frog treated with 12.0mg/kg, increased values of MN frequency was also found viz. 3.13±1.117 (at 24hrs), 4.97±1.244 (at 48hrs), 6.62±0.942 (at 72hrs) and 9.65± 1.776 (at 96hrs) respectively as shown in Table 21 and Fig. 54.

A comparison of data obtained in different tissues revealed that during first dose and duration of exposure (i.e. 1.5mg/kg at 24hrs), the result obtained were almost same in intestine and RBC. The result showed a significant increase in MN frequency with respect to dose and duration of exposure. Maximum frequency was recorded for RBC and intestine having almost same value of MN frequency and least in kidney.
Fig. 51 Micronuclei formation in intestine (B,C,D), RBC (F,G,H) and kidney (J,K,L) cells after cadmium treatment (A,E,I-normal cells)
Table 21: Frequencies of micronuclei (%) in different tissues of *Euphlyctis cyanophlyctis* exposed to Cadmium

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Total interphase cells studied</th>
<th>Frequency of micronuclei (Mean±SD)</th>
</tr>
</thead>
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<td></td>
<td></td>
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<td>Intestine</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>RBC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kidney</td>
</tr>
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<td>Contr.</td>
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<td>6000</td>
<td>0.95±0.404</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>0.3±0.346</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
<td>6000</td>
<td>0.3±0.346</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>4</td>
<td>6000</td>
<td>0.62±0.531</td>
</tr>
<tr>
<td>1.5</td>
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<td>6000</td>
<td>1.12±0.670&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>48</td>
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<td>6000</td>
<td>1.47±0.35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
<td>6000</td>
<td>1.97±1.419&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
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<td>4</td>
<td>6000</td>
<td>2.47±0.618&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>3.0</td>
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<td>6000</td>
<td>2.95±1.776&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>6000</td>
<td>3.47±1.765&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
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<td>6000</td>
<td>4.97±0.865&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>6.0</td>
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<td>6000</td>
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<td>6000</td>
<td>5.3±0.571&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>96</td>
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<td>6000</td>
<td>6.3±0.852&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>12.0</td>
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<td>6000</td>
<td>4.97±0.865&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>6.62±0.531&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>6000</td>
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<td></td>
<td>96</td>
<td>4</td>
<td>6000</td>
<td>10.8±1.458&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly differently from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control


Fig. 52: Frequencies of micronuclei in Intestine after treatment with Cadmium.

Fig. 53: Frequencies of micronuclei in RBCs after treatment with Cadmium.

Fig. 54: Frequencies of micronuclei in Kidney after treatment with Cadmium.
CHROMIUM TREATMENT

1. Intestine:

Values of MN determined in the intestinal tissue after different treatments and exposure periods are shown in Fig. 55(B-D) and Table 22). The values were found to be statistically highly significant (at 24, 48, 72 and 96hrs, \( p<0.01 \)). Frog treated in vivo with 2.0mg/kg of chromium showed an increase in the incidence of MN from 0.95±0.404 (at 24 and 48 hrs) to 1.12±0.670 (at 72 and 96hrs). At a sublethal concentration of 4.0mg/kg of test chemical, minimum (1.3±0.0) and maximum (2.97±0.865) frequency of MN was recorded at 24hrs and 96hrs of exposure duration respectively; however there was a decrease in value of MN frequency at 72 hrs of duration. An elevated response was observed during treatment with 8.0mg/kg wherein the values recorded were 1.65±0.404, 2.97±1.575, 4.47±1.147 and 4.8±1.151 at 24, 48, 72 and 96hrs respectively. Similarly, the incidences of MN were also found to increase in a time dependent manner from 2.65±1.22 (at 24hrs) to 9.97±1.97 (at 96 hrs) after treatment with 16.0 mg/kg of chromium through 4.95±0.867 (at48hrs) and 7.8±1.840 (at72hrs) (Table 22 and Fig.56).

2. RBCs:

Values of MN in the peripheral erythrocytes after different treatment and exposure periods have been shown in Fig. 55(F-H). In frog treated with 2.0m/kg, shown an significant increase in MN from 0.47±0.618 (at 24hrs) to 1.47±0.670 (at 96hrs) through 0.62±0.53 (at 48hrs) and 1.45±1.011 (at 72hrs) after 24, 48, 72 and 96hrs of exposure in treatment with 4.0mg/kg, frequency of MN were recorded as 1.62±0.865, 1.92±1.102, 1.95±0.750 and 4.15±1.276 respectively. At a sublethal concentration of 8.0mg/kg of test chemical, 1.3±0.57, 2.45±0.3, 4.97±1.757 and 4.97±0.865 frequencies of MN were observed after 24, 48, 72 and 96hrs of exposure respectively. Specimens exposed to 16.0mg/kg showed significant elevated incidence of MN at 24hrs (2.97±2.085), at48hrs (4.95±1.682), at 72hrs (7.47±1.117) and at 96hrs exposure (11.47±1.117). Data was found to be statistically significant compared to respective controls (at 24 and 48hrs, \( p<0.05 \); at 72hrs \( p<0.01 \) and at 96hrs, \( p<0.001 \)) as shown in the Table 22 and Fig. 57).

3. Kidney:

Frequencies of MN determined in the different treatments are summarized in Fig. 55(J-L) and Table 22). Frog exposed to different concentrations and durations of chromium showed a dose and
time dependent increase in the incidence of micronuclei which were significantly higher than that of controls (at24hrs, p< 0.05; at 48, 72 and 96hrs, p<0.01). In frog treated in vivo with 2.0mg/kg of chromium MN was recorded to be 0.77±0.35(at24hrs), 0.62±0.531(at48hrs), 1.3±0.571(at72 hrs) and 1.12±0.35(96hrs), the response was highest at 72hrs and lowest at 48hrs of exposure. An elevated response was observed in the frequency of MN from 24 to 96hrs of exposure, 1.3±0.571 (at24hrs), 1.6±1.154 (at48hrs), 1.82±0.35 (at72hrs) and 3.3±1.947 (at96hrs) with a dose of 4.0mg/kg of test chemical. Specimens exposed to 8.0mg/kg, 0.95±0.404, 1.97±0.531, 3.82±1.929 and 3.8±1.749 MN frequency were recorded at 24, 48, 72 and 96hrs of exposure respectively. Treatment with 16.0mg/kg showed a highly significant increase of micronuclei in relation to the respective controls at all the exposure periods. The values recorded were 2.97±0.865 (at24hrs), 4.62±0.942 (at48hrs) 5.3±1.947 (at72hrs) and 8.3±1.610 (at96hrs) (Table 22 and Fig. 58).

Result of MNT in different tissues of frog treated with various sublethal concentrations of chromium revealed prominent effect of test chemical in RBCs followed by intestine and least in kidney.
Fig. 55 Micronuclei formation in intestine (B,C,D), RBC (F,G,H) and kidney (J,K,L) cells after chromium treatment (A,E,I-normal cells)
Table 22: Frequencies of micronuclei (%) in different tissues of *Euphlyctis cyanophlyctis* exposed to Chromium.

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Total interphase cells studied</th>
<th>Frequency of micronuclei (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Intestine</td>
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<td>Contr.</td>
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<td>0.45±0.3</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>0.3±0.346</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
<td>6000</td>
<td>0.15±0.3</td>
</tr>
<tr>
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<td>6000</td>
<td>0.45±0.3</td>
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<td>6000</td>
<td>0.95±0.404</td>
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<td>6000</td>
<td>1.12±0.670</td>
</tr>
<tr>
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<td>96</td>
<td>4</td>
<td>6000</td>
<td>1.12±0.670</td>
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<td>4.0</td>
<td>24</td>
<td>4</td>
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<td>1.3±0.0</td>
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<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>1.97±0.531</td>
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<td>72</td>
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<td>6000</td>
<td>1.8±0.627</td>
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<td>96</td>
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<td>2.97±0.865</td>
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<td>6000</td>
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<td>4.95±0.867</td>
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<td>4</td>
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<td>7.8±1.840</td>
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<tr>
<td></td>
<td>96</td>
<td>4</td>
<td>6000</td>
<td>9.97±1.970</td>
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</table>

a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly differently from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control
Fig.56: Frequencies of micronuclei in Intestine after treatment with Chromium.

Fig.57: Frequencies of micronuclei in RBC after treatment with Chromium.

Fig.58: Frequencies of micronuclei in Kidney after treatment with Chromium.
COPPER TREATMENT

1. Intestine:

Frequencies of micronuclei determined in the different treatments are summarized in Fig. 59(B-D) and Table 23). Frogs exposed different concentrations and durations of copper showed a dose and time dependent increase in the incidence of micronuclei which were significantly higher than that of controls (at 24hrs, p<0.05; at 48, 72 and 96hrs, p<0.01). In frog treated in vivo with 3.5mg/kg of copper, frequency of MN was recorded to be 0.97±0.865(at24hrs) with a slight decrease to 0.95±0.404 (at 48hrs), followed by an increase in value at 72hrs (1.12±0.670) and at 96hrs (1.13±0.942). An elevated response was observed in the frequency of MN (exposed to 5.0mg/kg) from 24 to 96hrs of exposure (1.14±0.834 after 24hrs, 1.97±0.531 after 48hrs, 2.15±0.3 after 72hrs and 3.47±1.114 after 96hrs). Specimens exposed to 6.5mg/kg showed significantly elevated incidence of MN at 24hrs (1.47±0.35), at48hrs (2.12±0.618), at 72hrs (3.97±1.102) and at 96hrs (3.97±1.717). Values of MN frequency were same at 72 and 96hrs of exposure. Treatment with 8.0mg/kg showed a highly significant increase of micronuclei in relation to the respective controls at all the exposure periods. The values recorded were 3.32±0.942 (at 24hrs), 5.47±1.117 (at 48hrs), 8.12±0.618 (at 72hrs) and 11.62±0.865 (at 96hrs) as shown in Table 23 and Fig 60).

2. RBCs:

Values of MN in the peripheral erythrocytes after different treatments and exposure periods have been shown in Fig. 59 (F-H) and Table 23). Frog treated in vivo with 3.5mg/kg of copper revealed minimum value (0.8±0.627) of MN frequency at 24hrs and maximum value (1.47±0.35) of MN frequency at 72hrs. 0.62±0.531 and 1.12±0.670 are the values of MN recorded at 48hrs and 96hrs respectively. An elevated response was observed during treatment with 5.0mg/kg wherein the values obtained were 1.32±0.942, 1.62 ±0.865,1.8±1.0 and 3.3 ±1.205 after 24, 48, 72 and 96hrs of exposure respectively. Exposure to 6.5mg/kg induced 0.97±0.865, 1.97±0.531, 3.47±0.35 and 4.8±1.232, MN frequency after 24, 48, 72 and 96hrs respectively. Frogs exposed to sublethal concentration of 8.0mg/kg revealed significant induction of MN frequency after all the exposure periods in comparison to control. Minimum frequency (2.97±0.865) and maximum frequency (11.8 ±0.848) was recorded after 24 and 96hrs of exposure through 5.62 ±0.865 and 7.47±2.232 at 48 and 72hrs of exposure respectively. Data was found to be statistically significant in all treatment doses
versus respective controls (at 24hrs, p<0.05; at 48, 72 and at 96hrs, p<0.01) as shown in Table 23 and Fig. 61.

3. Kidney:

Frequencies of MN recorded in the kidney tissue after different treatments and exposure periods have been shown in Fig. 59(J-L) and Table 23. MN frequencies observed in frog treated in vivo with 3.5mg/kg increased from 0.62±0.531 at 24hrs to 1.47±0.67 at 96hrs through 0.95±0.404 at 48hrs and 1.47±0.35 at 72hrs, the value being same at 72 and 96hrs of exposure. Increased levels of MN were observed after all the exposure periods in experimental frog treated with 5.0mg/kg. Maximum frequency of MN was obtained after exposure period of 96hrs (3.45±1.350) and minimum frequency was obtained after exposure of 24hrs (1.62±0.865). Significant difference (at 24hrs, p<0.05, at 48 and 72hrs, p<0.01 and at 96hrs, p<0.001) in the incidence of MN between experimental and control values were also observed after all the exposure periods in treatment with 6.5mg/kg and 8.0mg/kg. Maximum value recorded was 4.65±1.223 and 9.8±1.840 after 96hrs of exposure in treatment with the respective concentrations. Similarly, minimum values (0.95±0.404 and 2.15±0.834 for 6.5 and 8.0mg/kg treatment respectively) were recorded after 24hrs of exposure (Table 23 and Fig. 62).

In general, the data revealed a time dependent increase in micronucleus frequencies with the increase in doses of copper in intestine, RBCs and kidney. With respect to the dose, maximum effect was found to be induced at the highest dose of exposure i.e. 8.0mg/kg while prominent effect with respect to the duration of exposure was induced after the maximum period of exposure i.e. 96hrs. Comparison between the micronucleus frequencies induced in the various tissues revealed highest MN frequencies in RBCs, followed by a narrow margin in intestine and least in kidney.
COPPER TREATMENT

4. Intestine:

Frequencies of micronuclei determined in the different treatments are summarized in Fig. 59(B-D) and Table 23). Frogs exposed different concentrations and durations of copper showed a dose and time dependent increase in the incidence of micronuclei which were significantly higher than that of controls (at 24hrs, p<0.05; at 48, 72 and 96hrs, p<0.01). In frog treated *in vivo* with 3.5mg/kg of copper, frequency of MN was recorded to be 0.97±0.865(at24hrs) with a slight decrease to 0.95±0.404 (at 48hrs), followed by an increase in value at 72hrs (1.12±0.670) and at 96hrs (1.13±0.942). An elevated response was observed in the frequency of MN (exposed to 5.0mg/kg) from 24 to 96hrs of exposure (1.14±0.834 after 24hrs, 1.97±0.531 after 48hrs, 2.15±0.3 after 72hrs and 3.47±1.114 after 96hrs). Specimens exposed to 6.5mg/kg showed significantly elevated incidence of MN at 24hrs (1.47±0.35), at48hrs (2.12±0.618), at 72hrs (3.97±1.102) and at 96hrs (3.97±1.717). Values of MN frequency were same at 72 and 96hrs of exposure. Treatment with 8.0mg/kg showed a highly significant increase of micronuclei in relation to the respective controls at all the exposure periods. The values recorded were 3.32±0.942 (at 24hrs), 5.47±1.117 (at 48hrs), 8.12±0.618 (at 72hrs) and 11.62±0.865 (at 96hrs) as shown in Table 23 and Fig 60).

5. RBCs:

Values of MN in the peripheral erythrocytes after different treatments and exposure periods have been shown in Fig. 59 (F-H) and Table 23). Frog treated in vivo with 3.5mg/kg of copper revealed minimum value (0.8±0.627) of MN frequency at 24hrs and maximum value (1.47±0.35) of MN frequency at 72hrs. 0.62±0.531 and 1.12±0.670 are the values of MN recorded at 48hrs and 96hrs respectively. An elevated response was observed during treatment with 5.0mg/kg wherein the values obtained were 1.32±0.942, 1.62 ±0.865,1.8±1.0 and 3.3 ±1.205 after 24, 48, 72 and 96hrs of exposure respectively. Exposure to 6.5mg/kg induced 0.97±0.865, 1.97±0.531, 3.47±0.35and 4.8±1.232, MN frequency after 24, 48, 72 and 96hrs respectively. Frogs exposed to sublethal concentration of 8.0mg/kg revealed significant induction of MN frequency after all the exposure periods in comparison to control. Minimum frequency (2.97±0.865) and maximum frequency (11.8 ±0.848) was recorded after 24 and 96hrs of exposure through 5.62 ±0.865 and 7.47±2.232 at 48 and 72hrs of exposure respectively. Data was found to be statistically significant in all treatment doses
versus respective controls (at 24hrs, p<0.05; at 48, 72 and at 96hrs, p<0.01) as shown in Table 23 and Fig.61.

6. Kidney:

Frequencies of MN recorded in the kidney tissue after different treatments and exposure periods have been shown in Fig. 59(J-L) and Table 23. MN frequencies observed in frog treated in vivo with 3.5mg/kg increased from 0.62±0.531 at 24hrs to 1.47±0.67 at 96hrs through 0.95±0.404 at 48hrs and 1.47±0.35 at 72hrs, the value being same at 72 and 96hrs of exposure. Increased levels of MN were observed after all the exposure periods in experimental frog treated with 5.0mg/kg. Maximum frequency of MN was obtained after exposure period of 96hrs (3.45±1.350) and minimum frequency was obtained after exposure of 24hrs (1.62±0.865). Significant difference (at 24hrs, p<0.05, at 48 and 72hrs, p<0.01 and at 96hrs, p<0.001) in the incidence of MN between experimental and control values were also observed after all the exposure periods in treatment with 6.5mg/kg and 8.0mg/kg. Maximum value recorded was 4.65±1.223 and 9.8±1.840 after 96hrs of exposure in treatment with the respective concentrations. Similarly, minimum values (0.95±0.404 and 2.15±0.834 for 6.5 and 8.0mg/kg treatment respectively) were recorded after 24hrs of exposure (Table 23 and Fig. 62).

In general, the data revealed a time dependent increase in micronucleus frequencies with the increase in doses of copper in intestine, RBCs and kidney. With respect to the dose, maximum effect was found to be induced at the highest dose of exposure i.e. 8.0mg/kg while prominent effect with respect to the duration of exposure was induced after the maximum period of exposure i.e. 96hrs. Comparison between the micronucleus frequencies induced in the various tissues revealed highest MN frequencies in RBCs, followed by a narrow margin in intestine and least in kidney.
Fig. 59 Micronuclei formation in intestine (B,C,D), RBC (F,G,H) and kidney (J,K,L) cells after copper treatment (A,E,I-normal cells)
Table 23: Frequencies of micronuclei (%) in different tissues of *Euphlyctis cyanophlyctis* exposed to Copper.

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Total interphase cells studied</th>
<th>Frequency of micronuclei (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intestine</td>
<td>RBCs</td>
</tr>
<tr>
<td>Contr.</td>
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<td>4</td>
<td>6000</td>
<td>0.3±0.346</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>0.45±0.3</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
<td>6000</td>
<td>0.15±0.3</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>4</td>
<td>6000</td>
<td>0.47±0.618</td>
</tr>
<tr>
<td>3.5</td>
<td>24</td>
<td>4</td>
<td>6000</td>
<td>0.97±0.865&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>0.95±0.404&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
<td>6000</td>
<td>1.12±0.670&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>4</td>
<td>6000</td>
<td>1.13±0.942&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.0</td>
<td>24</td>
<td>4</td>
<td>6000</td>
<td>1.14±0.834&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>1.97±0.531&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
<td>6000</td>
<td>2.15±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>4</td>
<td>6000</td>
<td>3.47±1.117&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6.5</td>
<td>24</td>
<td>4</td>
<td>6000</td>
<td>1.47±0.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>2.12±0.618&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
<td>6000</td>
<td>3.97±1.102&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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<td>6000</td>
<td>3.97±1.717&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>8.0</td>
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<td>4</td>
<td>6000</td>
<td>3.32±0.942&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>5.47±1.117&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
<td>6000</td>
<td>8.12±0.618&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>4</td>
<td>6000</td>
<td>11.62±0.865&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly differently from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control
Fig. 60: Frequencies of micronuclei in Intestine after treatment with Copper.

Fig. 61: Frequencies of micronuclei in RBC after treatment with Copper.

Fig. 62: Frequencies of micronuclei in Kidney after treatment with Copper.
LEAD TREATMENT

1. Intestine:

Frequency of MN determined in intestine after different treatments is summarized in Fig. 63(B-D) and Table 24. Frog exposed to different concentrations and durations of lead acetate showed a dose and time dependent increase in the incidence of micronuclei which were significantly higher than that of controls (at 24hrs, p<0.05; at 48 and 72hrs, p<0.01; 96hrs, p<0.001 versus respective controls in all treatment groups). In frog treated in vivo with 4.5mg/kg lead acetate, the frequency of MN was recorded to be 1.15±0.83 (at 24hrs), 1.45±0.83 (at 48hrs), 1.65±0.40 (at 72hrs) and 2.13±1.45 (at 96hrs) wherein the incidence of MN increased along with the duration of the same dose. An elevated response was observed up to 96hrs of exposure in the frequency of MN (2.30±0.85 at 24hrs, 3.30±0.57 at 48hrs, 3.65±1.38 at 72hrs and 4.30±0.35 at 96hrs) after treatment with 9.0mg/kg. Specimens exposed to 18.0mg/kg showed significantly decrease in MN at 24hrs (1.65±0.40) but then elevated increase in frequency of MN were observed at 48hrs (3.63±0.87), 72hrs (4.98±1.62) and 96hrs (5.80±0.63). Treatment with 36.0mg/kg showed a highly significant increase of micronuclei in relation to respective controls at all the exposure periods. The values recorded were 2.30±0.35 (at 24hrs), 4.13±1.49 (at 48hrs), 6.40±0.62 (at 72hrs) and 8.63±2.12 (at 96hrs) as shown in Table 24 and Fig. 64.

2. RBCs:

Values of MN in the peripheral erythrocytes after different treatment and exposure periods have been shown in Fig. 63(F-H) and Table 24. Frogs treated in vivo with 4.5mg/kg showed a highly significant increase (at 24, 48, 72 and 96hrs, p<0.01 versus control values) in the incidence of MN versus respective controls at all the exposure periods. The values recorded were 0.60±0.00, 1.12±0.67, 1.12±0.67, 1.82±0.35 after 24, 48, 72 and 96hrs respectively, wherein the frequency of MN were same at 48hrs and 72hrs of treatment. Frogs exposed to sub-lethal concentration of 9.0mg/kg, 18.0mg/kg and 36.0mg/kg also revealed highly significant increase (p<0.01) at all the exposure periods versus respective control values in the incidence of MN. At 9.0mg/kg of treatment, the values recorded were 0.77±0.35, 4.12±0.62, 3.65±0.40 and 3.97±1.10 after 24, 48, 72 and 96hrs respectively, where maximum frequency of MN were recorded at 48hrs of treatment. Exposure to 18.0mg/kg included 1.65±0.40, 3.30±0.57, 4.95±0.40 and 4.45±2.15 MN frequencies after 24, 48, 72 and 96hrs, here the maximum frequency was observed at 72hrs treatment period. An elevated
response was observed during treatment with 36.0mg/kg wherein the values recorded were 2.47±1.15, 4.30±1.61, 5.45±1.83 and 9.48±1.48 after 24, 48, 72 and 96hrs of exposure respectively as shown in Table 24 and Fig. 65.

3. **Kidney:**

Values of frequencies of MN recorded in kidney tissue have been shown in Fig. 63(J-L) and tabulated in Table 24. Data was found to be significant in respective controls (24, 72 and 96hrs, p<0.01; at 48hrs, p<0.05). In frog, treated with 4.5mg/kg, frequency of MN were same at 24, 76 and 96hrs and value recorded was 1.30±0.57 where at 48hrs, the value recorded were 0.77±0.35. At sublethal concentration of 9.0mg/kg, MN frequencies were 1.12±0.67, 1.97±0.53, 1.80±0.62 and 2.97±1.16 after 24, 48, 72 and 96hrs respectively. Frogs treated with 18.0mg/kg revealed the MN frequencies to be 2.27±0.65, 2.30±1.15, 4.15±0.83 and 3.97±1.46 recorded at 24, 48, 72 and 96hrs respectively, where the MN frequencies showed a slight decrease at 96hrs of treatment. Frequencies of MN recorded after treatments with 36.0mg/kg were found to increase from 3.47±0.67 (at 24hrs) to 8.47±0.61 (at 96hrs) through 4.47±1.78 (at 48hrs) and 6.30±0.05 (at 72hrs) (Table 24 and Fig. 66).

Results of MNT in different tissues of frog treated with various sublethal concentrations and duration of lead acetate revealed prominent effect of test chemical in RBCs followed by intestine and least in kidney.
Fig. 63 Micronuclei formation in intestine (B,C,D), RBC (F,G,H) and kidney (J,K,L) cells after lead treatment (A,E,I-normal cells)
Table 24: Frequencies of micronuclei (%) in different tissues of *Euphlyctis cyanophlyctis* exposed to Lead.

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Total interphase cells studied</th>
<th>Frequency of micronuclei (Mean±SD)</th>
<th>Intestine</th>
<th>RBC</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contr.</td>
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<td>6000</td>
<td>0.60±0.00</td>
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<td>0.62±0.53</td>
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<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
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<td>72</td>
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<td>6000</td>
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<td>96</td>
<td>4</td>
<td>6000</td>
<td>0.30±0.36</td>
<td>0.45±0.30</td>
<td>0.45±0.30</td>
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</tr>
<tr>
<td>4.5</td>
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<td>4</td>
<td>6000</td>
<td>1.15±0.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.60±0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.30±0.57&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>1.45±0.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.12±0.67&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
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<td>4</td>
<td>6000</td>
<td>1.65±0.40&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>6000</td>
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<td>6000</td>
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<tr>
<td></td>
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<td>6000</td>
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<td>6000</td>
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<td></td>
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<td>6000</td>
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<td>3.97±1.10&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>6000</td>
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</tr>
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</table>

a=p<0.05,b=p<0.01,c=p<0.001,ns= non-significant(represent values significantly differently from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control
Fig. 64: Frequencies of micronuclei in Intestine after treatment with Lead.

Fig. 65: Frequencies of micronuclei in RBC after treatment with Lead.

Fig. 66: Frequencies of micronuclei in Kidney after treatment with Lead.
MANGANESE TREATMENT

1. Intestine:

Frequencies of micronuclei recorded in intestinal tissue have been shown in Fig. 67(B-D) and tabulated in Table 25. Values of MN were found to be statistically significant except 24hrs of exposure in all treatment groups from the respective controls (at 48, 72 and 98hrs, p<0.01). In frog treated with 4.0mg/kg of manganese, frequency of MN recorded were 0.62±0.531, 1.65±0.404, 1.62±0.865 and 1.97±0.531 at 24, 48, 72 and 96hrs respectively, wherein a slight decrease in value of MN frequency was observed at 72hrs of exposure followed by an increase in MN frequency at 96hrs of exposure of test chemical. Values of MN frequency recorded with 8.0mg/kg of chromium were 1.3±0.571 (at 24hrs), 2.3±0.852 (at 48hrs), 2.47±1.147 (at 72hrs) and 3.3±1.061 (at 96hrs). An increasing trend in the frequency of MN was found in the treatment with 16.0mg/kg of test chemical viz. 0.77±0.35, 2.47±0.618, 4.15±1.660, 4.62±1.147 after 24, 48, 72 and 96 hrs of exposure respectively. Similarly at a concentration of 32.0mg/kg, incidence of MN increased from 2.65±0.750 (at 24hrs) to 9.10±2.012 (at 96hrs) through 4.62±0.531 (at 48hrs) and 6.3±0.852 (at 72hrs) as shown in the Table 25 and Fig. 68).

2. RBCs:

Fig. 67(F-H) and Table 25 show the values of MNT in RBCs of both experimental as well as control frog. Data showed that the values were significantly different from those of the respective controls after all the treatments (at 24hrs, p<0.05; at 48 and 72hrs, p<0.01 and at 96hrs, p<0.001). At a sublethal concentration of 4.0mg/kg, values of MN obtained were 0.77±0.35, 1.47±0.35, 1.47±1.117 and 1.8±0.627 after 24, 48, 72 and 96hrs of exposure. Frog treated with 8.0mg/kg of test chemical revealed an increase in the values of MN frequency i.e. 2.3±0.852, 3.3±0.808, 3.8±0.627 and 4.45±0.834 after 24, 48, 72 and 96hrs of exposure period respectively. In treatment with 16.0mg/kg, 1.97±0.531, 4.15±0.834, 4.65±1.223 and 5.12±1.477 were recorded after 24, 48, 72 and 96hrs respectively. An elevated level of MN frequency was also observed after all the exposure periods in experimental frog treated with 32.0mg/kg of test chemical. Maximum frequency (9.20±0.942) was recorded after 96hrs whereas minimum value (2.65±1.22) was observed at 24hrs as shown in Table 25 and Fig. 69.

3. Kidney
Frequencies of MN observed in kidney are shown in Fig. 67(J-L) and Table 25. Values of MN were found to be statistically significant from the respective controls except at 24 and 48hrs of exposure periods (at 72 and 96hrs, p<0.01). In frog treated with 4.0mg/kg of manganese, MN frequencies were 1.12±0.35, 1.46±0.670, 1.65±0.404 and 2.15±0.3 after 24, 48, 72 and 96hrs respectively. At a concentration of 8.0mg/kg, frequency of MN was found to increase from 2.97±1.377 (at 24hrs) to 4.62±1.466 (at 96 hrs) through 2.95±1.377 (at 48hrs) and 4.15±1.833 (at 72hrs). Exposure to 16.0mg/kg induced 1.97±0.531, 3.82±1.670, 5.3±0.571 and 5.95±0.943 MN frequencies after 24, 48, 72 and 96hrs respectively. A time dependent increase from 24 to 72 hrs in the MN frequency was observed in kidney after treatment with 32.0mg/kg of test chemical i.e. 4.62±1.970, 4.97±0.865, 6.65±0.943 and 7.25±3.487 respectively as shown in the Table 25 and Fig. 70.

The results showed a significant increase in MN frequency with dose and duration of exposure except for intestine and kidney with 24hrs of exposure period. A comparison between micronucleus frequencies in all tissues revealed highest MN frequencies in RBCs, followed by kidney and least in intestinal tissue.
Fig. 67 Micronuclei formation in intestine (B,C,D), RBC (F,G,H) and kidney (J,K,L) cells after manganese treatment
(A, E, I-normal cells)
Table 25: Frequencies of micronuclei (%) in different tissues of *Euphyctis cyanophyctis* exposed to Manganese.

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Total interphase cells studied</th>
<th>Frequency of micronuclei (Mean±SD)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Intestine</td>
<td>RBC</td>
</tr>
<tr>
<td>Contr.</td>
<td>24</td>
<td>4</td>
<td>6000</td>
<td>0.45±0.3</td>
<td>0.62±0.531</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>0.15±0.3</td>
<td>0.3±0.346</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
<td>6000</td>
<td>0.3±0.346</td>
<td>0.45±0.3</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>4</td>
<td>6000</td>
<td>0.45±0.3</td>
<td>0.45±0.3</td>
</tr>
<tr>
<td>4.0</td>
<td>24</td>
<td>4</td>
<td>6000</td>
<td>0.62±0.531&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>0.77±0.35&lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>1.65±0.404&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.47±0.35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>72</td>
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<td>6000</td>
<td>1.62±0.865&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.47±1.117&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>6000</td>
<td>1.97±0.531&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.8±0.627&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8.0</td>
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<td>6000</td>
<td>1.3±0.571&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>2.3±0.852&lt;sup&gt;ns&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>48</td>
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<td>2.3±0.852&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>3.8±0.627&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>6000</td>
<td>3.3±1.061&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.45±0.834&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>6000</td>
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<td>4.15±0.834&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>5.12±1.477&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>4.97±2.005&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>6000</td>
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<td>5.8±1.458&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>6000</td>
<td>9.10±2.012&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.20±0.942&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly differently from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control
Fig. 68: Frequencies of micronuclei in Intestine after treatment with Manganese.

Fig. 69: Frequencies of micronuclei in RBC after treatment with Manganese.
Fig. 70: Frequencies of micronuclei in Kidney after treatment with Manganese.

MERCURY TREATMENT

1. Intestine:

Frequencies of MN recorded in the intestinal tissue after different treatment and exposure periods have been shown in Fig. 71(B-D) and Table 26. MN frequencies observed in frog treated in vivo with 1.5μg/kg was 0.97±0.865, 0.97±0.65, 1.15±0.834 and 1.12±0.35 during 24, 48, 72 and 96hrs respectively. Values of MN were found to be same at 24 and 48hrs of exposure and there was a slight decrease in the frequencies of MN from 1.15±0.834 to 1.12±0.35 during 72 and 96hrs of the same dose treatment. MN frequency was found to increase from 1.3±0.0 (at 24hrs) to 2.3±0.346 (at 96hrs) through 1.65±0.404 (at 48hrs) and 1.97±531 (at 72hrs) after treatment with 3.0μg/kg of test chemical. Similarly, the incidence of MN was found to increase in a time dependent manner from 1.97±0.942 (at 24hrs) to 4.15±1.558 (at 97hrs) after treatment with 6.0μg/kg of mercury. Also, the frog treated with 12.0μg/kg of mercury showed an increase in MN frequency from 2.97±1.575 (at 24hrs) to 10.47±0.618 (at 96hrs) through 6.3±0.852 (at 48hrs) and 7.8±1.840 (at 72hrs). Data was found to be statistically significant versus respective controls after different exposure periods in all treatment doses (at 24hrs, p<0.0.5; at 48hrs and 72hrs, p<0.01; at 96hrs, p<0.001) as shown in Table 26 and Fig. 72.

2. RBCs:

Fig. 71(F-H) and Table 26 show the values of MN obtained in RBC after different treatment and exposure periods. In the frogs treated with 1.5μg/kg of test chemical, frequency of MN was determined as 0.77±0.35, 0.95±0.404, 1.3±0.0 and 1.3±0.571 at 24, 48, 72 and 96hrs respectively. Values of MN frequencies were same at 72 and 96hrs of treatment. MN frequency was found to increase from 1.47±0.670 (at 24hrs) to 3.65±0.404 (at 96hrs) through 1.82±0.35 (at 48hrs) and 1.97±0.531 (at 72hrs) after treatment with 3.0μg/kg of mercury. Minimum (0.95±0.404) and maximum (4.15±0.834) frequency of MN after treatment with 6.0μg/kg of test chemical was recorded at 24 and 96hrs of exposure periods respectively. Similarly, the incidence of MN was found to increase in a time dependent manner from 2.47±0.68 (at 24hrs) to 11.62±1.244 (at 96hrs) after treatment with 12.0μg/kg of mercury. Data was found to be statistically significant versus respective controls after exposure periods in all treatment doses (at 24 and 48hrs, p<0.01 and at 72 and 96hrs,
p<0.001) as shown in the table. The value of p revealed highly significant result after treatment and exposure periods as compared to respective controls as shown in Table 26 and Fig. 73).

3. **Kidney:**

Fig. 71(J-L) and Table 26 show the values of MN obtained in kidney at different sublethal concentrations and durations of exposure. Values of MN were found to be significant from the respective controls (at 24, 48, 72 and 96hrs, p<0.01) as shown in the Table 26 and Fig. 74. At sublethal concentrations of 1.5 µg/mg, MN frequencies were 0.45±0.3, 1.3±0.571, 1.12±0.35 and 1.47±0.35 at 24, 48, 72 and 96hrs respectively. Frequencies of MN recorded after treatment with 3.0µg/kg were found to increase from 1.65±0.404 (at 24 hrs) to 3.12±0.670 (at 96 hrs) but minimum MN frequency (1.3±0.571) was recorded at 96 hrs. Similarly, values of MN frequency at 6.0µg/kg of treatment of test chemical showed an increase from 2.62±0.531 (at24hrs) to 3.3±0.571 (at 96 hrs) but a minimum value of MN frequency (2.47±0.994) was observed at 96hrs. However, with the increase in dose concentration i.e. at 12.0µg/kg treatment of test chemical, the values of MN frequencies were found to increase significantly from 3.47±0.670 (at24hrs) to 7.65±2.344 (at 96 hrs) through 4.12±0.618(at 48hrs) and 4.97±1.595(at72hrs).

In general, the data revealed that with respect to the dose, maximum effect was found to be induced at the highest dose of exposure i.e.12.0mg/kg while prominent effect with respect to the duration of exposure was induced after maximum period of exposure i.e. 96hrs. Maximum frequency of MN was recorded in RBC, followed by intestine and least in kidney.
Fig. 71 Micronuclei formation in intestine (B,C,D), RBC (F,G,H) and kidney (J,K,L) cells after mercury treatment (A,E,I-normal cells)
Table 26: Frequencies of micronuclei (%) in different tissues of *Euphlyctis cyanophlyctis* exposed to Mercury.

<table>
<thead>
<tr>
<th>Conc. (μg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Total interphase cells studied</th>
<th>Frequency of micronuclei (Mean±SD)</th>
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<td>Intestine</td>
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<td>Contr.</td>
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<td>6000</td>
<td>0.45±0.3</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>0.3±0.346</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
<td>6000</td>
<td>0.62±0.531</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>4</td>
<td>6000</td>
<td>0.8±0.627</td>
</tr>
<tr>
<td>1.5</td>
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<td>4</td>
<td>6000</td>
<td>0.97±0.865&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
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<td>48</td>
<td>4</td>
<td>6000</td>
<td>0.97±0.65&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>4</td>
<td>6000</td>
<td>1.15±0.834&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>4</td>
<td>6000</td>
<td>1.12±0.35&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
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<td>6000</td>
<td>1.3±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4</td>
<td>6000</td>
<td>1.65±0.404&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>72</td>
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<td>6000</td>
<td>1.97±0.531&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>96</td>
<td>4</td>
<td>6000</td>
<td>2.3±0.346&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>6.0</td>
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<td>1.97±0.942&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>6.3±0.852&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>96</td>
<td>4</td>
<td>6000</td>
<td>10.47±0.618&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a=p<0.05,b=p<0.01,c=p<0.001,ns= non-significant(represent values significantly differently from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control
Fig. 72: Frequencies of micronuclei in Intestine after treatment with Mercury

Fig. 73: Frequencies of micronuclei in RBC after treatment with Mercury

Fig. 74: Frequencies of micronuclei in Kidney after treatment with Mercury
1. Intestine:

Frequencies of MN in intestine at different sublethal concentrations and durations of exposure have been shown in Fig. 75(B-D) and Table 27. In frog treated with 2.5mg/kg of test chemical, frequency of MN was determined as 0.62±0.531, 1.12±0.670, 1.47±0.35 and 1.82±0.35 at 24, 48, 72 and 96 hrs respectively. MN frequency was found to increase from 1.8±0.627 (at 24hrs) to 4.32±1.757 (at 96hrs) through 3.27±0.942 (at 48hrs) and 3.3±0.571 (at 72hrs) after treatment with 5.0mg/kg of test chemical. Minimum (1.97±0.531) and maximum (6.3±0.852) frequency of MN after treatment with 10.0mg/kg was recorded at 24 and 96hrs of exposure respectively. Similarly, the incidence of MN was found to increase in a time dependent manner from 2.97±0.865 (at 24hrs) to 8.3±1.151 (at 96hrs) after treatment with 20.0 mg/kg of nickel. The data was found to be statistically significant only at 72 and 96hrs of exposure periods versus controls (at 72 and 96hrs, p<0.01) as shown in the Table 27 and Fig. 76.

2. RBCs:

Fig. 75(F-H) and Table 27 show the values of MN obtained in RBCs after different treatments and exposure periods which were found to be statistically significant from the respective control (at 24hrs, p<0.05, at 48hrs, p<0.01, at 72 and 96hrs p<0.001) in frog treated with 2.5 mg/kg of test chemical, frequency of MN was found to be same at 24 hrs and 48 hrs of exposure (1.3±0.571) and also at 72 hrs & 96 hrs of exposure (1.47±0.350). Minimum 1.97±0.53 and maximum 2.95±1.135 were found to induce after 48 and 96hrs of exposure respectively in treatment with 5.0 mg/kg of test chemical. In treatment with 10.0 mg/kg incidence of MN increased from 2.65±0.750(at 24 hrs) to 5.8±1.15(at 96 hrs) through 4.15±0.834(at 48hrs) and 4.97±0.865(at 72hrs).In frog treated with 12.0 mg/kg of test chemical, the incidence of MN was found to be 2.97± 0.865(at 24hrs), 6.62±1.419(at 48hrs), 8.32±1.164(at 72 hrs) and 8.63±1.151(at 96 hrs), also increasing trend in the frequency of MN was found in the treatment with 20.0 mg/kg of nickel 2.97±0.865, 6.62±1.419, 8.32±1.164 and 8.63±1.151 after 24, 48, 72 and 96 hrs of exposure respectively as shown in Table 27 and Fig. 77.

3. Kidney:

Frequencies of micronuclei in kidney are tabulated in Fig. 75(J-L) and Table 27. The data was found to be statistically non-significant in all treatment groups at 24 and 48hrs but was found to be significant at 72 and 96 hrs of exposure compared to respective controls (at 72 and 96 hrs, p<0.01). In
the frog treated with 2.5 mg/kg of test chemical, frequency of MN increased from 0.62±0.531 (at 24 hrs) to 1.3±0.571 (at 96hrs) through 0.8±0.627 (at 48 hrs) and 1.12±0.670 (at 72 hrs). An elevated response was also observed during treatment with 5.0 mg/kg wherein the values recorded were 1.65±0.404, 1.95±0.943, 2.47±0.994 and 3.47±1.117 after 24, 48, 72 and 96 hrs of exposure. Frog exposed to sublethal concentrations of 10.0 mg/kg, recorded maximum (4.12±1.147) frequency of MN after 96 hrs whereas minimum value 0.97±0.865 was observed at 24 hrs of exposure. Exposure to 20.0 mg/kg of test chemical induced 3.3±1.205, 4.62±1.466, 5.47±2.202 and 6.3±0.852 of MN frequency after 24, 48, 72 and 96 hrs of exposure respectively. The data revealed insignificant results (at 24hrs, p>0.05) but significant (at 48hrs, p<0.05; at 72 and 96hrs, p<0.01) induction of MN frequency after all exposure periods in comparison to the controls (Table 27 and Fig. 78).

Data obtained in different tissues after exposure to different doses of nickel showed that micronuclei frequencies did increase but it was not a prominent effect in intestine and kidney with respect to the minimum doses and minimum durations of exposure. Highest frequency of MN was recorded in RBCs followed by intestine and least in kidney.
Fig. 75 Micronuclei formation in intestine (B,C,D), RBC (F,G,H) and kidney (J,K,L) cells after nickel treatment (A,E,I-normal cells)
<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Total interphase cells studied</th>
<th>Frequency of micronuclei (Mean±SD)</th>
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<td>RBC</td>
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<td>1.12±0.670&lt;sup&gt;ns&lt;/sup&gt;</td>
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a=p<0.05,b=p<0.01,c=p<0.001,ns= non-significant(represent values significantly differently from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control
Fig. 76: Frequencies of micronuclei in Intestine after treatment with Nickel

Fig. 77: Frequencies of micronuclei in RBC after treatment with Nickel

Fig. 78: Frequencies of micronuclei in Kidney after treatment with Nickel
ZINC TREATMENT

1. Intestine:

Values of MN obtained in intestine after different treatments and exposure durations have been shown in Fig. 79(B-D) and tabulated in Table 28. Data was found to be statistically significant except for 24 and 48hrs of exposure (p<0.01 for 72 and 96hrs for all sublethal concentrations used for the test) from the respective controls. Minimum (1.30±0.57) and maximum (2.48±0.99) MN were found to be induced after 24 and 96hrs of exposure respectively in treatment with 3.0mg/kg of test chemical. In treatment with 6.0mg/kg, the incidence of MN was found to be 2.30±0.85 (at 24hrs), 2.98±0.85 (at48hrs), 2.80± 1.0 (at72hrs) and 3.30±1.20 (at 96hrs) where value recorded at 72hrs of duration was low as compared to values observed at 48 and 96hrs. In the frog treated with 12.0mg/kg, the frequency of MN was found to increase from 1.63±0.07 (at 24hrs) to 4.80±1.15 (at 96hrs) through 2.45±0.30 (at48hrs) and 4.13±0.62 (72hrs). Similarly, the incidence of MN was found to increase in a time dependent manner from 2.95±0.40 (at24hrs) to 8.13±1.43 (at96hrs) through 4.48±0.62 (at 48hrs) and 5.98±0.94 (at72hrs) after treatment with 24.0mg/kg of zinc (Table 28 and Fig. 80).

2. RBCs:

Frequency of MN determined after different treatments and exposure periods in RBCs have been shown in the Fig. 79(F-H) and Table 28. MN frequencies observed in frog treated in vivo with 3.0mg/kg increased from 0.98±0.87 (at24hrs) to 1.83±0.35 (at 96hrs) through 1.30±0.00 (at 48hrs) and 1.48±0.35 (at72hrs). Values of MN recorded in frogs treated with 6.0mg/kg after different exposure periods showed an increase from 1.98±0.53 (at24hrs) to a maximum value of 2.30±0.85 (at96hrs) and then decrease to a minimum of 1.65±0.40 (at72hrs) and further increase to 2.15±0.85 (at96hrs). Similarly, minimum MN value was recorded was 0.95±0.40 (at24hrs) and maximum 4.62±1.46 (at 72hrs) and the MN value was 2.48±1.38 (at48hrs) and 4.45±1.48 (at98hrs) after treatment with 12.0mg/kg of zinc. In the specimens treated with 24.0mg/kg, the incidence of MN was found to increase in a time dependent manner from2.63±0.53 (at24hrs) to 8.13±1.49 (at96hrs). The data was found to be statistically significant at all exposure periods versus controls except for 24hrs of exposure with all treatment doses (at 48, 72 and 96hrs, p<0.01) (Table 28 and Fig. 81).

3. Kidney:
Frequency of MN recorded in kidney after different treatments are shown in Fig. 79 (J-L) and Table 28. The data was found to be statistically non-significant in all treatment groups at 24 and 48 hrs but found to be significant at 72 and 96 hrs of exposure compared to respective controls (at 72 and 96 hrs, p<0.01). At a sublethal concentration of 3.0 mg/kg, values obtained were 0.80±0.63 (at 24 hrs), 0.95±0.40 (at 48 hrs), 1.30±0.57 (at 72 hrs) and 1.65±0.40 (at 48 hrs). Frogs treated with 6.0 mg/kg of test chemical revealed 1.48±0.67, 1.98±0.53, 2.45±0.30 and 3.48±0.67 MN after 24, 48, 72, and 96 hrs of exposure period respectively. In treatment with 12.0 mg/kg, MN frequency recorded were 1.83±0.35, 2.80±0.63, 4.13±0.62 and 4.80±0.63 after 24, 48, 72 and 96 hrs respectively at a sublethal concentration of 24.0 mg/kg, values of MN recorded at 24, 48, 72 and 96 hrs were 3.30±0.57, 4.13±0.62, 6.13±0.62 and 7.80±0.63. Thus in kidneys, the effect of zinc was found to be dose and time dependent (Table 28 and Fig. 82).

In general, data showed that micronucleus frequency increased with an increase in dose and duration of exposure in all the tissues but it was not a prominent effect in intestine and kidney. Also, at a dose concentration of 6.0 mg/kg where minimum value was recorded after 72 hrs of exposure in RBCs and a decrease in trend of MN frequency was observed in intestine at the same dose and duration of exposure. Highest frequency of MN was recorded in intestine and RBCs which had same frequencies of MN at 96 hrs of exposure after treatment with the highest dose of zinc.

The results and the extent of frequencies of micronucleus in different tissues of the frog examined after the different heavy metal treatment revealed that all the heavy metals tested during the present investigation were found to be potentially genotoxic except zinc and nickel. Based on the MNT, genotoxic potential of aforesaid toxicants was recorded as Cu > Hg > Ch > Cd > Pb > Mn > Ni > Zn.

In general, the forgoing results indicated that the frequencies of micronuclei in various tissues of the frog examined were observed to be much lower than that of chromosome aberrations in the tissues analyzed for chromosomal aberration test.
Fig. 79 Micronuclei formation in intestine (B,C,D), RBC (F,G,H) and kidney (J,K,L) cells after zinc treatment (A,E,I-normal cells)
Table 28: Frequencies of micronuclei (%) in different tissues of *Euphlyctis cyanophlyctis* exposed to Zinc.

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (Hrs)</th>
<th>No. of specimens</th>
<th>Total interphase cells studied</th>
<th>Frequencies of micronuclei (Mean±SD)</th>
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<td>Intestine</td>
<td>RBC</td>
<td>Kidney</td>
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a=p<0.05, b=p<0.01, c=p<0.001, ns= non-significant (represent values significantly differently from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control
Fig. 80: Frequencies of micronuclei in Intestine after treatment with Zinc.

Fig. 81: Frequencies of micronuclei in RBC after treatment with Zinc.

Fig. 82: Frequencies of micronuclei in Kidney after treatment with Zinc.
Chapter 5

Discussion
Heavy metals in aquatic environment have been studied extensively in recent years due to their drastic impact on the ecosystems along with a large number living beings and components of trophic food web. The toxicity of heavy metals to various biological subjects and comparison of their sensitivity stimulated this work and to understand the toxic effects of these metals on members of these ecosystems. The heavy metal salts as test chemicals were selected based on their mutagenic and carcinogenic capacity and on the earlier results with some of these metals on different living systems. The effect of any substance on a living system is always dependent on the concentration of it available to its tissues and cells. There are no substances that are always toxic. What we need to evaluate toxicity are dose-response data i.e. qualitative dose- response paradigms in toxicology. During the present work, an attempt has been made to assess the time-dose-dependent, tissue-specific peculiarities of heavy metal genotoxicity in frog as an aquatic organism since individual tissues differ in their response to different contaminants even the heavy metals, depending upon their sensitivity and potential to metabolize, concentrate and store waterborne contaminants. In view of these facts, the present work was carried out to assess the genetic damage in a various tissues of the frog, *E. cyanophlyctis* (the skittering frog).
5.1 Effect of heavy metals as environmental genotoxicants on amphibians

Freshwater is one of the world’s scarcest resources, and anything that threatens it threatens all forms of life, including humans. The pollution of lakes and rivers with chemicals of anthropogenic origin have adverse consequences, the water becomes unsuitable for drinking, irrigation, fish cultivation, household purposes and the animal communities living in them have suffered seriously (Clickman and Lech, 1982).

The ecological assessment of territories exposed to anthropogenic influences must involve evaluation of the mutagenic potential of the environment. The presence of chemical mutagens in natural ecosystems and human communities is a genetic hazard to present and future generations. The assessment of genetic effects of environmental pollution on man is methodologically difficult and expensive; hence, it is expedient to use indicator animal species for ecogenetic monitoring. Many species of anurans are suitable for this purpose, as specific features of their life cycle make them convenient for assessing the state of both terrestrial and aquatic ecosystems. In the course of ecogenetic monitoring, it is often difficult to choose appropriate control populations, because all ecosystems are more or less exposed to the influence of pollutants (including genotoxic agents) as a result of pollution and local anthropogenic effects.

The organisms, inhabiting these ecosystems tends to accumulate a wide variety of pollutants in their tissues. It is known that the effects induced by the environmental contaminants are most frequently initially displayed at the molecular level (biochemical, genetical) level and as a result of such disturbances, various cytological, morphological, physiological and other changes occur. At the later stages, changes may occur at higher biological levels and affect the ability of the organisms to grow, reproduce and to survive. Genotoxic damage in cells can initiate carcinogenesis in various tissues and organs. Agents that cause genetic effects may be present at a very low, sublethal concentration in the environment but may produce their long term effects through biomagnifications in the food chain (Anderson et al., 1994).

Anthropogenic impact appears to be one of the factors responsible for the decrease in the abundance of amphibians. This phenomenon is regarded as a signal of impending ecological disaster (Biek et al., 2002). Amphibian populations from ecotopes with different pollution levels differ in morphological specificity and the degree of concordance with processes at tissue and cell levels (Kryukov, 2000). The
biological and toxic roles of heavy metals/metalliods have been studied extensively in recent years due to the drastic impact they can have on the ecosystem (Loumbourdis et al., 2007). Metal pollution is problematic because it is not easily eliminated from aquatic environment like organic pollutants (Frostner and Wittmann, 1981) and they can be present in water, suspended sediment and bottom sediments of aquatic systems. Heavy metals are stable, persistent and non biodegradable contaminants (Kaewsarn and Yu, 2001) and can be accumulated by some organisms either directly or through the food chain (bioaccumulation and biomagnifications), eventually posing a serious health risk to inhabitants of an ecosystem, including humans (Galloway et al., 1982; Angelone and Bini, 1992; Chan et al., 2003). The bioaccumulation of toxicants, such as heavy metals, by living organisms is often a good integrative indicator of exposure, and has been extensively used to assess contamination levels of heavy metals in polluted ecosystems (Phillips and Rainbow, 1994). The eight most common pollutant heavy metals listed by the Environmental Protection Agency (EPA) are: As, Cd, Cr, Cu, Hg, Ni, Pb and Zn.

Heavy metals are among the major environmental hazards due to their affinity for metal sensitive groups, such as thiol groups. Heavy metals block functional groups of proteins, displace and/or substitute essential metals, induce conformational changes, denature enzymes and disrupt cells and organelle integrity (Hall, 2002). It is also known that heavy metals can cause oxidative DNA damage (Hartwig, 1995). Formation of the reactive oxygen species leads to oxidative DNA damage, to the induction of DNA strand breaks, DNA-protein cross links and therefore, to the formation of chromosomal aberrations. On the other hand, heavy metals arsenic and mercury are mitotic spindle poisons and can induce aneugenic effects as a result of desegregation of chromosomes (Kirsch-Volders, 1986). Compounds of chromium, nickel, cobalt, cadmium and iron are genotoxic to organisms (Hartwig, 1995). The hazardous effects on vertebrates also include teratogenesis and anomalies in reproduction (Loumbourdis and Wray 1998). Availability of chemical species, delivery route and delivery rate of individual metals are influenced by many factors (Bochenek et al., 2008; John and Leventhal 1995; Peijnenburg and Vijver 2007) and play crucial roles in their uptake by an organism (Linder and Grillitsch 2000). Heavy metals, whether present separately or together, reduced the survival, growth and fright response of tadpoles. Cd is generally adsorbed to organic materials and easily accumulated by organisms. Pb on the other
hand, is transferred into solid phases and has lower transportation capacity and lower solubility (Bochenek et al., 2008; Dong et al., 2007). Some studies have shown that amphibians accumulate Pb and Cd proportionately to concentrations in their surrounding environment (Birdsall et al., 1986) and are adversely affected by these heavy metals either in natural or experimental exposures. While many heavy metals are nutrients at trace levels, Pb, Cd and Hg are non-essential and recognized as important industrial hazards, causing toxic effects in higher animals upon acute or chronic exposure. These three elements are highly persistent and in the bivalent form stable inorganic and organic complexes in biological systems.

Heavy metals released into the environment by mining industries, motorcar exhaust fumes, fossil fuel combustion, leakage from laboratories, electrical equipments, metal works, petroleum refineries, fertilizers, latex paints and other human activities and by-products (Cid and Caviedes-Vidal, 2010; Jarup 2003; Norton, 2007; Lindsquist et al., 1991). Industries such as laundries, soft drinks and ice-cream manufacturers discharge effluent enriched in Cu, textile dyeing and laundry waste may emit Cr and Ni is associated with bakery wastes (Frostner and Wittmann, 1981). Natural sources include bedrock, especially slate, which is enriched with heavy metals (Faure, 1998) and atmospheric processes, such as geological weathering, mining activities, volcanoes and storm water runoff, play a major role in the dispersal of heavy metal deposits everywhere. Direct volatilization from surface waters may also transfer heavy metals to the air (Ferrara et al., 1991). Thus, a wide range of combustible and putrescible substances, hazardous wastes, explosives, petroleum products, industrial and domestic effluents including organic and inorganic compounds are strikingly responsible for contamination of the aquatic environment which is often the ultimate recipient of this increasing range of anthropogenic contaminants, a large proportion of which are potentially genotoxic and carcinogenic substances and the aquatic environment plays vital roles for ecosystem functioning, human health and civilization.

It is likely that amphibians are more susceptible to genotoxic pollutants than other vertebrates because they may breed, develop, feed, and overwinter in intimate contact with contaminated water and sediments (Dunson et al. 1992). In addition, adult and larval amphibians, which primarily feed on insects and plant material, respectively, provide information about the earliest stages of trophic transfer of contaminants to vertebrate populations. Future studies assessing the uptake of trace elements in biota
should include both adult and larval anurans because they are an important trophic link to birds, mammals, reptiles, and predatory fish. The fact that many amphibian populations are apparently declining (Wyman 1990; Wake 1991) should provide further impetus for researchers to more closely examine and monitor the quantities of trace elements and other pollutants in these organisms.

5.2 Amphibians, sentinel organisms for environment genotoxicity

Amphibians possess several characteristics that may make them more sensitive to environmental disturbances than other wildlife (Rowe et al., 2003). Their permeable integument, which is critical for both gas exchange and osmoregulation, makes them particularly sensitive to changes in hydric conditions as well as contaminants. Moreover, the reliance of many amphibians on both aquatic and terrestrial habitats places them in “double jeopardy” because a disturbance to the quality or availability of either habitat can disrupt their life cycle and affect populations (Dunson et al., 1992). With regard to environmental pollution, the high conversion efficiency of amphibians should also be associated with high rates of contaminant bioaccumulation compared with other animals of similar trophic position. This theoretical framework was recently supported in a food web study using stable isotopes (Unrine et al., 2007).

Amphibians are critical components of both aquatic and terrestrial communities; therefore anthropogenic factors that negatively affect amphibians may influence entire ecosystems. Amphibians occupy diverse trophic niches, from planktivores to carnivores, and often serve as abundant and important prey for wildlife. Moreover, the ectothermic physiology of amphibians allows them to exploit energy poor resources and thus serve as critical links between the lowest and highest trophic levels within a community. Certain species of amphibians represent the most abundant vertebrates in many aquatic and terrestrial communities, reaching terrestrial and aquatic densities of 2,500 and 40,000 individuals/hectare, respectively (Burton and Likens, 1975; Petranka and Murray, 2001). Such high biomass, coupled with the typical voracious appetites of amphibian larvae (Taylor et al., 1988) and their high conversion efficiency (Burton and Likens, 1975), enables amphibians to play important roles in the transfer of energy and nutrients through food webs (Beard et al., 2002; Ranvestel et al., 2004; Seale, 1980; Wyman, 1998). Additionally, many amphibians have complex life cycles similar to that exhibited by many invertebrates, which occupy aquatic habitats during early life stages.
but ultimately metamorphose into terrestrial forms. A forthcoming study indicates that a single 10-hectare isolated wetland produced >360,000 metamorphic amphibians (>1400 kg of biomass) in 1 year (Gibbons et al., 2006). This and other recent work suggest that metamorphosing amphibians provide significant energy and nutrient subsidies to surrounding terrestrial habitats (Regester et al., 2006).

Over the last 30 years, it has become clear that amphibian populations around the globe are declining at unprecedented rates (Stuart et al., 2004), representing the greatest mass extinction of land vertebrates since the dinosaurs. Despite continued deliberation over which environmental factors are most important in specific declines, most scientists now agree that underlying causes for declines are extremely complex and are due largely to anthropogenic disturbances (e.g., Collins and Storfer, 2003; Kiesecker et al., 2001; Pounds et al., 2006). Many also agree that the health of other animals, including humans, is affected by many of the same factors that are injurious to amphibians. Therefore, it is plausible that amphibian models can serve as useful sentinels of environmental problems that other interdependent living systems face.

Russell Mittermeier, President of Conservation International, said: “Amphibians are one of nature’s best indicators of overall environmental health. Their catastrophic decline serves as a warning that we are in a period of significant environmental degradation.” (Conor, 2004)

5.3 Frog as a genotoxic model

Amphibians including frogs, toads, salamander and caecilians, are an interesting group to study exposure to and effects from environmental contaminants due to their physiology and ecology. Frogs and toads constitute about 90% of all amphibians (Mc Diarmid and Mitchell, 2000). They are the main components of aquatic and terrestrial ecosystems (Unrine et al., 2007) and are important link humans and ecosystem health (Hayes et al., 2002). However, they have highly permeable skins and different life cycle stages making them sensitive to environmental perturbations both in terrestrial and aquatic habitats (Alford and Richards, 1999). From among frog and toad, toad are more terrestrial in nature during adult phase of their life-cycle and require the aquatic habitat for breeding whereas frog typically live in fresh waters: first an aquatic larval form followed by a terrestrial or semi-aquatic adult form and so are subject to the changes in pollutant load and also they are sensitive to a wide range of water borne
contaminants. They are therefore the bioindicators organisms used to determine the water quality and also to follow the changes in their habitats and in ecotoxicological studies (Henry, 2000). Because of the aquatic nature of frogs, their populations around the world have shown increasing sign of stress in recent years. Inspite of the facts that frog are important components of many ecosystems and also important energy- trophic link between invertebrates and other vertebrates, some species have disappeared and others are no longer found where they used to be. More than 500 populations of frogs are in decline and many are listed as of special conservation concern (Alford and Richards, 1999).

The use of frogs as sentinel organisms of metal pollution is also becoming more common (Burger and Snodgrass, 1998) and are increasingly being studied for accumulation of contaminants especially the heavy metals for their toxicity at low concentrations (Welsh and Ollivier, 1998; Johansson et al., 2001; Loumbourdis et al., 2007). Saleh and Zeytinoglu, (2001) showed that frogs are excellent animals for the study of the mutagenic and/or carcinogenic potential of contaminants. Amphibians as model organisms for study environmental genotoxicity was documented by Burlibasa and Gavrila (2011) favoring the use of African clawed frog, *Xenopus laevis* as an excellent model organism, major contributor to our understanding of cell biological and biochemical processes, including: (1) chromosome replication; (2) chromatin, cytoskeleton and nuclear assembly; (3) cell cycle progression and intracellular signaling. Amphibian embryos remained the embryos of choice for experimental embryologists for many decades (Burlibaşa et al, 2005). Various toxicological aspects of different heavy metals have been studied by many workers using different species of frog as a model animal. Rollins-Smith *et al.*, (2004) used *Xenopus laevis* to test the effect of xenobiotic chemicals on the development of the hematopoietic system. *Xenopus laevis* embryos and tadpoles as models for testing for pollution by zinc, copper, lead and cadmium was investigated by Haywood *et al.*, (2004). Mouchet *et al.*, 2006; Lienesch *et al.*, 2000) also conducted toxicity studies on *Xenopus laevis*. Sublethal effects of lead on northern leopard frog, *Rana pipiens* tadpoles were studied by Chen *et al.*, (2006). Brodeur *et al.*, (2009) evaluated the acute and sub chronic toxicity of arsenite and zinc to tadpoles of *Rhinella arenarum*, both alone and in combination. Short term toxic effects of lead nitrate, lead acetate and mercuric acetate on tadpoles of *Rana tigrina* was observed by Wast *et al.*, (2012). Unrine *et al.*, (2004)

Some other frog species were also utilized as model for genotoxic testing such as *Rana clamitans* (Bank *et al.*, 2007), *Lithobates catesbeianus* (Hothem *et al.*, 2010; Burger, 1990; Bank *et al.*, 2007), *Haplobatracus occipitalis* (Ezemonye and Enuneku, 2011a,b,c, 2012), *Rana ridibunda* (Selvi *et al.*, 2003; Sura *et al.*, 2006), *Ptychadena bibroni* (Ezemonye and Enuneku, 2005), *Pelophylax ridibundus* (Stepanyan *et al.*, 2011), *Rana pipiens* (Chang *et al.*, 1974), *Haplobatracus tigerinus* (Vinod and Naik, 1999,2000), *Rana ridipunda* (Saleh and Zeytinoglu, 2001), *Rana luteiventris* (Lefcort *et al.*, 1998). However, these frogs so far provided standard model for genotoxicological studies. Inspite of the above facts, the use of frog chromosomes in genotoxic investigations has been less practiced and various ecotoxicological studies have been performed on them especially at the tadpole stage to test for the levels of contaminants such as heavy metals and pesticides (Broomhall, 2004). Acute and genotoxic studies of various pesticides have been done on a variety of frog’s species and sublethal effect of various heavy metals have been performed on them resulting in negative effect on their growth and development which may cause various morphological deformities and abnormalities. Such effects have also been witnessed in their natural contaminated sites throughout the world compelling the interest and concern of researchers.

Frogs are interesting organisms as far as their cytogenetic is concerned. Studies are carried out worldwide, suggesting they can be good models because of their low and conserved diploid number, less differentiated sex chromosome systems, co-existence of primitive and bimodal karyotypes, occasional polyploidy, very few chances of accessory chromosomes and fairly large size of chromosomes. Although the amphibians of the state are in least concerned category in IUCN Red list but if anthropogenic disturbances remain at the same rate, our state may witness a serious and
irreversible declining trend in populations of these species in near future. In order to design strategies for conservations of the amphibian population of the state, work need to be done at the earliest.

In order to accomplish the above mentioned aspects regarding the threat to amphibian populations throughout the world, the present work was taken with *E. cyanophlyctis* as a genotoxic model animal. Firstly, it is abundant and collection of the organism did not affect the survival of the species in nature. It is found at five of six large water bodies, in puddles, ponds, rain-fed pools, farm ponds, wetlands and even a concrete tank. It occurs in cultivated and uncultivated parts of estate. Secondly, it is always seen in water and have low dispersal rate. Male appear to be territorial, usually maintaining distance from each other. Thus, they best represent the aquatic ecosystem and influence of aquatic contaminants can be interpreted by experimenting on this test specimen. Thirdly, as test animal it has straight forward maintenance, high sensitivity to environmental pollutants and well known taxonomically. The live specimens of same size of the frog were effortlessly collected from the fresh unpolluted water bodies from different areas of Jammu region. The genomic size was small (2n=26) and the chromosome size was large enough to facilitate the study of genomic effects. Out of the three frog species available, *E. cyanophlyctis* (Skittering frog) is the potential test organism for conducting investigations in the area of fish genotoxicity because out of the other two species, *H. tigerinus* (Indian Bull frog) is more secretive in nature, larger in size and is witnessed after a heavy monsoon shower in water logged areas whereas *Chrysopaa sternosignata* (Karez frog) is found in the hilly districts of Jammu. Also several studies have reported that some pesticides reduce cholinesterase (ChE) activity in the frogs; *Rana tigrina* (Khan *et al.*, 2002a,b and 2003a) and *Rana cyanophlyctis* (Khan *et al.*, 2003b,c,d ; Khan and Yasmeen, 2005; Khan *et al.*, 2006; Khan *et al.*, 2007) and reported that *R. cyanophlyctis* is more sensitive to these chemicals than *R. tigrina*. On the basis of present results after genotoxic bioassays with this species, it is suggested that it could prove to be an excellent model specimen for the study of the genotoxic potential of water samples under laboratory and field conditions. In addition, the development of biological monitoring techniques based on this frog offers the possibility of checking water pollution with fast responses on low concentrations of direct acting toxicants. Development of chemical and biological methods for effectively
monitoring environmental levels of heavy metals is a subject of interest and critical state of environmental waste management.

5.4 Genotoxicity of Heavy metals and Mechanism of their genotoxic effect:

Cadmium is recognized as one of the major environmental pollutants and produces toxic effects in living organisms, both aquatic and terrestrial biota (De Conto Cinier et al., 1998). Cadmium (Cd) is a heavy metal with no known biological function. Cadmium is introduced into the aquatic environment primarily by human activities including mining, fertilizer application and industrial discharges (James and Little, 2003; Ezemonye and Enuneku, 2005; Volgiatzis and Loumbourdis, 1997). Grains, cereal products, and leafy vegetables usually constitute the main source of cadmium in food. The disease Itai-Itai resulting from consumption of cadmium contaminated rice in Japan is a well known case of cadmium toxicity (Ernest-Hodgson et al., 2004). Other sources of human exposure to cadmium include food, cigarette smoke and alcoholic beverages (Jarup et al., 1998).

Adult frogs can acquire cadmium through their skin or orally by consumption and respiration. Once absorbed, it can be found in numerous amphibian tissues especially the liver, kidney, gonads, placenta, brain and bones (Sobha et al., 2007). Cadmium has been shown to stimulate free radical production, deplete antioxidant levels resulting in oxidative deterioration of lipids, proteins and DNA and initiating various pathological conditions in animals and humans (Sarkar et al., 1997; Shaikh et al., 1999). It promotes an early oxidative stress and afterward contributes to the development of serious pathological conditions because of its long retention in some tissues (Bagchi et al., 2000). Cadmium may cause the deterioration of cell membranes by binding to metallothionein or glutathione and consequently interfere with the ability of these proteins to avoid oxidative stress. Cadmium can also replace essential metals such as copper and zinc in several metalloproteins, altering the protein conformation and affecting their activity because this element interacts ubiquitously with sulphydryl groups of amino acids, proteins and enzymes (Serafim and Babianno, 2007). Thus, the toxic effects of cadmium are related to changes in natural physiological and biochemical processes in organisms. Cadmium is also known to induce genotoxicity via oxidative stress, DNA binding or inhibition of DNA repair activities (Hartwig, 1998). It was suggested that the mechanism of cadmium genotoxicity is mainly conditioned by
single strand breaks in DNA through direct cadmium-DNA interactions as well as by the action of incision nucleases and/or DNA glycosylase during DNA repair (Privezentsev et al., 1996).

Chromium is a transition metal with three oxidation states: +2, +3 and +6, forming various colorful compounds. Trivalent chromium is essential in human nutrition, acting in glucose metabolism, however the hexavalent chromium is the less stable and more biologically reactive form, highly toxic, genotoxic and carcinogenic (Csuros and Csuros, 2002) and may come to have a lethal effect if absorbed through the skin, ingested or inhaled (Harmel, 2004). Chromium VI, as a metallic ion or in the chromate and dichromate salts, acts by inducing mutations through oxidative damages, similar to reactive oxygen species and to ultraviolet light (Lewin, 2000). Chromium chemistry is an important determinant of its genotoxicity. Hexavalent chromium exists as an oxy-anion at physiological pH and is believed to be transported into living cells via sulphate anion transport system (Standeven and Wetterhahn, 1989). Within a short period of time, hexavalent chromium becomes reduced to the trivalent form which is highly stable, less prone to legend substitution and thus often described in the toxicological literature as ‘kinetically inert’. Intermediate chromium oxidation states, such as Cr (V) and Cr (IV), are formed during the process of intracellular reduction (Standeven and Wetterhahn, 1989).

Studies on chemicals speciation of chromium reveals that Cr (III) is less toxic, while Cr (VI) is extremely toxic as it is more mobile than trivalent form. Moreover, Cr (VI) is a strong oxidizing agent, diffuses readily in the tissues and easily penetrates cell membranes (Piscator, 1986; De-Flora and Wetterhahn, 1989). The toxic action results from its strong oxidative effect on membrane phospholipid proteins and nucleic acids (Chorvatovicova et al., 1993). However, genotoxic activity occurs through intracellular reduction of Cr (VI) to Cr (III), the most stable form of chromium ion and reactive intermediates such as Cr (IV) and Cr (V). During reduction process, oxygen is generated as highly reactive free radical species that can react with DNA (Tsalev and Zaprianov, 1983; Mirsalis et al., 1996).

The chromium associated with DNA is usually found in the trivalent form (Hughes et al., 1995), may form single strand breaks (Sugiyama et al., 1986), DNA cross-links (Manning et al., 1994), alkali-labile sites (Cantoni and Costa, 1984), DNA-
DNA cross links (Xu et al., 1996) and DNA-protein crosslinks (Costa, 1990). Induction of DNA strand breaks in Cr (VI) exposed cells can result from either a direct DNA cleavage by intermediate chromium forms (Bose et al., 1999; Sugden, 1999) or through the attack of sugar-phosphate backbone by hydroxyl radicals (Shi et al., 1999). It is now well established that hexavalent Cr compounds produce DNA strand breaks (Synder, 1988). Chromium (VI) in the form of tetrahedral chromate anions (CrO$_4^{2-}$) crosses cell membrane using a non specific anion carrier, so called permease system which transports a number of anions with tetrahedral configuration such as SO$_4^{2-}$ and PO$_4^{3-}$ (Belagyi et al., 1999). Potassium chromate at physiological pH exists almost entirely as chromate (CrO$_4^{2-}$). Chromate selective accumulation in proximal convoluted tubule may occur due to its structural similarity to sulphate, which is primarily reabsorbed in PCT (Ruegg, 1997). Once inside the cell, Cr (VI) compounds are reductively metabolized by cellular reducers like glutathione, ascorbic acid or cysteine to trivalent species (Gaggelli et al., 2002). This reduction is an important mechanism to inactivate Cr (VI). However, during this process a wide range of genetic lesions are generated, namely Cr-DNA adducts, DNA-proteins cross links or single strand breaks (Zhitkovich, 2005).

Induction of chromosome aberrations, mitotic index and induction of micronuclei in frog, *E. cyanophlyctis* as a result of exposure to sublethal concentration of chromium in the present study are quite suggestive of the fact that Cr (VI) is a potent genotoxic agent. Chromium compounds are often mutagenic at a very narrow dose range. This may be due to persistent toxicity after the initial cell treatments because of the inability of reduced Cr (III) to exit the cells (Standeven and Wetterhahn, 1989). Other particulate chromium compounds, such as insoluble crystalline Cr oxide (Cr$_2$O$_3$) have also been reported to induce sister chromatid exchanges in cultured Chinese hamster cells (IARC, 1987).

Copper, in trace amounts, is essential for life found in small amounts in a variety of cells and tissues, with the highest concentrations in the liver (Turnlund, 1998). It functions as a cofactor and is required for structural and catalytic properties of a variety of important enzymes and hormones (Gaetke and Chow, 2003; Turnlund, 1999; Uauy et al., 1998). The accumulation of large amounts of copper in cells and organs can be toxic. One of the most accepted explanations for copper induced cellular toxicity comes from the assumption that copper ions are prone to participate in the
formation of ROS (Gaetke and Chow, 2003) that can occur when the ability of the cells to store excess copper in a benign form has been exceeded (Linder, 2001). Cupric and cuprous copper ions can act in oxidation and reduction reactions. The cupric ion (Cu (II)), in the presence of biological reductants such as ascorbic acid, can be reduced to cuprous ion (Cu (I)), which is capable of catalyzing the formation of reactive hydroxyl radicals (•OH) through decomposition of hydrogen peroxide via the Fenton reaction (Lloyd et al., 1997). The hydroxyl radical is very reactive and can further react with practically any biological molecules in the near vicinity via hydrogen abstraction, leaving behind a carbon-centered radical, as observed during the formation of a lipid radical from unsaturated fatty acids (Buettner and Jurkiewicz, 1996). Copper is also capable of inducing DNA strand breaks and the oxidation of bases via oxygen free radicals (Brezova et al., 2003). Copper in both oxidation states (cupric or cuprous) was more active than iron in enhancing DNA breakage induced by the genotoxic benzene metabolite 1, 2, 4-benzenetriol. DNA damage occurred mainly by a site-specific Fenton reaction (Brezova et al., 2003). The copper system also generated significant levels of DNA lesions. Guecheva et al.,(2001) also suggested that genotoxicity of Cu could appear via the action of induced ROS, while the inhibition of DNA repair enzymes could be caused by a non specific binding of Cu\(^{2+}\) to essential sites of the enzyme molecule. Copper is known to be as effective or even more effective than iron in causing DNA damage (Ozawa et al., 1993), protein or peptide modification (Uchida and Kawakishi, 1990), and oxidation of low-density lipoproteins (Esterbauer et al., 1990).

Genotoxic and mutagenic effects of copper compounds have been studied in a variety of test systems (Agarwal et al., 1990; Bhunya and Jena, 1996; Steinkellner et al., 1998) and it has been found that copper undergoes redox cycling resulting in the production of reactive oxygen species (Stohs and Bagchi, 1995). A decrease in the mitotic index as recorded in the present investigation indicates that copper chloride interferes in the normal sequences of mitosis (Inceer et al., 2003). Such a reduction in mitotic activity could be due to inhibition of DNA synthesis (Beu et al., 1976).

Lead (Pb) is a non essential hazardous heavy metal. It is of great risk both for the environment and human health, having harmful effects that in some cases may exceed those of other inorganic toxicants. Lead is a weak mutagen in mammalian cell systems, but it is a strong mitogen. Metal-induced genome damage includes DNA
single-strand and double-strand breaks, DNA-DNA cross links, induction of reactive oxygen intermediates (Ariza et al., 1998), DNA-protein links, and base modifications. Indirectly, metals may inhibit DNA repair enzymes or DNA replication (Zelikoff et al., 1988), and consequently act as co-clastogens or co-mutagens (Miadoková et al., 2000). There are several possible mechanisms how lead (II) might interfere with DNA repair process. Besides a direct interaction with repair enzymes, lead ions may also interfere with calcium regulated processes involved in the regulation of DNA replication and repair.

It was reported that lead had an effect on genetic system through chromosomal abnormalities (Fahmy, 1999; Ahmed et al., 2012a,b) and micronucleus (Celik et al., 2005a,b). The mechanisms for these genotoxic responses may involve direct damage to DNA affecting the stabilization of chromatin (Johansson and Pellicciari, 1988) or integration with repair processes (Hartwig et al., 1990). Lead is believed to be covalently interact with tertiary phosphate ions in nucleic acids and proteins (Bremer, 1974; Holtzman et al., 1984) and affect the fidelity of DNA synthesis in vitro (Sirover and Loeb, 1976). Studies in laboratory animals have shown that exposure to lead at levels 10 mg Pb²⁺/ml of blood leads to chromosomal aberrations (tetraploidy, mitotic anomalies, chromatid breaks) and these effects may be related to interference with the mechanism of replication, transcription and DNA repair (Goyer and Moore, 1974). Part of this effect on chromosomes may be related to the ability of lead to form bridges with structural proteins that can adversely affect the degree of chromosomal condensation. Mutagenic or clastogenic activities of lead are related to disturbances in enzyme regulation that probably affect replication, translation and repair of genetic material (Goyer and Moore, 1974). The inhibitory effect of lead on cell cycle than the induction of cell repair mechanism was proposed by Al-Sabti and Hardig (1990) and Poongothai et al., (1996). Thus, lead may affect both chromosomes and key enzymes involved in metabolic changes in the cell.

Lead is also an electrophile that avidly forms covalent bonds with the sulphydryls groups of cysteine in proteins. Thus, proteins in all tissues exposed to lead will have lead bound to them. Some proteins become labile because of binding of lead which causes the tertiary structure of the proteins to change; cells of the nervous system are particularly susceptible to this effect because they are exposed to relatively high lead concentration during clearance (Burtis and Ashwood, 1994).
Mercury (Hg) is a xenobiotic metal that is a highly deleterious environmental contaminant that has generated widespread concern due to its global ubiquity and known toxicity to humans and wildlife (Eisler, 2006). Mercury (Hg) enters aquatic ecosystems via atmospheric deposition (Mierle, 1990; Wiener et al., 1990) and is biologically and chemically converted to methylmercury (MeHg), a biologically active and toxic form. Acidified freshwater ecosystems with high temperatures and dissolved organic carbon levels facilitate MeHg bioaccumulation and biomagnification (Wiener et al., 2003). From a toxicological perspective, Hg is most widely known for its neurotoxicity, a characteristic responsible for altering behavior and impairing cognition in vertebrates (Wolfe et al., 1998). However, there is also growing evidence that Hg can affect the endocrine and reproductive systems of vertebrates (Scheuhammer et al., 2007; Crump and Trudeau 2009; Tan et al., 2009).

It was found during the present study, that frog exposed even to sublethal concentrations of Hg (II) resulted in a significant increase in the frequencies of chromosome aberrations and micronuclei in all the exposure periods compared with controls. Mercury compounds induce a general collapse of antioxidant mechanisms in the cell by binding to the sulfhydryl groups of glutathione, a radical scavenger. Such a collapse results in cell degeneration, loss of membrane integrity and finally cell necrosis (Schurz et al., 2000). Necrosis can be indicated by a decrease in mitotic index, as shown in the present results. A decrease in mitotic index followed by an increase in the generation of reactive oxygen species was detected in human body lymphocytes exposed to CH$_3$HgCl (Ogura et al., 1996). Binding of mercury metal to sulfhydryls groups of glutathione blocks its function as a free radical scavenger (Shenker et al., 2000). Thus, free radicals become available to cause DNA damage (Ogura et al., 1996). These mechanisms can lead to double-strand breaks (Harvey et al., 1997) that can be visualized as the chromatid gaps observed in the present study and/or give rise to more evident chromosome alterations such as breaks, rearrangements and so on (Morgan et al., 1998).

Another mechanism that may contribute to cell death induced by mercury compounds is apoptosis. Shenker et al., (2000) reported that CH$_3$HgCl caused a significant increase in cytochrome-C in the cytosol of T-cells. In contrast, HgCl$_2$ did not alter the levels of cytosolic cytochrome-C, suggesting that the apoptotic pathway triggered by HgCl$_2$ compounds is independent of cytochrome-C release. Results
obtained by Bahia et al., (1999) for TK6 cells support the higher cytotoxic effect of CH$_3$HgCl compared to HgCl$_2$.

Toxicity of manganese is related to biological transformation of Mn$^{2+}$ to Mn$^{3+}$ with autoxidation of dopamine and the production of cytotoxic free radicals (Chu et al., 1995). Water soluble nickel chloride can enter into cytoplasm and nucleus by pinocytosis or ion channel and induce DNA damages by directly binding to chromatin, nucleic acids and nuclear protein (Ciccarelli and Wetterhahn, 1984) or indirectly catalysing the formation of DNA-protein cross links through the formation of oxygen radicals (Huang et al., 1993). All of the compounds tested in the present study were genotoxic except manganese and zinc, although in case of Ni (II) this effect was weak since it was seen only at the highest concentration whereas in case of zinc the effect was of non genotoxic type. Toxic and carcinogenic effects of nickel compounds are associated with nickel-mediated oxidative damage to DNA and proteins and to inhibition of cellular antioxidant defenses thereby indicating the genotoxic potential of this heavy metal. Some nickel compounds are weakly mutagenic in a variety of test systems, but much of the evidence is inconclusive or negative.

Metallothionein (MT), a low molecular weight protein, binds a variety of essential (Zinc, copper, etc.) and non essential metals (mercury, arsenic and cadmium) (Kagi and Nodberg, 1979). As a stress response protein, metallothionein confers protection against a variety of stress factors, including toxic metals and oxidative stress (Miles et al., 2000). Since metallothionein is a cysteine-rich protein, mercury might bind to this protein (Hg-MT) and protect against the toxic effects of the metals.

Present study finds its relevance in the above context by revealing the fact that heavy metal pollutant viz. Cd (II), Cr (VI), Cu (II), Pb (II), Mn (II), Hg (II) Ni (II), and Zn (II), by virtue of their presence in aquatic habitat damage genetic profile of the model, *E. cyanophlyctis* thus posing a threat for the loss of amphibian biodiversity, which is currently a major international concern. Out of the 20 countries with highest number of threatened amphibian species Indian comes at 9th place with 66 threatened species. Recently genome sequence of *Xenopus tropicalis* which has been worked out filling a major gap among the vertebrates sequenced to date, imaging the fate of frogs in future; one can conceive tragic effects humans will face. It is therefore, recommended that effluents containing heavy metals should only be disposed off after
their proper treatment. Further, there should be strict and regular monitoring of these toxicants in water bodies to check possible environmental hazards.

5.5 Discussion on present observations

5.5.1 Chromosomal aberration test (CAT) and Mitotic Index (MI):

The present results of the chromosomal aberration test and mitotic index obtained after treatment of the frog with sublethal concentrations of various heavy metals revealed significantly higher frequencies of chromosomal aberrations and mitodepressive effect than that of the controls, evidencing the genotoxic effect of heavy metals. The result of chromosome aberrations and mitotic index induced by individual heavy metal salts are discussed in detail as under:

CADMIUM TREATMENT

During the present investigation, genotoxicity of Cadmium in E cyanophlyctis was clearly evidenced with the high percentage of different chromosomal aberrations induced after different doses and durations of exposure. Among various chromosomal abnormalities scored higher percentage at 72 and 96hrs of exposure compared to 24 and 48hrs of exposure in various tissues of exposed specimens. Predominant aberrations recorded in intestine were ring chromosomes and stickiness followed by terminal chromatid deletions and centromeric gaps whereas clumping, terminal association of chromosome and stretching was recorded in least frequencies. In case of bone marrow, terminal association of chromosomes, clumping, stretching and pycnosis were found to be predominant aberrations whereas terminal chromatid deletions followed by centromeric gaps were recorded in least percentage. Total percentage of chromosomal aberrations was found to be highest in bone marrow followed by intestine. Analysis of MI after treatment with various sublethal concentration of cadmium revealed mitodepressive effect of the heavy metal studied. The effect was predominant in intestine followed by bone marrow which is reverse as in case of CAT (Table 5 and 6; Fig. 11, 12, 13, 14 and 15).

The present result evaluates the toxic potential of cadmium. However, from the literature in hand no record of evidence of chromosome aberrations in any frog species after cadmium treatment was found but its genotoxic and stress inductive potential in larvae of frog Xenopus laevis was analyzed by Mouchet et al., (2006) after 12days
exposure to environmentally relevant contamination levels. These results show clastogenic and/or aneugenic effects in the circulating blood with 2, 10, 30µgL⁻¹; Cd uptake by the whole organism and by the liver as a response to Cd exposure conditions and up regulation of the genes involved in detoxification process and response to oxidative stress, while genes involved in DNA repair and apoptosis were repressed. Although cadmium is suspected to have a spindle activity and is known for its likely carcinogenic, mutagenic and teratogenic activity (I.A.R.C., 1987), cytogenetic bioassay data are conflicting (Hartwig, 1994; Forni, 1992) probably because of the indirect effects of Cd. Indeed, even if the mechanisms underlying the toxic effects of cadmium are still not well understood, Cd is known to induce DNA strand breaks and chromosomal aberrations but its mutagenic potential is rather weak (Hartwig, 1994). Cadmium is also known to induce genotoxicity via oxidative stress (Filipic and Hei, 2004; Devi et al., 2001), DNA binding (Hassoun and Stohs, 1996), or inhibition of DNA repair activities (Lutzen et al., 2004; Hartwig, 1998). Sura (2008) and El-Damerdash et al., (2004) also found that cadmium is known to alter antioxidant levels and induces oxidative stress in living systems. Selvi et al., (2003) also investigated the acute toxicity of cadmium chloride metal salt and behavioral changes it caused on water frog, Rana ridibunda. The 96-hr LC₅₀ value for water frog was estimated to be 51.2mg/L. Frogs subjected to Cd may show various behavioral disorders such as loss of balance, respiratory difficulty, slowness in motion, capsizing in water, sinking to the bottom and increased mucus secretion (Selvi et al. 2003).

Aberrations scored in the present investigation can be compared with the findings of worker on other genotoxic models. Chandra and Khuda-Bukhsh (2001) evaluated the genotoxic effects of cadmium chloride and their alleviation by vitamin-C on fish Oreochromis mossambicus for two different concentrations, 0.003% and 0.05%. They divided the chromosome aberrations into ‘major types’ and ‘other types’. As ‘major types’ which included gaps, breaks, ventric fusion, pulverization, rings, fragments, polyploidy and terminal association and ‘other types’ included stickiness, precocious separation, constriction, C-mitosis, pycnosis and stretching. Their data revealed that the total percentage of aberrations were maximum in 0.05% CdCl₂ at all fixation intervals, gradually rising from 6hrs to 48hrs, after that is gradually declined through 72hrs to 96hrs. 0.003% CdCl₂ yielded aberration frequencies less than that of 0.05% cadmium treatment, but followed by the same trend of showing the peak at
48hrs and then declining. However, during the present investigations, among various individual abnormalities the percentage some show an increase while others decreased with the dose and duration of exposure but total chromosomal aberrations was found gradually increase with the increasing dose of exposure at each sublethal concentration of cadmium chloride tested (Table 5 and 6; Fig.11,12,13,14 and 15).

Bauchinger et al., (1976) carried out research work on human lymphocytes, on exposure to cadmium and lead at zinc smelting plant. They observed a significant increase in percentage of aberrant cells as compared to control workers which confirms the present finding regarding significant increase in aberrations compared to controls at each concentration of cadmium chloride.

Seoane and Dulout (1999) analyzed the aneugenic and clastogenic abilities of cadmium sulphate (at concentration of 0.03 mM, 0.067 mM and 0.134 mM) using the anaphase-telophase test on Chinese hamster ovary cells. Frequency of cells with Chromatin bridges, lagging chromosomes and lagging chromosomal fragments was scored. The mitotic index was also determined and was expressed as percentage of the number of mitotic plates. Cadmium was found to be clastogenic and aneugenic and increased the frequencies of three types of aberrations scored, although this was significantly different from the untreated controls only at the highest dose (at 0.134 mM). Variations of mitotic index were inversely correlated with the dose (r= 0.0868, p=0.068).

From the results of the present investigations, it is thus concluded that cadmium has clastogenic and aneugenic effects on the frog cells exposed in laboratory conditions and thereby it becomes a matter of concern that water pollution especially with heavy metals may lead to serious health risks among various other aquatic organisms through chronic exposure and ultimately effecting the ecosystem and the man itself.

**CHROMIUM TREATMENT**

Present results of chromosome aberrations in intestine and bone marrow after using chromium as test chemical have revealed that the frequencies of total aberrations were found to be predominant in bone marrow compared to intestine. In case of intestine, the predominant aberrations recorded were ring chromosomes and stretching while in bone marrow, the maximum frequency was found for chromosome
fragmentation and stretching, all having the same percentage value. The maximum frequency of chromosome aberrations was recorded only after treatment with maximum sublethal concentration of test chemical at 96hrs of exposure period which was regarded as the effective sublethal concentration at 96hrs of exposure period. The frequency clumping was found to be least in both intestine as well as bone marrow. Maximum decrease of mitotic index was observed in intestine compared to bone marrow (Table 7 and 8; Fig.16, 17, 18, 19 and 20).

The aneugenic and clastogenic effects of potassium dichromate was also revealed by Seoane and Duout (1999) at different sublethal concentrations of 1.0µM, 2.0 µM, 3.0 µM and 4.0 µM using anaphase- telophase test on Chinese hamster ovary cells. The cells with various chromosomal aberrations like chromatin bridges, lagging chromosomes and lagging chromosomal fragments were scored for their frequencies. The frequency of lagging chromosomes was found to increase in all the four doses whereas frequency of lagging chromosomal fragments increased significantly at three highest doses and the frequency of chromatin bridges increased significantly at two higher dose concentrations. However the variations of mitotic index were inversely correlated with the dose concentration (r=0.899, p=0.013).

Krishnaja and Rege (1982) also observed the induction of chromosomal aberrations in fish *Boleophthalmus dussumieri* after in vivo exposure to mitomycin-C and heavy metals including chromium. The fish was treated by giving intramuscular injection of 1.0 and 5.0mg/kg and also by directly dissolving 24.0 and 30.0ppm of Na2Cr2O7 in water of aquarium for 72 and 96hrs respectively. The frequencies of chromosomal aberrations such as chromatid and isochromatid breaks, gaps, rings, fragments and exchanges were found to increase in all the doses.

Yadav and Trivedi (2006) also studied the genotoxic effect of chromium (VI) in *Channa punctatus* after its treatment with a sublethal concentration of 7.689 mg/kg of Cr (VI), dissolved in water for 24, 48, 72 and 96hrs of exposure. A significant increase in chromosomal aberrations like chromatid breaks, chromosome breaks, chromatid deletions, fragments, acentric fragments, ring and dicentric chromosomes, along with chromatid and chromosome gaps, after exposure of Cr (VI) for 72hrs. Similarly in the present study, the frequencies of total chromosomal aberrations increase with the duration of exposure for a particular treatment dose of chromium.
However, chromium treatment had been observed to have a negative impact on the mitotic index of intestine and bone marrow of exposed frogs in the present study as shown in the Fig.16, 17, 18, 19 and 20; Table 7 and 8.

Chromium also shows toxicity towards different animals (Fatima et al., 2005; Geetha et al., 2005). Exposure of *Rana ridibunda* to either Cr or a mixture of Cr and Cd caused a decrease in liver GST and P450-MO and renal GST activities (Kostaropoulos et al., 2005). It was observed that treatment of rats with chromium (0.8mg/100g body weight/day) for a period of 28days caused significant increase in chromium content while lowering the body weight along with the organ (kidney, testis, spleen, cerebrum and cerebellum) weight, (Dey et al., 2003). Beside this there was a significant decrease in total protein content was also observed (Dey et al., 2003). The application of higher dose of chromium in drinking water during embryonic development in mice results in reduced fetal weight, regarded fetal development and higher incidence of dead fetuses (Junaid et al., 1995). Animal studies on the carcinogenicity of various chromium species have generally suggested that water- soluble species, CaCrO$_4$ in particular (Levy et al., 1986), are the causative agent of respiratory cancers (Gad, 1989). Studies have showed the toxicity of chromium picolinate in renal impairment, skin blisters and pustules, anemia, hemolysis, tissue edema, liver dysfunction; neuronal cell injury, impaired cognitive, perceptual and motor activity; enhanced production of hydroxyl radicals, chromosomal aberration, depletion of antioxidant enzymes in mice (Bagchi et al., 2002). In mice, potassium dichromate treatment (20mg CrKg$^{-1}$), as Cr (VI) compound, significantly elevate the level of lipid peroxidation as compared with the control group. This was accompanied by significant decrease in nonprotein sulfhydryls (NPSH) level, superoxide dismutase (SOD) and catalase (CAT) enzyme activities as well as a significant chromium accumulation (Bosgelmez and Guvendik, 2004). Hexavalent chromium compounds are approximately 1000-fold more cytotoxic and mutagenic than trivalent chromium compounds in cultured diploid human fibroblasts, but both hexavalent and trivalent chromium compounds in cultured diploid human fibroblasts, but both hexavalent and trivalent chromium compounds induce dose-dependent anchorage independence in human diploid fibroblasts (Biedermann and Landolph, 1987).
These results of the present investigations thus reveal that chromium is a potent genotoxic heavy metal and it brings about chromosomal aberrations and decreases mitotic index in frog, *E. cyanophlyctis* exposed to sublethal concentrations of Cr (VI).

**COPPER TREATMENT**

Chromosomal aberrations recorded in different tissues revealed a dose and time dependent increase in their frequencies which were significantly different from the respective controls at all exposure periods (Table 9 and 10; Fig. 21, 22, 23, 24 and 25), but the highest percentage of different chromosomal aberrations were induced only after treatment with maximum dose of 8.0mg/kg of test chemical in both the tissues of the exposed specimens. Different aberrations which were found to be predominant at different doses of exposure were minutes followed by chromosomal fragmentation and terminal association of chromosomes (in intestine); chromosome fragmentation and terminal chromatid deletion (in bone marrow) while stickiness (in intestine) and centromeric gaps, clumping and pycnosis were found to have least frequency. Total percentage of chromosomal aberrations was found to be highest in intestine followed by bone marrow. Analysis of MI after treatment with various sublethal concentrations of copper revealed mitodepressive effect of heavy metal studied. The effect was predominant in bone marrow than in intestine.

The results of the present investigations have been correlated with the work of various other workers having reported similar types of observations in other living systems. Stouthart *et al.*, (1996) reported that copper produced teratogenic effect in common carp even at low concentrations i.e. 0.051mg/L and 0.019mg/L. Agarwal *et al.*, (1990) also investigated that a single intraperitoneal injection of copper (II) sulphate pentahydrate in mice induced a dose related significant increase in the incidence of chromatid type chromosome aberrations in the bone marrow after 6hrs when given a dose between 0.28 and 1.7mg/kg body weight. Yadav and Trivedi (2009) exposed the fish *Channa punctatus* to sublethal concentrations i.e. 10% of 96hrs LC$_{50}$ (0.407mg/l) of copper sulphate for a period of 24, 48, 72, 96 and 168hrs. Chromosomal aberrations such as chromatid and chromosome breaks, chromatid and chromosome gaps, ring and dicentric chromosomes were observed in the kidney cells of the exposed fish. A significant increase over negative control in the frequency of chromosomal aberrations was also observed in fish exposed to Cu (II). Krishna and Gupta (2002)
also reported chromosome aberrations in fish, *Labeo rohita* when treated with 0.5, 1.5 and 3.0 ppm of copper sulphate. The genotoxicity of copper sulphate was also elucidated by Guecheva *et al.*, (2001) in planaria by means of comet assay. Planaria which were pre-exposed to methylmethane sulphonate when treated with copper had elevated levels of DNA strand breakage and inhibition of DNA repair. They suggested that genotoxicity of copper could appear via the action of induced reactive oxygen species, while the inhibition of DNA repair enzymes could be caused by a non-specific binding of Cu$^{2+}$ cations to essential sites in the enzyme molecule.

The results reported by Mohamed *et al.*, (2008) with regard to mitotic index and induction of chromosomal aberrations in fish, *Oreochromis niloticus* after its exposure to copper sulphate and lead acetate also reveals the genotoxic effect of these heavy metals. In the gill cells of the treated fish, these two heavy metals displayed lower mitotic activity than that of the control group, both the heavy metals were found to be positive inducer of macro-DNA damage which represented different types of aberrations viz. chromatid deletions, chromatid breaks, gaps, fragments, stickiness, translocations, ring chromosomes and centromeric attenuation. Chromatid deletions, fragments and stickiness were most frequent than other chromosomal aberrations in the gill cells of the treated fish. Inceer *et al.*, (2003) also observed mitodepressive action of copper chloride on root tip cells of *Helianthus annuus* after giving treatment with sub lethal concentrations of 10, 25, 50 and 100mg/L (ppm) of copper chloride for 24hrs. The results revealed that mitotic abnormalities increased and mitotic index decreased depending on the concentration of copper chloride applied. Copper chloride affected the spindle and decreased anaphase and telophase stages while metaphase stage showed an increase. Chromosomal aberrations were observed to increase with increase in concentration and the chromosomal aberrations found included fragmentation, disturbed chromosomes, sticky chromosomes and chromatin bridges, unequal distribution of chromatin and condensed chromatin. A decrease in the mitotic index indicates that copper chloride interferes with normal process of mitosis. Such a reduction in mitotic activity could be due to inhibition of DNA synthesis (Beu *et al.*, 1976). Srivastava and Srivastava (2004) also reported maximum chromosome fragmentation in plant, *Helianthus annuus* after it was treated with copper chloride.

All the above effects of copper whether in the form of copper sulphate or copper chloride on fish as well as plant cells lead us to the conclusion that copper have
genotoxic potential as it brings out chromosomal abnormalities as well as affect the mitotic index in them. This justifies the present work done on the frog, *E. cyanophlyctis* that copper has harmful effects on chromosomes of the cells of different tissues studied and also have mitodepressive effect on them even when the frog was treated with sublethal doses of coppersulphate.

**LEAD TREATMENT**

In the present study, toxicity of lead acetate in the frog, *E. cyanophlyctis* was clearly evidenced by the increased chromosomal aberrations along with a reduction of mitotic index after treatment with different doses and duration of exposure. Results of the chromosomal aberrations test (Table 11 and 12; Fig. 26, 27, 28, 29 and 30) showed that lead acetate treatment in the tissues of frog induced a whole spectrum of chromosome aberrations which could be broadly classified into clastogenic and physiological type. The former aberrations include chromosomal fragmentation, ring chromosomes, terminal chromatid deletion, minutes and centromeric gap and the later include terminal association of chromosomes, stickiness, clumping, pycnosis and stretching. Among the various chromosome abnormalities scored in intestine, clumping followed by chromosomal fragmentation and pycnosis showed the highest frequency and the frequency of stickiness was found to be least. In case of bone marrow, predominant aberrations were found to be pycnosis followed by ring chromosome and stretching while clumping and stickiness were the least observed aberrations. Therefore, the highest percentage of individual aberrations has been recorded among the physiological type of aberrations i.e. clumping and pycnosis and also the least percentage was also observed in physiological type of aberrations showing that this type of aberrations had a wider range of the values recorded. The maximum percentage of chromosomal aberrations was observed in case of intestine than in bone marrow.

Cytogenetic effect of lead acetate treatment on mitotic division in different cells of the frog was also studied. Mitotic index decreased with increasing concentration of lead in the tissues of the treated frog specimens. The results of mitotic index showed a general trend of decrease in both the treated tissues versus respective control values. Maximum decrease in the mitotic index was evident in case of intestine compared to bone marrow.
Vinod and Naik (1999) analyzed the effect of lead acetate on the bone marrow chromosomes of frog, *Haplobatracus tigerinus* and found that significant increase in chromosome aberrations even after 48hrs with high dose and also after exposure of frogs to the higher dose of solution for 14 days.

Various other workers have investigated the genotoxicity of lead with their findings on plants as well as other animals. Cestari *et al.*, (2004) reported an increase in various type of chromosomal abnormalities in the metaphase plates of a fish, *Hoplias malabaricus* after it was fed lead contaminated food for 18 and 41 days. The chromosomal aberrations identified were chromatid gaps and breaks, chromosomal fragmentation, chromatin decondensation and pericentric inversions. Chromatid breaks were the predominant chromosomal aberrations and a quantitative relationship between the occurrence of chromosomal aberrations and the intake of lead was demonstrated as has also been revealed during the present investigations. The effect of lead on mitotic index and induction of chromosomal aberrations in another fish, *Oreochromis niloticus* have also been investigated by Mohamed *et al.*, (2008). They reported that gill cells of the treated fish displayed lower mitotic activity than that of control group. Lead was found to be positive inducer of macro-DNA damage which represented different types of aberrations viz. chromatid deletions, chromatid breaks, gaps, fragments, stickiness, translocations, ring chromosomes and centromeric attenuation. Chromatid deletion, stickiness and fragments were observed to be the most frequent chromosomal aberrations. Mathew and Jahageerdar (1999) have also found that when fish, *Channa punctatus*, exposed to lead at a very low concentration of 0.012mg/L and just 96hrs of exposure period, it induced chromosomal aberrations in the fish.

Truta *et al.*, (2011) reported that lead induced genotoxicity in wheat. The changes induced in cytogenetic parameters from root meristems of *Triticum aestivum* seedlings have been studied after treatment with lead acetate and lead nitrate solutions, at four concentrations (10, 25, 50, 100 μM) containing 2.07, 5.18, 10.36, respectively 20.72 μg ml⁻¹ Pb²⁺. Lead induced mitosis disturbances in root meristematic cells of wheat seedlings, expressed mainly in decrease of mitotic index and changes in preponderance of division phases. This heavy metal has genotoxic effects, expressed in the occurrence of many chromosomal aberrations in all Pb²⁺ treated variants. It was also earlier reported that lead depresses mitotic activity in *Allium sativum* (Mukherjee and Sharma, 1987) and *Vallisneria spiralis* (Mukherjee *et al.*, 1990). Srivastava and
Srivastava (2004) also reported that lead nitrate was toxic for the chromosomes of *Helianthus annus*. Among the four heavy metals studied (Pb, Cu, Hg, Zn), lead nitrate was found to be mutagenic agent and it induced tetraploidy in sunflower which is in conformity with the present findings.

Syarif *et al.*, (2008) investigated the induction of chromosomal aberration by lead acetate in mouse bone marrow with different concentrations of lead acetate i.e. 1008mg/kg bw, 1327mg/kg bw, 1747mg/kg bw, 2299mg/kg bw and 3025mg/kg bw. The lead acetate was given orally (gavage) for 24hrs. The results indicated that the maximum chromosomal aberration (62.20%) was shown in 3025mg/kg bw and the lowest in control (17.80%). The chromosomal aberration analyzed were stickiness, broken fragment, fragment chromosome, gap chromosome, acentric chromosome, triradials chromosome, ring chromosome, double point chromosome and numeric aberration. The frequency of chromosomal aberrations increased along the lead acetate concentration. Similar chromosomal aberration caused by lead acetate in bone marrow cells of mice were also observed by Fahmy (1999) and by lead nitrate in somatic and germ cells of rat (*Rattus rattus*) were reported by Verma *et al.*, (2004).

Ahmed *et al.*, (2012b) analyzed the toxic effect of prolonged lead exposure on chromosomal and testicular tissue of male rabbits. The lead acetate doses of 15, 20 and 30mg/kg b. wt were given orally for 12 days. The results revealed significant increase in the percentages of chromosomal aberrations in bone marrow of male rabbit. High incidence of chromosomal aberrations were observed after treatment with highest treated dose of 30mg/kg b. wt. concerning the different types of chromosomal aberrations, gaps were the most sensitive type of aberrations induced. Fragments, breaks, deletions and centric fusions were also recorded. Numerical chromosomal aberrations in form of polyploidy were observed with all the three doses. In another work done by Ahmed *et al.*, (2012a) on female rabbit, it became apparent that prolonged lead exposure causes DNA damage and tissue apoptosis in reproductive and vital organs in female rabbit. Lead acetate doses of 15 and 30mg/kg b.wt were orally given for 8 weeks to female rabbits and the results obtained after DNA extraction of the tissues and using DNA electrophoresis revealed that lead acetate induced DNA fragmentation, a hallmark of necrosis.
These observations clearly show that lead acetate is mutagenic, capable inducing chromosomal aberration. Chromosomal stickiness is due to improper folding of chromosome fiber into single chromatid and chromosomes (Ahmad and Yasmin, 1992). It was reported that lead had an effect on genetic system through chromosomal abnormalities (Fahmy, 1999; Ahmed et al., 2012a) and micronucleus (Celik et al., 2005a,b). The mechanisms for these genotoxic responses may involve direct damage to DNA affecting the stabilization of chromatin Johansson and Pellicciari, 1988) or interacting with repair processes (Hartwig et al., 1990). Lead is believed to covalently interact with tertiary phosphate ions in nucleic acids and proteins [Bremer, 1974; Holtzman et al., 1984]) and affect the fidelity of DNA synthesis in vitro (Sirover and Loeb, 1976).

Metal-induced genome damage includes DNA single-strand and double-strand breaks, DNA-DNA cross links, induction of reactive oxygen intermediates (Ariza et al., 1998), DNA-protein links, and base modifications. Indirectly, metals may inhibit DNA repair enzymes or DNA replication (Zelikoff et al., 1988), and consequently act as co-clastogens or co mutagens (Miadoková et al., 2000). There are several possible mechanisms how lead (II) might interfere with DNA repair process. Besides a direct interaction with repair enzymes, lead ions may also interfere with calcium regulated processes involved in the regulation of DNA replication and repair.

From the results of the present investigations, it is quite suggestive that lead has carcinogenic effects on the frog cells exposed in laboratory conditions. In additions to these findings, increase in soil and water pollution can lead to certain irreversible cytogenetic effects in aquatic animals and even in higher organisms.

MANGANESE TREATMENT

The chromosomal aberrations recorded in different tissues revealed a dose and time dependent increase in their frequencies which were significantly different from their respective controls at all exposure periods (Table 13 and 14; Fig. 31, 32, 33, 34 and 35). Most frequent aberrations recorded in intestine after treatment with manganese were stretching, pycnosis and clumping whereas in bone marrow, ring chromosome and stretching were the most frequent aberrations recorded. Stretching was observed to be frequent abnormality recorded in both the tissues however, maximum percentage of chromosomal aberrations was found in intestine followed by bone marrow. Maximum
decrease of MI was found in intestine and the minimum effect of manganese treatment on MI was revealed in bone marrow.

Manganese chloride produced an increased frequency of mutations in *Salmonella typhimurium* strain TA1537, but negative results in other strains; however manganese sulphate was reported to be positive and negative in separate studies in *Salmonella* strain TA97, but negative in other strains. Positive results were obtained with various manganese compounds in *Photobacterium fischeri* and *Escherichia coli*, as well as *Saccharomyces cerevisiae*, mouse lymphoma cells and hamster embryo cells (IPCS, 1999).

Lima *et al.*, (2008) evaluated the genotoxic and cytotoxic effects of manganese chloride in cultured human lymphocytes treated in different phases of cell cycle. Cultured human lymphocytes were treated with 15, 20 and 25μM manganese chloride during the G1, G1/S, S (pulses of 1 and 6hrs), and G2 phases of the cell cycle. All tested concentrations were cytotoxic and reduced significantly the mitotic index in G1, G1/S and S (1 and 6 hrs) treatments, while in G2 treatment only the higher concentrations (20 and 25μM) showed cytotoxic effects. Clastogenicity and DNA damage were found only in treatments with the highest concentration (25μM). Chromosome aberrations were found exclusively in the G2 phase of the cell cycle. The absence of polyploidy in mitosis, suggests that manganese does not affect the formation of the mitotic spindle with the concentrations tested. The genotoxicity found in G2 phase and in the comet assay can be related to the short time of treatment in both cases.

Hirata *et al.*, (1998) investigated the induction of apoptosis by manganese in PC12 cells. PC12 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 7% horse serum and 4% fetal bovine serum and were treated with manganese chloride (MnCl₂) in complete growth medium for various periods and soluble DNA was then extracted and analyzed. Internucleosomal DNA fragmentation characteristic for apoptosis was observed 16h after treatment with 1nM MnCl₂ and 24hrs after application of 0.1nM MnCl₂. The effects were dose dependent. Cell death was observed at 0.1 and 1nM but not at 0.01nM revealing that the concentrations that caused cell death also produced DNA fragmentation so that DNA fragmentation is closely correlated with subsequent cell death. Desole *et al.*, (1996, 1997) have also reported the induction of apoptosis by manganese in PC12 cells using the TUNEL technique and flow cytometry. They suggested involvement of an oxidative stress mechanism.
Samanta et al., (2005) documented that manganese and zinc were probably less harmful to the aquatic ecosystem which is in conformity with the least frequencies of chromosomal aberrations observed presently in the tissues of frog treated with manganese compared with other heavy metals tested.

**MERCURY TREATMENT**

Results of chromosomal aberration test showed that higher percentage of different chromosomal aberrations at 72 and 96hrs of exposure compared to 24 and 48hrs of exposure in intestine as well as bone marrow of exposed specimens. In case of intestine, the aberrations which were found to be predominant were ring chromosome, and pycnosis while terminal chromatid deletion and minutes were recorded to have the least percentage. Predominant aberrations recorded in bone marrow were ring chromosome, terminal chromatid deletion and chromosome fragmentation while stickiness and clumping were found to have minimum frequency. Total percentage of chromosome aberrations was found to be highest in bone marrow than in intestine. The result of mitotic index showed a general trend of decrease in both the treated tissues versus respective control values. Maximum decrease in the mitotic index was evident in case of bone marrow followed by intestine (Table 15 and 16; Fig. 36, 37, 38, 39 and 40).

Perry et al., (1988) investigated mutagenic effects of mercury chloride on the fish, *Fundulus heteroclitus* and also observed chromosomal aberrations and micronuclei formation in fish exposed to methyl chloride. In vivo exposure of mercury to fish, *Boleophthalmus dussumieri* induced significantly high frequency of chromosomal aberrations was reported by Krishnaja and Rege (1982). Directly by giving intramuscular injection of 0.59 and 1.19mg/kg and indirectly by dissolving 0.010, 0.056, 0.10 and 0.56 ppm of mercury in aquarium water and noticed increased frequency of chromosomal aberrations at all doses. Aberrations noticed include chromatid and isochromatid breaks, gaps, rings, fragments and exchanges. Manna and Mukherjee (1989) exposed the fish (males and females) to mercuric chloride, keeping the concentration constant for different durations ranging from 15min. to 96h. They arbitrarily classified the aberrations as individual types involving chromatid breaks, acentric fragments, translocation involving breaks; physiological types like constrictions, gaps; gross types involving polyploidy and aneuploidy and miscellaneous
ones like stickiness, pycnosis etc. They reported 8% increase in the total abnormalities after 96h exposure as compared to control. Yadav and Trivedi (2009) exposed the fish, *Channa punctatus* to sublethal concentrations (10% of 96h LC$_{50}$) of mercuric chloride (0.081mg/L) for 24, 48, 72, 96 and 168h. Chromosomal aberrations observed in the kidney cells of exposed fish were chromatid and chromosome breaks, chromatid and chromosome gaps along with ring chromosomes. A significant increase over negative control in the frequency of chromosomal aberrations was observed in fish treated with mercury which confirms the present work done on frog after treatment with mercury.

Bonacker et al., (2004) studied genotoxicity of inorganic mercury salts based on disturbed microtubule function, resulting from interactions with cytoskeletal proteins resulting into clastogenic and aneugenic effects of mercury leading to chromosomal genotoxicity which get supported with the view of Leonard (1988) that mechanisms affecting chromosomal distribution are also relevant for chromosomal genotoxicity of mercury compounds. Dose response relationships also play a major role in risk assessment and regulatory toxicology since they allow the identification of no-observed effect concentrations of chemical exposure (Bolt 2003).

Akiyama et al., (2001) investigated the genotoxic effects of Hg released from dental amalgams. Chromosomal aberration test was conducted using original extracts and their dilutions of conventional type amalgam and high copper amalgam. The concentrations of Hg, Cu and Ag in the original extract of high copper amalgam were 17.64, 7.97 and 43.90 microM, respectively. Those in the original extract of conventional type amalgam were 20.63, 7.87 and 14.79 microM, respectively. 10 and 30 microM Hg (2+) were also used for comparison. Result revealed that the conventional type amalgam induced chromosome aberrations with quadruple dilution where cell viability was about 80% and that the high copper amalgam induced a high level of chromosome aberrations with the two-thirds dilution. Silva-Pereira et al., (2005) determined the changes induced *In vitro* by two mercury compounds (HgCl$_2$ and CH$_3$HgCl) in cultured human lymphocytes. Cultures were separately and simultaneously treated with low doses (0.1 to1000 μg/l) of HgCl$_2$ and CH$_3$HgCl and incubated at 37°C for 48 h. Genotoxicity was assessed by chromosome aberrations and polyploid cells. Mitotic index was used as a measure of cytotoxicity. A significant increase (P < 0.05) in the relative frequency of chromosome aberrations was observed for all concentrations of CH$_3$HgCl when compared to control, whether alone or in an
evident sinergic combination with HgCl₂. The frequency of polyploid cells was also significantly increased (P < 0.05) when compared to control after exposure to all concentrations of CH₃HgCl alone or in combination with HgCl₂. CH₃HgCl significantly decreased (P < 0.05) the mitotic index at 100 and 1000 μg/l alone, and at 1, 10, 100, and 1000 μg/l when combined with HgCl₂, showing a synergistic cytotoxic effect.

Ehrenstein et al., (2002) evaluated methyl mercury uptake and associations with the induction of chromosomal aberrations in Chinese hamster ovary (CHO) cells and observed that at a concentration of methyl mercury in the culture medium of 1.0×10⁻⁶M where the frequencies of chromosomal aberration and sister chromatid exchanges are significantly elevated, the intracellular concentration was 1.99×10⁻¹⁶ mol/cell. However, Kanamadi and Saidapur, (1992) investigated the effects of exposure to sublethal mercuric chloride on testis (germ cells) and fat body of frog, E. cyanophlyctis.

Therefore, as far as the genotoxicity of mercury compounds is concerned, Leonard (1988) primarily addressed effects on chromosome distribution, e.g. aneugenicity. Later, De Flora et al., (1994) reviewed clastogenic effects of mercury compounds, and attributed these to the generation and activity of reactive oxygen. This view is seconded by others (Schurz et al., 2000; Rao et al., 2001). In cellular in vitro systems, mercury (II) compounds cause glutathione depletion that facilitates toxic effects of redox cyclings (De Flora et al., 1994), whereas ascorbic acid prevents genotoxicity of mercury (II) chloride (Rao et al., 2001). This points to a relevance of reactive oxygen mechanisms which are being viewed as connected to clastogenicity.

In conclusion, mercury has harmful effects on different cells of the frog, E. cyanophlyctis studied even when exposed to sublethal concentrations of mercury. Thus, the cytotoxic effect may be considered an earlier indication of cellular damage with possible biological consequences and should be taken into account in the preliminary evaluation of the risks to populations exposed in vivo, as already suggested by others.

**NICKEL TREATMENT**

The results of chromosomal aberration test clearly evidenced the higher percentage of different chromosomal aberrations at 72 and 96hrs of exposure compared to 24 and 48hrs of exposure in both the tissues of frog specimens exposed to different doses of nickel. Predominant aberrations recorded in intestine were ring chromosomes...
and pycnosis followed by chromosome fragmentations and terminal chromatid deletion while in bone marrow the percentage of chromosome fragmentations was found to be maximum. In case of intestine, the least observed frequency was found to be for stretching whereas clumping followed by pycnosis was the least recorded aberrations in case of bone marrow. Total percentage of chromosome aberrations was found to be highest in bone marrow followed by intestine. After treatment with various sublethal concentrations of nickel, analysis of MI revealed mitodepressive effect of the heavy metal studied except for 24hrs of exposure in bone marrow where the results obtained were not statistically significant from the respective controls. However the overall effect was predominant in intestine compared to bone marrow (Table 17 and 18; Fig. 41, 42, 43, 44 and 45).

Reinecke and Reincke (2004) investigated the heavy metal genotoxicity in earthworm (*Eisenia fetida*). The primary cell culture of earthworm coelomocytes were exposed in vitro to nickel chloride and the DNA damage caused was determined by the comet assay (single gel electrophoresis). Results indicated DNA single strand breaks in soil invertebrate cells caused by exposure to a nickel compound which is in agreement with the present findings of chromosomal aberrations in the frog.

Howard *et al.*, (1991) reported that in Chinese hamster ovary cells, nickel chloride increased the frequency of chromosomal aberrations and sister chromatid exchanges. Cells with aberrations increased from 8% at about 6 μg Ni/L to 21% at about 6 mg Ni/L in a dose-dependent manner. Seoane and Dulout, (1999) also analyzed the aneugenic and clastogenic effects of nickel chloride on Chinese hamster ovary cells using anaphase-telophase test at concentrations of 0.013μM, 0.027μM, 0.040μM and 0.054μM. frequency of cells with different chromosomal aberrations were scored. The aberrations observed were chromatin bridges, lagging chromosomes and lagging chromosomal fragments, but the frequency of lagging chromosomes increased with nickel treatment. However, significant differences between the cells treated with the second (0.027μM) and fourth doses (0.054μM). Mitotic index was also determined and was expressed as a percentage of number of mitotic plates. No effect on mitotic index was observed, except at higher dose; regression analysis was not significant (r=0.50, p=0.181). Similarly, the present findings regarding the mitotic index effect of this heavy metal on frog after treatment with various sublethal concentrations of nickel
revealed that nickel did not cause significant mitodepressive effect on the tissues studied.

Yadav et al., (2001) reported the chromosomal damage in lymphocytes of stainless steel welders exposed to welding fumes generated by manual metal arc (MMA), metal inert gas (MIG) and oxyacetylene welding. The chromosomal damage was the induction of chromatid (gaps, breaks and isochromatid exchanges) and chromosome type (dicentrics, rings, acentric fragments, translocations, chromosome gaps, chromosome breaks and diplochromosomes) aberrations. It was concluded that the welding fumes, containing chromium (VI) and nickel, are genotoxic as well as carcinogenic and lead to the chromosomal damage. It was also reported by Sobti et al., (2006) that nickel chloride, nickel sulphate and nickel nitrate are toxic to bone of mice at higher doses.

Sunderman (1993) investigated the possible molecular mechanisms in the genotoxicity of nickel using South African frogs (Xenopus laevis) as an experimental model for the teratogenic effects of bivalent nickel ions (Ni$^{2+}$). A Ni$^{2+}$ binding protein, pNiXa, was identified in Xenopus oocytes and embryos. The possibility that pNiXa plays a key role in Ni$^{2+}$ teratogenesis is indicated by (i) the avidity of pNiXa for Ni$^{2+}$ (ii) the presence of pNiXa when the embryos are susceptible to Ni$^{2+}$ teratogenesis, and (iii) the potential to form Ni$^{2+}$ complexes that could catalyze the formation of oxygen free radicals and thereby damage deoxyribonucleic acid (DNA) and chromosomes.

Toxic and carcinogenic effects of nickel compounds are associated with nickel-mediated oxidative damage to DNA and proteins and to inhibition of cellular antioxidant defenses thereby indicating the genotoxic potential of this heavy metal. Some nickel compounds are weakly mutagenic in a variety of test systems, but much of the evidence is inconclusive or negative.

**ZINC TREATMENT**

In the present study, toxicity of zinc in the treated frog was clearly evidenced from the results of the chromosomal aberrations recorded in different tissues which were significantly different from the respective controls (Table 19 and 20; Fig. 46, 47, 48, 49 and 50). The results of CAT revealed higher percentage of vary chromosomal aberrations at 96hrs of exposure compared to 24, 48 and 96hrs of exposure in both the tissues of exposed specimens. In case of intestine, aberrations which were found to be
predominant were pycnosis followed by ring chromosomes and terminal chromatid deletion while terminal association of chromosome had minimum incidence but in case of bone marrow, the percentage of terminal association of chromosomes followed by ring chromosomes was found to be maximum and the frequency of stickiness was recorded in least percentage. Since maximum frequency of total chromosomal aberrations was induced only after treatment 24mg/kg of zinc at 96hrs of exposure period, so it could be regarded as the effective sublethal dose and period of exposure of zinc for induction of chromosome type aberrations in intestine and bone marrow. Total percentage of chromosome aberrations was found to be highest in intestine in comparison to bone marrow. Analysis of MI treatment with various sublethal concentrations of zinc revealed that zinc did not cause significant mitodepressive effect on the tissues studied. This is because the effect of zinc in treated series was not significantly different from the respective controls in all the exposure groups in both intestine and bone marrow.

Single stranded DNA break induced by zinc sulfate in mice has been studied In vivo using Alkaline Single Cell Gel Electrophoresis (Comet assay) by Banu et al., (2001). Mice were administered orally with doses of 5.70, 8.55, 11.40, 14.25, 17.10 and 19.95mg/kg body weight of zinc sulfate respectively. Results indicated a significant DNA damage at all the doses after treatment with zinc sulfate when compared to controls showing a clear dose-dependent response (p<0.05). A gradual decrease in the tail-lengths from 48 hr post-treatment onwards was observed indicating a time dependent decrease in the DNA damage. The study confirms that zinc sulfate causes significant DNA damage at the doses used as revealed by comet assay.

Genotoxicity and cytotoxicity of zinc oxide and titanium dioxide in Human Epithelial Cells (HEp-2 cells) was determined using the Comet and the cytokinesis-blocked micronucleus assays and found concentration- and time-dependent cytotoxicity and an increase in DNA and cytogenetic damage by Osman et al.,(2010). Sharma et al., (2011) also revealed zinc oxide nanoparticles induce oxidative stress, DNA damage and cytotoxicity in Human Liver Cells (HepG2).

Kumari et al., (2011) reported cytogenetic and genotoxic effects of zinc oxide nanoparticles on root cells of Allium cepa. The effects of ZnO nanoparticles (NPs) on the mitotic index (MI), micronuclei index (MN index), chromosomal aberration index, and lipid peroxidation were determined through the hydroponic culturing of A. cepa. A.
*cepa* roots were treated with the dispersions of ZnO NPs at four different concentrations (25, 50, 75, and 100µg/ml). With the increasing concentrations of ZnO NPs, MI decreased with the increase of pycnotic cells, on the other hand MN and chromosomal aberration index increased. Thus the results demonstrated that ZnO NPs can be a clastogenic/genotoxic and cytotoxic agent.

Zinc is a ubiquitous and essential element. Zinc toxicity to aquatic organisms is dependent on the physical and chemical forms of zinc, the toxicity of each form, and the degree of interconversion among the various forms. Aquatic plants and fish are relatively unaffected by suspended zinc, but many aquatic invertebrates and some fish may be adversely affected from ingesting enough zinc-containing particulates (U.S. EPA 1987). However, zinc is an effective mutagen and clastogen when presented to a susceptible cell population in an appropriate form (Thompson et al. 1989). Zinc acetate produced dose-related positive responses in the mouse lymphoma assay and also in a cytogenetic assay with Chinese hamster ovary cells; however, results of mutagenicity assays with inorganic zinc were negative in the *Salmonella* mutation assay and in unscheduled DNA synthesis on primary cultures of rat hepatocytes (Thompson et al., 1989). Structural chromosome aberrations, particularly chromatid gaps and increased frequency of fragment exchange, were observed in rat bone marrow cells after 14 days of exposure to 240mg Zn/L drinking water (Kowalska-Wochna et al., 1988). Chromosomal aberrations were observed in bone marrow cells of mice fed diets equivalent to 650mg Zn/kg BW daily in mice exposed to zinc oxide by inhalation, and in mice maintained on a low calcium diet (U.S. PHS 1989). Aberrations in bone marrow of mice given 5,000mg Zn/kg diet may be associated with calcium deficiency (Leonard and Gerber, 1989). Calcium is displaced by zinc in calcium-depleted conditions, leading to chromosomal breaks and interference in the repair process (U.S. PHS 1989).

Zinc chloride induces chromosomal aberrations in human lymphocytes *in vitro* (Elinder, 1986). A higher incidence of chromosome anomalies in leukocytes occurs among workers exposed to zinc (Elinder, 1986), but these aberrations are probably due to other (unspecified) mutagenic factors in the work environment (Leonard and Gerber, 1989). Excess zinc is teratogenic to frog and fish embryos, possibly by inhibition of DNA synthesis (Dawson *et al.*, 1988; Fort *et al.*, 1989).
Zinc inhibits the mutagenic action of some carcinogens because it is a constituent of mutagen detoxifying enzymes or because it acts directly on the microsomal monooxygenases forming the ultimate carcinogen (Leonard and Gerber, 1989). Zinc significantly reduced a genotoxic effect of lead in rat bone marrow cells (500mg Pb/L drinking water followed by 240 mg Zn/L for 2 weeks) and also protected against lead accumulations in erythrocytes and lead-induced inhibition of delta-amino levulinic acid dehydratase (Kowalska-Wocha et al., 1988). The beneficial role of zinc against cadmium induced testicular damage has also been reported (Batra et al., 1998; Claveria et al., 2000; Rostan et al., 2002; Xu et al., 2005). Samanta et al., (2005) documented that manganese and zinc were probably less harmful to the aquatic ecosystem which is in conformity with the least frequencies of chromosomal aberrations observed presently in the tissues of frog treated with zinc compared with other heavy metals tested. Rao and Madhyastha (1987) also conducted bioassays to determine the relative acute toxicities of five heavy metals (mercury, cadmium, copper, manganese and zinc) to 1-week-old and 4-week-old tadpoles of the frog, Microhyla ornata. Toxic effects were calculated on the basis of LC$_{50}$ for 24 h, 48 h, 72 h and 96 h exposures at 25.5–26.0°C. Mercury was the most toxic and zinc was the least toxic of the heavy metals tested.

Based on the results obtained for CAT after the exposure of frog (*E. Cyanophlyctis*) to different sublethal concentrations of different heavy metals, all the heavy metals tested had proved to be potentially genotoxic except nickel. The toxic potential of heavy metal toxicant was evident as Pb >Hg>Cu>Cd>Cr> Ni >Mn> Zn.

### 5.5.2 Micronucleus test (MNT)

The present results of the micronucleus test obtained after treatment of the frog with sublethal concentrations of various heavy metals revealed significantly higher frequencies of micronuclei than that of the controls, evidencing the genotoxic effect of heavy metals. The control fish specimens also showed some incidences of micronuclei but the frequency was negligible compared to the treated specimens. The result of micronucleus test obtained after treatment with individual heavy metal salts are discussed in detail as under:
CADMIUM TREATMENT

During the present investigation, results of micronucleus test showed that treatment with sublethal concentrations of cadmium clearly increased the micronuclei frequencies in intestine, kidney and RBCs which were dose and time dependent, compared to respective controls. This clearly revealed that cadmium had genotoxic effect in frog, *E. cyanophlyctis* and induced the formation of micronuclei in its different tissues. A comparison between the micronuclei frequencies in the tissues studied illustrated that highest frequency of MN was recorded in intestine and RBCs which had almost same frequencies of MN at 96hrs of exposure after treatment with the highest dose of cadmium and least was observed in kidney (Table 21; Fig. 51, 52, 53 and 54).

Haematological changes in *Bufo maculatus* treated with sublethal concentrations (0.25, 0.50, 1.00 and 2.00mg/l) of cadmium chloride was investigated by Ezemonye and Enuneku (2011b). The hematologic alterations based on the examination of blood indices during the 28 days of exposure showed that total erythrocyte count (TEC), hematocrit (Hct) and hemoglobin (Hb) concentration decreased (P<0.05) relative to controls. The decline was concentration-dependent as concentration of cadmium increased. The decline in hemoglobin and hematocrit in the experimental organism could be due to a decrease in the synthesis or release of erythrocytes into the circulation or an increase in the rate of erythrocyte destruction inflicted by cadmium toxicity. There was significant (P<0.05) elevation in total leukocyte count (TLC) with increase in the concentration of cadmium.

Mouchet *et al.*, (2006) analyzed several genotoxic and detoxification mechanism in *Xenopus laevis* larvae including the clastogenic and/or aneugenic effects in the circulating blood by micronucleus (MN) induction after 12 days of exposure to environmentally relevant contamination levels, close to those measured in the river Lot (France) and observed micronucleus induction at environmental levels of Cd contamination (2, 10, 30μg/L).

Zhu *et al.*, (2004) reported that micronuclei frequencies increased with the rise in cadmium concentrations during exposure periods after the carp was treated with different concentrations of cadmium (as CdCl$_2$; doses of 0.001, 0.01 and 0.1 mg/L) for 2, 4 ,6, 9, 12, 17, 22, 32 and 42 day of exposure periods which revealed a positive
relationship between metal concentrations and micronuclei frequencies confirming the *in vivo* induction of micronuclei by cadmium exposure in present investigation. Similarly, Jiraungkoorskul *et al.*, (2007) revealed the effects of cadmium and ascorbic acid on the red tailed barb (*Puntius altus*) using the micronucleus test and nuclear abnormality tests for the period of 24, 48, 72 and 96hrs. The micronuclei frequencies in erythrocytes, gill, liver and fin cells were analyzed comparatively to evaluate the sensitively and suitability of these different cells. In erythrocytes, the nuclear abnormalities (NA) were scored as blebbed nuclei (BL), lobbed nuclei (LB), notched nuclei (NT) and binuclei (BN). The highest value of both micronuclei and NA cells were significantly increased in the cadmium treated group followed by the combination of cadmium and ascorbic acid treated group. Micronuclei and NA frequencies in erythrocytes were most sensitive to the treatment and provide valuable information than those in gill, liver and fin cells and also highest number of micronuclei and NA were observed after 48hrs of treatment in all cases which decreased with longer time of exposure. In present study, micronuclei frequencies increased with the increase in dose and duration of exposure.

Rosa *et al.*, (2003) investigated that cadmium induced DNA damage via generation of reactive oxygen species in *Vicia faba* upholding the view that micronuclei induction may be interpreted as a consequence of oxidative stress. In another study by Unyayar *et al.*, (2006) in *Vicia faba* root cells, after treatment with different concentrations of cadmium found that cadmium might delay mitosis by damaging the transport mechanism due to the attack of free radicals on fatty acid component of membrane lipids. Reactive oxygen species overproduction coupled with deficiency of antioxidant defence mechanisms may be an important factor contributing to the increase in micronuclei. Mayer *et al.*, (2000) also demonstrated a positive correlation between lipid peroxidation status and genotoxicity as reflected by increased micronuclei formation in lymphocytes. However, Ezemonye and Enuneku, (2005) also evaluated acute toxicity of cadmium and lead to amphibian tadpoles of toad (*Bufo maculatus*) and frog (*Ptychadena bibroni*) and differential toxicity of Cd and Pb was observed in both the species with Cd showing much toxicity than Pb. In another study performed by Stepanyyan *et al.*, (2011), it was indicated that in experimental environment containing molybdenum, chrome and cadmium, the morphology of erythrocytes of the marsh frog (*Pelophylax ridibundus*) changed with respect to
control. In experimental environment containing molybdenum and chrome, the morphology of young frog erythrocytes slightly differed from controls but the effects of long-time exposure to cadmium on morphology of erythrocytes were substantially different than those of chrome and molybdenum.

**CHROMIUM TREATMENT**

In the present study, the incidence of bodies noted as micronuclei in the cytoplasm of the intestine, RBCs and kidney of the frog after chromium treatment showed a clear dose related increase in all the treatment periods after treatment with four sublethal concentrations of chromium. Result of micronucleus test in different tissues of frog treated with various sublethal concentrations of chromium revealed prominent effect of test chemical in RBCs followed by intestine and least in kidney (Table 22; Fig. 55, 56, 57 and 58).

Al-Sabti *et al*., (1994) evaluated the genotoxic effects of Cr (VI) in Prussian carp (*Carassius auratus gibelio*) and recorded a significant increase in frequencies in micronuclei after it was exposed to 10, 50 and 100mg/L of this metal. De lemos *et al*., (2001) also observed significantly increased frequencies of micronuclei in peripheral erythrocytes of the fat minnow, *Pimephales promelas* exposed to sublethal concentrations (2.5mg/L) of chromium taken as potassium dichromate for 7, 14 and 21 days of exposure periods and recorded that micronuclei induction was found to decrease after 21 days of exposure and observed a statistically significant difference between all the exposure periods except between 7 and 21 days and between 14 and 21 days of exposure periods. Cavas and Ergene-Gozukara (2005) investigated the micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents. Results revealed that both effluents had genotoxic potential.

Talapatra and Banerjee (2007) also reported highly significant frequencies of micronuclei in gills and kidney erythrocytes of fish, *Labeo bata* grown in sewage fed fish farms that contain chromium. Mersch *et al*., (1996) also reported induction of micronuclei in haemocytes and gill cells of zebra mussels, *Dreissena polymorpha*, exposed to potassium chromate under laboratory conditions and found that micronuclei induction capacity of potassium chromate in the tissues studied. Positive relationship between the metal concentration and micronuclei frequencies during present
investigations is similar to that observed by Zhu et al., (2004) in the carp after it was exposed to different concentrations (0.001, 0.01 and 0.1mg/L) of chromium as potassium dichromate for 2, 4, 6, 9, 12, 17, 22, 32 and 42 day of exposure periods and found micronuclei frequencies increased with exposure. However, the micronuclei frequencies increase to a smooth level after a peak value with the rise of chromium concentrations. The frequencies of micronuclei induced were observed to be significantly higher in treated groups than those of control group confirming the results of present investigations.

Norman et al., (2008) also investigated the clastogenic effects of potassium dichromate in armored catfish (*Hypostomus plecotorum*) after it was subjected to 12mg/L of the test chemical for 15 days, yielding the formation of micronuclei in red blood cells of the fish. Da Rocha et al., (2011) analyzed the frequencies of micronuclei and other nuclear abnormalities in peripheral blood of Nile tilapia (*Oreochromis niloticus*) treated with potassium dichromate (12mg/L) for 24 and 48hrs and found a significant increase in micronuclei frequencies between control and exposed groups confirming the potential adverse effects of the chemical on the animal. Genotoxic potential of chromium can also be related with the studies of other workers. Dillon and his collaborators (1998) detected, in V79 cells, micronuclei and other alterations resulting from genotoxic action of chromium complexes. Kohlpoth et al., (1999) found significant formation of micronuclei in RTG-2 cells exposed to industrial effluents containing between 20 and 100 μM of potassium dichromate. Vaglenov and collaborators (1999) also found, in measurements made in metallurgic workers, high red blood cell concentrations of chrome, besides a significant increase in the number of red blood cells having micronuclei. Messer et al., (1999) also associate nuclear morphological alterations, reflecting genomic alterations, in gingival fibroblasts subjected to chrome.

**COPPER TREATMENT**

The data from the present observations revealed a time dependent increase in micronucleus frequencies with the increase in doses of copper in intestine, RBCs and kidney. With respect to the dose, maximum effect was found to be induced at the highest dose of exposure i.e. 8.0mg/kg while prominent effect with respect to the duration of exposure was induced after the maximum period of exposure i.e. 96hrs.
Comparison between the micronucleus frequencies induced in the various tissues revealed highest MN frequencies in RBCs, followed by a narrow margin in intestine and least in kidney (Table 23; Fig. 59, 60, 61 and 62).

The present findings are in agreement with the earlier study carried out by Bhunya and Pati (1987), who reported a significant dose related increase in the incidence of micronuclei in bone marrow cells of mice after two injections of different doses (5, 10 and 20mg/kg b.w.) of the chemical. For MN assay each dose was injected (i.p.) twice at an interval of 24 hours and the animals were sacrificed 6 hours after the 2nd injection. The results were dose, time dependent and an increase in the incidence of micronuclei in bone marrow cells was observed although some cases of nuclear lysis were also recorded. Highest percentage of micronuclei was recorded in PCEs (polychromatic erythrocytes). The micronuclei were either dot or ring shaped and size varied from 1/7 to 1/12 of the cell size. All the treated results differed significantly from the controls.

Bagdonas and Vosyliene (2006) revealed increased frequencies of micronuclei in fish treated with sublethal concentrations (0.16, 0.08 and 0.04mg/L) of copper sulphate for 96hrs of exposure and observed significant increase in micronuclei frequency in fish exposed to highest concentrations of copper. Villella et al., (2006) also reported the genotoxicity of coppersulphate in golden mussel, *Limnoperna fortune*. Talapatra and Banerjee (2007) observed highly significant (p<0.001) frequencies of micronuclei in gill and kidney erythrocytes of fish, *Labeo bata* grown in sewage fed fish farms having metal like copper.

Da-Rocha et al., (2012) performed micronucleus test using copper sulphate on tadpoles of the amphibian *Lithobates catesbeianus*, and reported the induction of micronuclei and other nuclear abnormalities in the positive control and in the two experimental groups exposed to 0.2 and 0.4mg/L of coppersulphate revealing the potential adverse effects of copper on amphibian erythrocytes in aquatic and agricultural ecosystems.

**LEAD TREATMENT**

Present experimental results of treatment with various sublethal concentrations of lead have shown that among different tissues used for micronucleus test, frequencies
of micronuclei were predominant in RBCs followed by intestine and least in the kidney cells revealing the prominent genotoxic effect of lead in the RBCs (Table 24; Fig. 63, 64, 65 and 66).

The micronuclei formation in present results are similar to those found by Kasuba et al., (2004) who determined the toxicity of lead as lead acetate in six-day-old suckling Winter rats. They evaluated the genotoxic effect of lead acetate in the early period of life, by exposing six-day-old suckling Wistar rats to lead (as acetate) either orally for 9 days (daily dose 2 mg Pb/kg b. wt., 18mg/kg b.wt. total dose) or by a single intraperitoneal injection (5 mg Pb/kg b. wt.). DNA damage was investigated using the comet assay and in vivo micronucleus test. The results of the comet assay showed statistically significant differences between the control (unexposed) animals and the two groups of exposed animals by ANOVA weighted for unequal variance (heterogeneity of variances was found by Levene’s test), followed by Tukey’s post hoc test at the level of significance of \( p < 0.05 \). The two groups of lead-exposed animals were also significantly different from each other. Orally lead-exposed animals showed a significant increase of micronuclei frequencies in reticulocytes and erythrocytes compared to unexposed animals (ANOVA, \( p < 0.05 \)).

Celik et al., (2005b) evaluated the frequency of micronuclei in the peripheral blood of female rats treated with three different cumulative doses (140, 250 and 500mg/kg b.w) of lead acetate by gavage once per week for ten weeks. Mitomycin C (MMC) 2mg/kg b.w. was used as a positive control. The cytotoxic and genotoxic effects of lead acetate on peripheral blood reticulocytes was investigated using the micronucleus test following chronic exposure and results revealed a decrease in the number of polychromatic erythrocytes in the peripheral blood and an increase in the frequency of micronucleated reticulocytes.

Vaglenov et al., (1997) found a significant increase in MN frequency among the lead-exposed staff (millers and assemblers) of a battery plant and again in 2001, Vaglenov et al. observed a significant increase in the frequencies of binucleated cells with micronuclei in occupational exposure to lead and found that lead exposure at levels higher than 1.20 µ may pose an increase in genetic risk. Governa et al., (1987) found an impairment of chemotactic and phagocytic activities in human lead-exposed PMN \textit{in vitro} and have explained their results as a consequence of the effect of the
metal on the cytoskeleton rather than on the cell membrane structure. Chen et al., (2006) evaluated the genotoxic effects of workers exposed to lead using micronucleus assay, comet assay and TCR gene mutation test. The results of MN test showed that the mean micronuclei rate (MNR) and mean micronucleated cells rate (MCR) in workers were significantly higher than those in controls.

Kryukov (2000) analyzed the effect of lead as lead nitrate on the induction of micronuclei in anuran larvae. Groups of seven tadpoles were placed for 6, 12, 18, and 24 h in lead nitrate solutions with the final lead concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/l. The microscopic analysis of the slides prepared from blood smears revealed cells with all types of micronuclei and the frequencies of cells with micronuclei increased proportionally to the lead concentration and duration of its action on the animals which is in conformity with present findings wherein maximum induction of micronuclei was recorded after 96hrs of exposure with the highest sublethal dose of lead.

**MANGANESE TREATMENT**

Present data from different tissues that micronucleus frequencies did increase in different tissues of the frog after treatment with various sublethal concentrations of manganese but revealed poor effect of the test chemical. In general, the results showed a significant increase in MN frequency with dose and duration of exposure except for kidney with 24hrs of exposure period. However, not much increase in frequencies of micronuclei was recorded at 24, 48 and 72hrs of exposure in all the tissues studied. Frequencies of micronuclei were found to be elevated only after 96hrs of exposure. A comparison between micronucleus frequencies in all tissues revealed highest MN frequencies in RBCs, followed by intestinal tissue and least in kidney (Table 25; Fig. 67, 68, 69 and 70).

Samanta et al., (2005) documented that manganese and zinc were probably less harmful to the aquatic ecosystems as in the present work less frequencies of micronuclei were recorded in various tissues of frog treated with manganese. From the literature in hand, no record of evidence of micronuclei induction in adult frogs after manganese treatment was found. However its toxicity has been reported by various workers. Hirata et al., (1998) reported activation of JNK pathway and induction of apoptosis by manganese in PC12 cells. Imam and Chandra (1975) reported
histochemical alterations in rabbit testis produced by manganese chloride and Elbetieha (2001) investigated the effects of long-term exposure to manganese chloride on fertility of male and female mice.

**MERCURY TREATMENT**

A dose and a time dependent increase in the micronuclei frequency were observed in the various tissue of the frog studied after treatment with various sublethal concentrations of mercury. In general, the data revealed that with respect to the dose, maximum effect was found to be induced at the highest dose of exposure i.e. 12.0mg/kg while prominent effect with respect to the duration of exposure was induced after maximum period of exposure i.e. 96hrs. Maximum frequency of MN was recorded in RBC, followed by intestine and least in kidney (Table 26; Fig.71, 72, 73 and 74).

The present results obtained after mercury treatment are in agreement with the earlier studies of various workers. Nepomuceno et al., (1997) have observed an increase in the frequencies of micronuclei induction when the fish, *Cyprinus carpio* were exposed to metallic mercury at concentrations of 2, 20 and 200mg/L of water for 31, 90 and 159 days of exposure periods. They observed that the effects were higher after 31 days of exposure periods followed by a slight stabilization and gradual decrease. Ayllon and Garcia-Vazquez (2000) also observed a significant increase in the frequency of micronuclei in fish, *Poecilia latipinna* intraperitoneally injected at 0.17 and 1.7mg/kg mercury nitrate. Das and Nanda (1986) observed similar observations in *Heteropneustus fossilis*, exposed to mitomycin-C (MMC) and paper mill effluents (PME). They reported an increased percentage of micronuclei with increased dose and durations.

Porto et al., (2005) studied the mutagenic effects of mercury pollution as revealed by micronucleus test to three Amazonian fish species. Distinct mean frequencies of micronuclei (MN) were observed in three trophically distinct characin fish species collected in two riverine environments in the Amazon Basin: the Madeira (polluted area) and the Solimoes (unpolluted area) rivers. Mean frequencies of MN observed in *Prochilodus nigricans* (detritivore), *Mylossoma duriventris* (omnivore), and *Hoplias malabaricus* (piscivore) from the Madeira River were significantly higher compared to the frequencies from the same species from the Solimoes River. In
addition, mean frequencies of MN from piscivore species were almost fivefold higher than the detritivore and/or omnivore species.

Rozgaj et al., (2005) studied the genotoxicity of mercuric chloride in rats following oral exposure. Female rats, aged 14 weeks were exposed to mercury chloride in oral doses of 0.068, 0.136 and 0.272 mg/kg body weight for five consecutive days and noticed the micronuclei frequency were significantly higher in the treated rats than in control. The work of Nagarani et al., (2009) also determined significant increase of micronuclei frequency in erythrocytes of fish, *Therapon jarbua* exposed to mercuric chloride in aquarium under controlled conditions. Concentrations of 0.25 ppm induced induced highest micronuclei frequency of 2.95 micronucleated cells per 1000 cells compared to 1 micronucleated cell per 1000 cells in control animals.

Inspite of showing some variations, the present work regarding the genotoxicity of lead can be correlated with the work of various workers. The formation of micronuclei was similar in all cases through their mechanism may be different.

**NICKEL TREATMENT**

Data obtained in different tissues after exposure to different doses of nickel showed that micronuclei frequencies did increase but it was not a prominent effect in intestine and kidney with respect to the doses and minimum durations of exposure. Maximum frequencies of micronuclei were induced at the highest period and dose of exposure. i.e at 96 hrs after treatment with 20 mg/kg of the test chemical. Highest frequency of MN was recorded in RBCs followed by intestine and least in kidney. (Table 27; Fig. 75, 76, 77 and 78).

Present results are not in conformity with the findings of Oller and Erexson (2007), who found negative results in terms of formation of micronucleated polychromatic erythrocytes in rat bone marrow exposed to nickel sulphate hexahydrate at any dose examined. Doses used in micronucleus assay were 125, 250 and 500 mg/kg/day. The maximum tolerated dose was estimated to be 500 mg/kg/day. The present results indicated the increase in frequencies of micronuclei after dose and duration of exposure, however results obtained were not significant at 24 and 48 hrs of exposure in kidney and in intestine results were not significant at 24 hrs of exposure. Similar observations were given by Das and Nanda (1986) in *Heteropneustes fossilis,*
exposed to mitomycin-C (MMC) and paper mill effluents (PME). They observed an increase in percentage of micronuclei in doses and duration of exposure.

**ZINC TREATMENT**

In the present study of the effect of zinc treatment on various tissues of frog using micronucleus test showed that micronucleus frequency increased with an increase in dose and duration of exposure in all the tissues but it was not a prominent effect in intestine and kidney with respect to doses and duration of exposure compared to respective controls. Also, at a dose concentration of 6.0mg/kg where minimum value was recorded after 72hrs of exposure in RBCs and a decrease in trend of MN frequency was observed in intestine at the same dose and duration of exposure. Highest frequency of MN was recorded in intestine and RBCs which had same frequencies of MN at 96hrs of exposure after treatment with the highest dose of zinc (Table 28; Fig.79, 80, 81 and 82).

Bagdonas and Vosyliene (2006) reported significant increase in the count of micronuclei in *Oncorhynchus mykiss* only at lowest sublethal concentration of zinc (0.238mg/L) and in all concentrations of mixture of copper and zinc. Present finding about the non genotoxic effects of zinc treatment get confirmed from the earlier reports demonstrating the antioxidant function of zinc as revealed by Rostan *et al.*, (2002) as an antioxidant trace element that is present in all organs, tissues and fluids of the body and is required for cell proliferation, differentiation, normal growth, immune functions and wound healing.

Kim *et al.*, (2000) analyzed the effect of zinc on the visual sensitivity of the Bullfrog’s eye and reported that zinc treatment elevated the dark-adapted electroretinogram (ERG) threshold, all of the peak amplitudes of ERG were increased and rhodopsin regeneration was accelerated during visual adaptation meaning thereby that zinc, which is abundant in the retina and the retinal pigment epithelium particularly, is essential factor for the visual process. Afonne *et al.*, (2002) revealed that zinc protects chromium induced testicular injury in mice. It is an essential element for spermatogenesis and a hepatocellular metallothionein inducer and zinc co-treatment protects tissues against free radicals and oxidative stress.
Samanta et al., (2005) documented that manganese and zinc were probably less harmful to the aquatic ecosystems as in the present work less frequencies of micronuclei were recorded in various tissues of frog treated with zinc. The non genotoxic nature of zinc against genotoxicity of hexavalent chromium was also confirmed by Tripathi and Dubey (2008) in Channa punctatus using micronucleus test. Zinc as zinc chloride had no effect in causing the formation of micronuclei compared to the respective control. On the other hand, chromium (VI) as potassium dichromate significantly induced the micronuclei compared to control. Concomitant exposure to both the metals significantly decreased the frequency of micronuclei compared to chromium.

Base on the micronucleus test, toxic potential of aforesaid heavy metal toxicants was recorded as Cu > Hg >Ch > Cd > Pb > Mn >Ni > Zn and based on the chromosomal aberration test, toxic potential of aforesaid metal toxicants was recorded as Pb >Hg>Cu>Cd>Cr> Ni >Mn> Zn. All the metals found to be potentially genotoxic except nickel and Zinc as was evident from the extent of frequency of micronuclei in frog, Euphlyctis cyanophylaxis treated with sublethal concentrations of metallic compounds. Zn was found to be least toxic in both the cases.

A study of Vinod and Naik (2000) with respect to the effect of nickel chloride, cadmium chloride and mercuric chloride on Haplobatractus tigerinus revealed that mercuric chloride was most toxic followed by cadmium chloride and nickel chloride. Nickel was found to less toxic as compared to other two heavy metals which justifies the present investigations. Bati et al., 1999 also revealed that copper is the most toxic followed by lead and zinc which confirms the order of their genotoxicity in the present study in terms of the micronucleus test. Chu and Chow, 2002 also conducted a study and analyzed that Hg, Pb and Cu have synergistic toxic effects compared to Cd, Co, Ni, Mn and Zn. Haywood et al., 2004 also revealed that out of four heavy metals (Zn, Cu, Pb and Cd), Pb was found to be most toxic and Zn to be the least toxic of all the heavy metals.

Bardiene et al., 2005 suggested the relevance of multi-tissue analysis to demonstrate genotoxic effects of different compounds. In the present study, bone marrow was found to have more frequency of chromosomal aberrations than intestine in response to sublethal treatment of the various heavy metals. The ability of the
chemicals to produce chromosomal damage in vivo especially in the bone marrow can be explained by the work of Heddle and Salamone, 1981 that bone marrow is a ready source of dividing cells, so it has been a favorite tissue for studying chromosomal aberrations in vivo. In the natural environment, some toxic metabolites cannot research the bone marrow cells if their lives are short-lived. Therefore the micronucleus test is a useful tool for risk assessment of the short-life toxic metabolites, which affect the blood lymphocytes (Zhang et al., 2012). Higher frequencies of micronuclei in RBCs could also be explained by the fact that blood is the most appropriate target organ when the dose is given intraperitoneally and also in nature because of the permeable skin frog absorb the water along with genotoxins. Variation in the micronuclei frequency with time seems to have related to the blood cell kinetics and erythrocyte replacement. The least frequency of micronuclei in the kidney can be explained because of the fact that kidney receives the chemical after a course of its circulation via blood.

The observed tissue-specific responses either observed through chromosomal aberration test or through micronucleus test may also be due to physiological activities distinctive to these organs, with respect to either the activation or detoxification of particular pollutant or the repair of different types of strand breaks.

In general, the present results indicated the frequencies of micronuclei in various tissues analyzed were observed to be much lower than that of chromosomal aberrations. Occurrence of lower frequency of micronuclei than that of chromosomal aberrations in treated frog specimens referred above could be explained in light that all forms of chromosomal aberrations could not contribute to formation of micronuclei. Micronuclei are generally formed by lagging chromosomes, acentric fragments and asymmetrical exchanges, while symmetrical exchanges and other simple or gross aberrations do not form micronuclei. Moreover, the micronucleated cells of the various tissues may get destroyed by the body’s defence mechanism before being scored during cytological preparations and hence do not correlate with the frequencies of chromosome aberrations, for this reason, frequencies of micronuclei in the present study could be lesser than that of chromosomal aberrations.

Thus the present study showed that cytogenetic techniques prove to be good tool in the assessment of genotoxic damage in the aquatic systems. In amphibians, contaminants are of major concern because many populations are declining
dramatically and the death of entire populations occurs increasingly frequently, in some cases due to anthropogenic changes in the environment. One of the key functions of such biomarkers (micronucleus and chromosomal aberration test) is to provide an ‘‘early warning” signal of significant biological effects (changes at the genetic/molecular level) with suborganismic (molecular, biochemical, and physiological) responses preceding those occurring at higher levels of biological organization such as cellular, tissue, organ, whole-body levels, and in line at population level. Biomarkers are also well suited as rapid and effective screening assays to assess ‘‘hot spots” of contamination. In this way, the use of the MNT and CAT may provide an important tool for the prediction of the potential long-term effects in amphibians in the environment.
Chapter 6

Summary
With fast economic development and industrialization, a vast range of genotoxic chemicals (metal and metalloid compounds) are produced and distributed into the environment leading to potential health hazard for all living organisms. These chemicals adversely affecting living organisms, often lead to serious diseases in human beings. Heavy metals are among the most common inorganic pollutants in water and are distributed over the earth's crust; therefore, represent one of the most toxic environmental pollutants of global concern owing to their non biodegradability. Heavy metals have been studied extensively in recent years due to the drastic effects they have on the ecosystems because of their toxicity, persistence, bioaccumulation and biomagnifications in aquatic organisms as aquatic environment is often the ultimate recipient of this increasing range of anthropogenic inorganic contaminants where the scenario becomes all the more worst as they are readily soluble too and thus environmentally mobile. Some of the most common pollutant heavy metals listed by Environmental Protection Agency (EPA) are: Cd, Cr, Cu, Hg, Ni, Pb and Zn.
Several of these heavy metals are mutagenic in nature. Genotoxicity as a result of heavy metal toxicity is also described to play a major role in inducing several cellular stress responses and damage different cellular components such as membranes, proteins, and DNA thereby highly toxic to biological organisms. They cause heavy mortality when present in lethal concentration; however, when present in sublethal concentration they exert undesirable alterations in genetic content of a living cell. Further, these mutagens may adversely affect the fertility and fecundity of living organisms. Moreover there is an ecological risk that may lead to heritable mutations and loss of total genetic diversity (either intra or inter species), with significant implications on long term survival of natural populations. Evaluation of the effect of heavy metals is an important factor not only from toxicology point of view in case of animals, but also for assessing potential impacts on public health.

Animals, the silent sentinels, stand watch over world’s environmental health. Every day, animals demonstrate intricate connection between them, us and our surroundings. Amphibians are in most cases, small, diverse, and sensitive to environmental pertuberations. They can be good indicators of habitat diversity, biological variety, and local stressors on the environment. Amphibians have been described as ‘biomarkers species’ or equivalent of ‘canaries of the coal mines’ meaning they provide an important signal to the health of biodiversity. Their skin, larvae, and unshelled eggs are constantly exposed and are in contact with the substances in their surroundings. Amphibians are biologically significant. They are perhaps one of the most successful groups of wildlife on the earth enjoying dual status, i.e. both aquatic and terrestrial and have different feeding ecologies at various stages of their life cycle. First ever land vertebrates and other higher land vertebrates have evolved after them, thus are “Connecting link” between aquatic and land vertebrates. Best model animals for analyzing embryological, biochemical processes (cryoprotectants for tissue preservations) genetic and cytogenetic studies (chromosome replication; chromatin, cytoskeleton and nuclear assembly; cell cycle progression and intracellular signaling). Approximately 10% of Nobel Prizes in Physiology and Medicine have resulted from investigations that used frogs. Recently Genome sequencing of an amphibian, *Xenopus* sp. has filled the gap between aquatic and terrestrial fauna and thus linked the evolutionary chain. Crucial part of ecosystem for maintaining ecological balance, economically significant for medicines, as food, other products of pharmaceutical and
industrial importance. Unfortunately, amphibians are threatened and declining worldwide at an alarming rate representing the great mass extinction of land vertebrates since the dinosaurs. Many amphibian species are on the brink of extinction, with 436 species (7.37%) listed as critically endangered, as compared with 179 birds (1.8%) and 184 mammals (3.8%). About 90% of all amphibians found in the world are frogs and toads, the most incredibly diverse animals (vertebrate) on the earth. Frog populations have been declining worldwide at unprecedented rates, and nearly 42.5% of the world's amphibian species are threatened with extinction and are therefore of global biodiversity concern.

Of the 11 amphibian species found in Jammu and Kashmir State which forms just 5.3% of the total amphibian population of the country, 6 species are found in Jammu. Anurans are the most neglected group of vertebrates of the state. Out of the 6 species of anurans, three species i.e. *Euphlyctis cyanophlyctis*, *Hoplobatrachus tigerinus*, *Chrysopaa sternosignata*, are of frogs belonging to family Dicroglossidae. This family is perhaps the most widely distributed family having 186 species in 13 genera (Frost, 2013). Out of the three, *H. tigerinus* is more secretive in nature, larger in size and is witnessed after a heavy monsoon shower in water logged areas whereas *Chrysopaa sternosignata* is found in the hilly districts of Jammu. *Euphlyctis cyanophlyctis*, also known as common skittering frog is one of the most widely distributed oriental frogs and is highly aquatic and littoral frog. The frog can tolerate a wide range of temperature, oxygen and pH variations, from fresh water to considerably brakish and polluted refuse water; it thrives equally well in sewer systems of towns and cities. *E. cyanophlyctis* is also claimed to be the native frog of Kashmir and is reported declining at an alarming rate especially in the urban and semi-urban areas of the valley, warning environmentalists. The research report regarding the native frog of Kashmir in the paddy field environment, appeared in the Journal of Centre of Research for Development, University of Kashmir, revealed that native population of frog – *Rana cyanophlyctis* – is sparsely found in the valley.

Aquatic animals have often been used in bioassays to biomonitor water quality of effluents and surface water. The use of frogs and toads as biological indicators of metal pollution is becoming common. Frog has proved to be a valuable indicator and sensitive model for environmental studies. Keeping in view the above facts, the present
study has been undertaken to bio-monitor the genotoxic potential of eight heavy metals viz. CdCl$_2$.1/2H$_2$O, K$_2$Cr$_2$O$_7$, CuSO$_4$.5H$_2$O, Pb(COOH)$_2$, MnCl$_2$, HgCl$_2$, NiCl$_2$, and ZnCl$_2$, using *Euphlyctis cyanophlyctis* as an amphibian test organism by utilizing various genotoxicity assays like chromosome aberration test (CAT), mitotic index (MI) and micronucleus test (MNT).

Cytogenetic analysis of male and female specimens of model species was carried out following the conventional colchicine-hypotonic-acetic-alcohol air-drying Giemsa staining technique (modified after Tijo and Whang, 1965). Different tissues like intestine, kidney and bone marrow were used for chromosomal preparation. Chromosomal and morphometric analysis of male and female specimens of test frog, *Euphlyctis cyanophlyctis*, included the determination of diploid chromosome number, length of long arm and short arm, arm ratio, total length percentage, relative length percentage and centromeric index. Idiogram and histogram was also prepared. The diploid number was recorded as 2n=26; chromosomal formula as 7M+6SM; fundamental arm number as 52.

C-banded and NOR-banded karyotyping was also done following C- banding (modified after Sumner *et al.*, 1971) and NOR-banding (after Howell and Black, 1980) techniques. C-banding of somatic metaphase complements revealed that centromeric C-bands are present in all of the chromosomes showing presence of heterochromatin. Some telomeric C-bands have also been observed in some chromosomes but these bands were very light. The Ag-NOR staining showed a well defined and conspicuous pair of nucleolar organizer regions on long arm of 10th submetacentric pair.

Toxicity tests were conducted in accordance with standard methods. Acclimatized, live, active and apparently healthy frog specimens were treated through intraperitoneal injection having a precise measurement of the amount of heavy metal, once in treatment period. The groups of frogs, treated with four sub-lethal concentrations of each heavy metal salt mentioned above for 24, 48, 72 and 96 hrs of duration. Frogs were not fed during experimental periods and the frogs of the control group (without the heavy metal treatment) were maintained on the normal food i.e. earthworms in similar experimental conditions.
For chromosomal aberration test (CAT), chromosome preparations were made from intestine and bone marrow after exposure to particular duration and concentration of heavy metal as well as in control specimens. Number and types of chromosome aberrations induced were recorded and expressed as mean frequency per 100 cells. Mitotic index (MI) was established in intestine and bone marrow tissue of both exposed and control specimens by determining the number of metaphases among the total cells observed. The Micronucleus test (MNT) was performed as per standard methods in peripheral erythrocytes (RBCs), intestine and kidney cells of both treated and control frogs so as to record the frequencies of micronuclei per 1000 interphase cells in the respective groups.

Data from CAT, MI and MNT were expressed as mean±SD. Comparative evaluation of genotoxicity in different tissues of frog (intestine, bone marrow, kidney and RBCs) was also done and the effect of dose and duration of exposure to a particular sublethal concentration of heavy metal was also determined. Statistical significance in the frequencies of chromosome aberrations, mitotic index and micronuclei between exposed and control groups after each dose and duration of exposure was evaluated and data was statistically analyzed by non-parametric Kruskal-Wallis test using the computer software called ‘PRIMERS-4.0’. p<0.05 was considered to be the level of significance.

Chromosomes of somatic metaphases obtained from intestine, kidney and bone marrow of control specimens showed clear morphology without any chromosomal aberrations. Frog treated with sublethal doses of heavy metal showed a wide spectrum of chromosomal aberrations (CA) in intestine and bone marrow cells. The various chromosomal abnormalities observed during present investigations on frog, *E. cyanophlyctis*, after treatment with different heavy metal salts were: Chromosome fragmentation (Cf), Ring chromosomes (Rc), Terminal chromatid deletion (Tcd), Minutes (M), Centromeric gaps (Cg), Terminal association of chromosomes (Tac), Stickiness (Stk), Clumping(C), Pycnosis (Py) and Stretching (Sth). These chromosomal aberrations recorded could be categorized as clastogenic and physiological type. The clastogenic effects on the chromosomes obtained after treatment with heavy metals included chromosomal fragmentation, ring chromosomes, terminal chromatid deletion, minutes and centromeric gaps. The physiological type of aberrations included terminal association of chromosomes, stickiness, clumping, pycnosis and stretching.
Treatment of the frog with four sublethal concentrations of different metal salts showed significantly increased frequencies of chromosomal aberrations except for exposure with manganese and zinc. The frequencies of chromosomal aberrations were found to gradually increase from 24hrs to 96hrs of exposure except in certain cases where it increased after highest dose and duration of exposure, as in treatment with zinc. Also, in some cases the frequencies of chromosomal aberrations were not significantly different than the respective controls. Following acute exposure to sub toxic doses of various heavy metal salts, bone marrow had more frequencies of chromosomal aberrations after exposure to cadmium, chromium, copper, mercury and nickel whereas lead, manganese and zinc induced more chromosomal aberrations in intestine, revealing the affect to genotoxic exposures. Furthermore, a decrease in mitotic frequency with an increase in concentration and duration of exposure but a reverse phenomenon was observed with respect to chromosomal aberrations. Based on the results obtained for CAT after the exposure of frog to different sublethal concentrations of different heavy metals, all the heavy metals tested had proved to be potentially genotoxic except zinc. The genotoxic potential of heavy metal toxicant was evident as Pb > Hg > Cu > Cd > Cr > Ni > Mn > Zn.

Frogs exposed to different concentrations and durations of heavy metals showed a dose and time dependent increase in the incidence of micronuclei which were significantly higher than that of controls. All the heavy metals tested during the present investigation were found to be potentially genotoxic except nickel and zinc as was evident from the extent of frequencies of micronuclei in different tissues (RBCs, intestine and kidney cells) examined. Among the various tissues examined for the induction of micronuclei, highest frequency of micronuclei was recorded from peripheral erythrocytes followed by intestine and least in kidney cells. Based on the MNT, genotoxic potential of aforesaid toxicants was recorded as Cu > Hg > Ch > Cd > Pb > Mn > Ni > Zn.

Generally, heavy metals presently evaluated have been found to be highly genotoxic. However, detailed studies using assay systems having different end points may be needed to reconfirm the genotoxic status of manganese and zinc and to explore the mechanism of their genotoxicity in aquatic organisms since these metals were found to exhibit very low and non-genotoxic effects respectively. It can also be concluded that the CAT and MNT showed high sensitivity in detection of genomic damage in cells of various tissues. RBCs showed more frequency of MN than other tissue studied and bone marrow had more frequencies of chromosomal aberrations revealing the affect to genotoxic exposures.
Present study finds its relevance in the context that heavy metal pollutants viz. Cd (II), Cr (VI), Cu (II), Pb (II), Mn (II), Hg (II), Ni (II), and Zn (II), by virtue of their presence in aquatic habitat can damage genetic profile of the model, *Euphlyctis cyanophlyctis* thus posing a threat for the loss of frog biodiversity and also directly or indirectly affecting our health as well. It is therefore, recommended that effluents containing heavy metals should only be disposed off after their proper treatment. Further, there should be strict and regular monitoring of these toxicants in water bodies to check possible environmental hazards as human survival depends on the preservation of other animals especially frogs because when a frog species disappear so does any promise it holds for improving human health. The development of biological monitoring techniques base on frog offers the possibility of checking water pollution with fast response on low concentrations of directly acting toxicants. Development of chemical and biological methods for effectively monitoring environmental levels of heavy metals is subject of interest and a critical step in development of environmental waste management.

From the present studies conducted under the title, “Genotoxic effect of some heavy metals on frogs (amphibian),” it can thus be interpreted that:

1. The skittering frog, *Euphlyctis cyanophlyctis* as having more of aquatic and littoral habit can best be exploited as a model for conducting investigations in the area of frog genotoxicity and also for biomonitoring genotoxic pollutants. It can therefore, serve as useful sentinel of environmental problems because of its trophic importance, environmental sensitivity, research tractability and impending extinction.

2. Following the treatment of frog with sub toxic doses various heavy metals salts, a decrease in mitotic frequency and an increase in the number of chromosomal abnormalities were observed except for zinc. Also significantly elevated frequencies of micronuclei were detected in various tissues examined after treatment with different heavy metal salts except for nickel and zinc compared to respective controls.

3. The chromosomal aberration test revealed that the most vulnerable or sensitive region in the chromosome appeared to be heterochromatic region of either centromere or telomere.

4. In general, the present results indicated that the frequencies of micronuclei in various tissues analyzed were observed to be much lower than that of the chromosomal aberrations.
5. Present data regarding the frequencies of CA and MN in the frog investigated can serve as a relevant database for future studies on assessment of effect of genotoxic pollutants and may represent a valuable tool as a biological dosimeter in polluted water reservoirs. In biomonitoring environmental genotoxins, the data obtained after these assays from present investigations are of biological significance as it was statistically analyzed by non-parametric Kruskal-Wallis test using the computer software called ‘PRIMERS-4.0’ and p<0.05 was considered to be the level of significance. Therefore, CAT and MNT can be successfully employed for determination of in vivo DNA damage caused to the frogs due to environmental genotoxins. Use of frog MNT is recommended for easy and faster evaluation of the pollution status of aquatic bodies compared to the CAT.

6. The chromosomal aberration test and micronucleus test are two most extensively used methods in the detection of genotoxicity of chemicals in the environment. Compared to other assays, they are sensitive, rapid and easy to handle and in the present study, the CAT and MNT showed high sensitivity in detection of genomic damage in cells of various tissues. RBCs showed more frequency of MN than other tissue studied and bone marrow had more frequencies of chromosomal aberrations revealing the affect to genotoxic exposures. Generally, heavy metals presently evaluated have been found to be highly genotoxic. However, detailed studies using assay systems having different end points may be needed to reconfirm the genotoxic status of manganese, nickel and zinc and to explore the mechanism of their genotoxicity in aquatic organisms since these metals were found to exhibit very low genotoxic effects.

The biological significance of present investigation is that the ecological assessment of territories exposed to anthropogenic influences must involve evaluation of the mutagenic potential of the environment. The assessment of genetic effects of environmental pollution on man is methodologically difficult and expensive; hence it is expedient to use indicator animal species for ecogenetic monitoring. Mass species of anurans are promising for this purpose, as specific features of their life cycle make them convenient for assessing the state of both terrestrial and aquatic ecosystems. Also, the purposes of genotoxicity testing are (i) to assess the mutagenicity of chemicals, in order to protect the human gene pool and (ii) to identify potential carcinogens. Genotoxic exposure can also act as a selective force by eliminating sensitive genotypes,
or by reducing the number of offspring that they contribute to the next generation. The result is a reduction in the total genetic variation within that population or a shift in genotypic frequencies and thus leading to increased rate of extinction as is evident in case of amphibians.

In amphibians, contaminants are of major concern because many populations are declining dramatically and the death of entire populations occurs increasingly frequently, in some cases due to anthropogenic changes in the environment. One of the key functions of such biomarkers (micronucleus and chromosomal aberration test) is to provide an “early warning” signal of significant biological effects (changes at the genetic/molecular level) with suborganismic (molecular, biochemical, and physiological) responses preceding those occurring at higher levels of biological organization such as cellular, tissue, organ, whole-body levels, and in line at population level. Biomarkers are also well suited as rapid and effective screening assays to assess “hot spots” of contamination. In this way, the use of the MNT and CAT may provide an important tool for the prediction of the potential long-term effects in amphibians in the environment.

Since CAT and MNT detect chromosome/genome mutations and also DNA primary damage, the combined use of both tests on the same biological model is recommendable for genotoxicity testing. The complexity of deleterious effects on an aquatic vertebrate such as the present model animal could then be better taken into account. However, techniques in genetic ecotoxicology are in a rapidly evolving state. Therefore, reliable tools are now available for addressing more complex environmental problems. The increasing availability of reliable diagnostic tools will greatly improve our ability to assess the sublethal effects of exposure to hazardous substances. We must envision their promise for addressing these problems and identify the most urgent directions for future research.

Science and technology, no doubt allow man considerable control of his immediate environment, but earth-inhabitant continuum cannot be denied. The secret of survival, therefore, is not to absolutely prohibit the use of any chemical, but to utilize each chemical rationally. The environment quantities of all minerals and their salts must be maintained at levels compatible with continued optimum health and existence. Humans tend to be unconcerned/unimpressed unless our own health is threatened or unless scientific evidence shows direct cause and effect. Yet the first step in addressing
environmental/ecosystem health is admitting that animals are indicating problems. We need to move forward with multi-disciplinary research that recognizes the value of animals as sentinels of environmental health. We also need to recognize that environmental protection measures which protect animal health often directly and indirectly protect our health as well. The burden of proof has now shifted to those who would continue to ignore their fate.
Chapter 7

Bibliography


Khan, M.Z., Maria, Z. and Fatima, F. (2003b). Effect of lamda cyhalothrin (Pyrethroid) and monocrotophos (Organophosphate) on cholinesterase activity in liver,


Appendix
Induction of micronuclei due to cytotoxic and genotoxic effect of Lead Acetate metal salt in *Euphlyctis cyanophlyctis* (Amphibia: Anura)

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Abstract

The aim of the present study was to investigate the genotoxic effects of lead acetate in different somatic tissues (Intestine and RBCs) of skittering frog (*Euphlyctis cyanophlyctis*) using micronucleus test. LC$_{50}$ value of lead acetate was calculated to be 42mg/kg. The four different sublethal concentrations of lead acetate (4.5, 9.0, 18.0 and 36.0mg/kg) were injected intraperitoneally once in the treatment period for 24, 48, 72 and 96hrs. It was observed that this heavy metal induced a significant increase in frequency of micronuclei at different concentrations in frog for 24, 48, 72 and 96hrs when compared with the control. These results lead us to the conclusion that lead may have genotoxic and cytotoxic properties due to the induction in the frequencies of MN in different tissues of the frog.

Keywords

Genotoxic, Lead acetate, Somatic, Micronucleus test, Intraperitoneal, Heavy metal, Cytotoxic

1.1 Introduction
Micronuclei are cytoplasmic chromatin-containing bodies that appear in the cell like a small satellite nucleus around the cell nucleus, due to chromosome fragments or entire chromosomes that are not incorporated in the main nucleus after cell division. The presence of micronuclei (MN) in cells is considered as a biomarker of damage to the DNA. The micronucleus test is an *in vivo* and *in vitro* short-time screening cytogenetic test, introduced by Heddle (1973) and Schmid (1975) is a widely used method for assessing genotoxicity of chemicals in organisms (Meier et al., 1999). The micronucleus test has been used because it is technically easy to master, reliable, least expensive and extremely rapid screening system. The micronucleus test for mutagenicity screening has been well established in several systems i.e. ovary, bone marrow, epithelial tissue, peripheral blood, liver, exfoliated buccal cells and fetus cells of several laboratory animals or human (Heddle, 1990; Saleh and Zeytinoglu, 2001). Micronuclei formation occurred in any dividing tissue of any species (Heddle et al., 1983; Zuniga et al., 1996). Micronucleus test was used to investigate environmental pollution in plants (Ma et al., 2005), fish (Cavas and Ergene Gozukara, 2005), birds (Bhunya and Jena, 1996) and frogs (Mouchet et al., 2005 and 2006). Furthermore, (Hayashi et al., 1998) evaluated the monitoring systems that use aquatic organisms to assess the genotoxicity of water in the field and in the laboratory. In a field study, micronucleus assay was shown to be applicable to micronucleus inducing agents in frogs (Saleh and Zeytinoglu, 2001). Aquatic animals have often been used in bioassays to monitor water quality of effluent and surface water (Fernandez and L Haridon, 1992). The development of biological monitoring techniques regarding frogs and fishes offer the possibility of checking water pollution with fast responses on low concentrations of direct acting toxicants (Al Sabti, 1986; Al Sabti and Metcalfe, 1995; Andrade et al., 2004). Amphibians have also proved to be valuable biological models for the study of micronucleus induction in erythrocytes, leading to the normalized micronucleus test MNT (Jaylet et al., 1986; Krauter et al., 1987; Gauthier, 1996; Ferrier et al., 1998; Jaylet and Zoll-Morreux, 1990; Zoll-Morreux et al., 1990; Djomo et al., 2000; Bekaert et al., 2002). Determining the direct and indirect effects of agro and industrial chemicals to amphibian species continues to be identified, as a research need. The conclusiveness of published information on the ecotoxicology of metal contaminants to amphibians is limited in many cases (Sparling et al., 2000).

As one of these metal contaminants, lead is extensively used as industrial materials such as in the manufacture of batteries, metal product, paints and ceramic glazes. The
largest source of lead in the atmosphere has been from lead gasoline combustion. Lead enters the organisms through food chain and absorbed mainly by ingestion and inhalation. Since it cannot be discharged, the lead accumulates in tissues and in over doses the lead has toxic effects (Uysal, 1997). Lead acetate is a chemical compound, which is also known as mutagen and carcinogen.

Amphibians are considered as excellent “bio indicators” of environmental health (Schuytema and Nebeker, 1999; Tejedo, 2003; Garcia-Munoz et al., 2010. The Indian Skipper Frog or Skittering Frog (Euphlyctis cyanophlyctis) is a common frog found in South Asia and is the native frog of Kashmir. They are often seen at the edge of bodies of water with their eyes above the water. E. cyanophlyctis was chosen in the present study to evaluate the genotoxic potential of lead acetate by using the micronucleus test.

1.2 Materials and Methods

1.2.1 Sample Collection

Healthy and actively living adult frog specimens of Euphlyctis cyanophlyctis were collected from unpolluted ponds and ditches in the vicinity of Jammu region. They were collected using hand nets to prevent injury to animals during capture since they are active animals. The weight of frogs ranged from 35-40g. These frogs were kept under laboratory conditions for about 5 days, at ambient temperature and natural photoperiod, according to the time of the year, in which each test was performed, for acclimatization before starting the assay. They were kept in 15L plastic containers. The water was changed daily to avoid accumulation of toxic substances.

1.2.3 Heavy metal treatment:

After adaptation, the frogs were separated in 5 experimental groups, 1 group as control and 4 treated groups in separate plastic containers of 10L capacity. Four frog specimens were kept for treatment with each heavy metal salt concentration (sublethal conc.) for 24, 48, 72, and 98 hrs. Separate batch of same number of frogs were kept as control for each duration of exposure i.e. 24, 48, 72, and 98hrs. LC50 value at 96hrs was calculated using standard method of Finney (1980) and was found to 42mg/kg. Based on the LC50 value of lead acetate, the four sublethal concentrations were arbitrarily chosen and the frogs were treated with these doses (i.e. 4.5mg/kg, 9.0mg/kg, 18.0mg/kg and 36.0mg/kg) through an intraperitoneal injection by using 1ml syringe, only once in the treatment period.

1.2.4 Slides preparation and staining
For each frog, experimental as well control, fresh blood samples were taken after each duration of exposure and smeared onto the clean slides. The slides were air dried for 1-2hrs and then fixed in absolute methanol for 10min. After fixing, the same slides were stained in Giemsa (2%) for about 30min.

1.2.5 Examining and scoring of MN slides

Red blood cells in lower vertebrates such as amphibians are nucleated and undergo cell division in the circulation. These cells are therefore suitable for micronuclei detection which can be readily counted in blood smears. The frequency of MN in erythrocytes was established by estimating the number of MN in at least 1500 interphase cells/specimen (total of 6000 interphase cells from four specimens used for conc. and duration). The micronucleated interphase erythrocytes were photomicrographed at 1000X magnification under a binocular microscope (Olympus). Only cells with intact cellular and nuclear membrane were scored. The following criteria were used as described by previous studies:

a) MN should be one-tenth and one-third diameter of main nucleus.
b) They should be on the same place of focus.
c) They should have the same colour, texture and refraction as the main nucleus.
d) They should be clearly separated from the main nucleus.

1.2.6 Statistical analysis

The frequency of micronuclei obtained from the experimental as well as controlled group were expressed as mean ±SD. Statistical analysis of the data was carried out using the non-parametric Kruskal-Wallis test and the computer software called ‘PRIMERS-4.0’ was used for it. Difference between means are regarded as significant if p<0.05.

1.3 Results

1.3.1 Effect of Lead acetate treatment on Intestine

Frequency of MN determined in different treatment is summarized in Table 1 and Fig 1 and 3. Frog exposed to different concentration and durations of Lead acetate showed a dose and time dependent increase in the incidence of micronuclei which were significantly higher than that of controls (at 24hrs, p<0.05; at 48hrs, 72hrs and 92hrs, p<0.010 verses respective controls in all treatment groups). In frog treated in vivo with 4.5mg/kg lead acetate, the frequency of MN was recorded to be 1.15±0.83 (24hrs), 1.45±0.83 (48hrs), 1.65±0.40 (72hrs) and 2.13±1.45 (96hrs) wherein the incidence of micronuclei increased along with the duration of the same dose. An elevated response
was observed up to 96hrs of exposure in the frequency of MN (2.30±0.85 at 24hrs, 3.30±0.57 at 48hrs, 3.65±1.38 at 72hrs and 4.30±0.35 at 96hrs) after treatment with 9.0mg/kg. Specimens exposed to 18.0mg/kg showed significantly decrease in MN at 24hrs (1.65±0.40) but an elevated increase in frequency of MN were observed at 48hrs (3.63±0.87), 72hrs (4.98±1.62) and 96hrs (5.80±0.63). Treatment with 36.0mg/kg showed a highly significant increase of micronuclei in relation to respective controls at all the exposure periods. The values recorded were 2.30±0.35 (24hrs), 4.13±1.49 (48hrs), 6.40±0.62 (72hrs) and 8.63±2.12 (96hrs).

1.3.2 Effect of Lead acetate treatment on RBCs

Values of MN in the peripheral erythrocytes after different treatment and exposure periods have been shown in Table 1 and Fig 2 and 4. Frog treated In vivo with 4.5mg/kg showed a highly significant increase (at 24, 48, 72 and 96hrs, p<0.010 versus control values) in the incidence of MN versus respective controls at all the exposure periods. The values recorded were 0.60±0.00, 1.12±0.67, 1.12±0.67, 1.82±0.35 after 24hrs, 48hrs, 72hrs and 96hrs respectively, wherein the frequency of MN were same at 48hrs and 72hrs of treatment. Frogs exposed to sub-lethal concentration of 9.0mg/kg, 18.0mg/kg and 36mg/kg also revealed highly significant increase (p<0.010 at all the exposure periods versus respective control values) in the incidence of MN. At 9.0mg/kg of treatment, the values recorded were 0.77±0.35, 4.12±0.62, 3.65±0.40 and 3.95±1.10 after 24hrs, 48hrs, 72hrs and 96hrs respectively, where maximum frequency of MN were recorded at 48hrs of treatment. Exposure to 18.0mg/kg induced 1.65±0.40, 3.30±0.57, 4.95±0.40 and 4.45±2.15 MN frequency after 24hrs, 48hrs, 72hrs and 96hrs, here the maximum frequency were observed at 72hrs treatment period. An elevated response was observed during treatment with 36.0mg/kg wherein the values recorded were 2.47±1.15, 4.30±1.61, 5.45±1.83 and 9.48±1.48 after 24, 48, 72 and 96hrs of exposure respectively.

1.4 Discussion

Over the past decades, the decline of the amphibian populations have been extensively reported (Houlahan et al., 2000; Stuart et al., 2004). Amphibian are reliable indicators of environmental pollution due to their biphasic life (aquatic and terrestrial) and semi-permeable skin (Lee and Stuebing, 1990). In some cases this phenomenon is associated with pollution by pesticides and heavy metals. Although environmental pollution may interfere with amphibian growth and development, the induction of genetic damage after chronic exposure to low doses of chemicals is perhaps the most important
biological effect. The blood of amphibians is very plastic tissue. In fact, variation of several hematological parameters in response to natural changes in the environment have widely described by researchers (Krauter, 1993; Stansley and Roscoe, 1996). Jayet et al. (1986) first adapted the MN test to amphibians. Many MN tests on amphibians have used genotoxic agents (Van Hummelen et al., 1989; Chen and Xia, 1993; Zoll Morreux and Ferrier, 1999). It has been used as a measure of genotoxicity in amphibians (Ferrier et al., 1998; Compana et al., 2003) and has shown potential for \textit{in situ} monitoring of water quality (Gauthier, 1996). MN derivative from chromosomal fragments or whole chromosomes which are not incorporated into main nucleus during cell division as a consequence of DNA fragmentation (clastogenic origin) or of alteration of the mitotic apparatus (an eugenic origin) (Schmid, 1975; Heddle et al., 1991; Norppa and Flack, 2003).

In the present study of genotoxic effect of Lead acetate using micronucleus test revealed that there is a significant induction of micronuclei in all the tissues (intestine and RBCs) of \textit{E. cyanophlyctis}, studied as compared to positive control groups. The frequencies of micronuclei in different tissues was evaluated (Table 1) and expressed as Mean±SD. A comparison between micronucleus frequencies in both the tissues revealed highest micronuclei frequencies at 96hrs duration in RBCs (Fig 4) followed by Intestine (Fig 3). In intestine, the value of MN recorded ranged from 1.15±0.83 (at 24hrs with a dose of 4.5mg/kg) to 8.63±2.12 (at 96hrs with a dose of 36.0mg/kg) showing a significant increase in the value of micronuclei with the increase in dose and duration of exposure excepting for 24hrs and 48hrs, each for 18mg/kg and 36.0mg/kg treatment dose, where a slight fall in value of the MN frequency was observed. In RBCs the MN frequency ranged from 0.60±0.00 (at 24hrs with a dose of 4.5mg/kg) to 9.48±1.48 (at 96hrs with a dose of 36.0mg/kg). There was significant increase in the MN frequency for all the exposure periods (24, 48, 72 and 96 hrs) at 4.5mg/kg but with the increase in dose (9.0 and 18.0 mg/kg) fluctuation in the MN value was observed with a maximum value (4.12±0.62) at 48hrs of duration with 9.0mg/kg treatment of the test chemical and with 18.0 mg/kg treatment, maximum value (4.95±0.40) was recorded at 72hrs exposure period. However, with the increase in dose of the test chemical the value of MN frequency showed a statistical increase. Thus, a positive relationship between metal concentrations and micronuclei frequencies were recorded for all the treatment groups with respect to their dose and the exposure periods compared to the control groups. The frog treated with different doses of the lead acetate
(4.5mg/kg, 9.0mg/kg, 18.0mg/kg, 36.0mg/kg) at 24hrs of duration showed significant results (p<0.05) but with increase in duration of the doses, the result obtained were highly significant (p<0.01).

1.5 Conclusion

Present results, thus suggested that sublethal doses of lead acetate caused detectable genome damage in various tissues of frog, *Euphlyctis cyanophlyctis*. Further studies should include more of the different lead doses and different stages of development of the frog to gain a better insight in the significance of present findings and to elucidate the mechanism of lead genotoxicity and also different genotoxic tests can be taken into account.

1.6 Acknowledgements

The first author is very indebted to UGC, New Delhi for providing assistance under Fellowship Development Programme. The author is also grateful to Prof. K.K.Sharma, Head, Department of Zoology for proving necessary lab facilities.

1.7 References


Mouchet, F., Gauthier, L., Mailhes, C., Jourdain, M.J., Ferrier, V., Triffault, G. and Devaux, A. 2006. Biomonitoring of the genotoxic potential of aqueous extracts of soils and bottom ash resulting from municipal solid waste incineration, using the comet and


### 1.8 Appendices

Table 1 Frequencies of micronuclei (%) in different tissues of *Euphlyctis cyanophlyctis* exposed to Lead acetate.

<table>
<thead>
<tr>
<th>Conc. (mg/kg)</th>
<th>Duration (hrs)</th>
<th>No. of Specimens</th>
<th>Total interphase cells studied</th>
<th>Frequency of micronuclei (Mean±SD)</th>
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<td>Intestine</td>
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<td>0.30±0.36</td>
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</tr>
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</tr>
<tr>
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<tr>
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<td>6000</td>
<td>8.63±2.12*</td>
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*a=p<0.05, b=p<0.01, c=p<0.001, ns = non-significant (represent values significantly differently from the respective controls; Kruskal-Wallis test; df=4), Contr.= Control*
Captions to Figures

Fig 1: Shows the micronuclei frequency in Intestinal cell.

Fig 2: Shows the micronuclei frequency in RBC.

Fig 3: Frequencies of micronuclei in Intestine after treatment with Lead acetate

Fig 4: Frequencies of micronuclei in RBCs after treatment with Lead acetate
Evaluation of acute toxicity of copper sulphate in different tissues of

*Euphlyctis cyanophlyctis*

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Department of Zoology, University of Jammu, Jammu-180006, Jammu & Kashmir, India

Abstract
Copper sulphate, CuSO$_4$.5H$_2$O (Fine Chemical, India), Purity (99%) and CAS No. 7758-98-7, a broad-spectrum inorganic pesticide, has many-fold uses as fungicide, molluscicide and weedicide. The adult Indian skittering frog, *Euphlyctis cyanophlyctis* was treated *In vivo* with different sublethal concentrations of CuSO$_4$ (3.5, 5.0, 6.5 and 8.0mg/kg) after calculating the LC$_{50}$ value which was found to be 10mg/kg. Each dose was injected intraperitoneally once in the treatment period for 24, 48, 72 and 96hrs to study its potential toxic effect using micronucleus test. It was observed that copper sulphate induced a significant increase in frequency of micronuclei at different concentrations in frog for 24, 48, 72 and 96hrs when compared with the control. The results lead us to the conclusion that there is a dose-effect relationship in the induction in the frequencies of MN in different somatic tissues of the frog due to its genotoxic and cytotoxic properties.

Keywords
Genotoxicity, Copper sulphate, *Euphlyctis cyanophlyctis*, LC$_{50}$ value, Micronucleus test

1. INTRODUCTION
Chemical stressors such as acid deposition, industrial chemicals, pesticides, heavy metals, salts and nitrogen fertilizers are possible causes for the decline of some amphibian populations. The global phenomena of localized amphibian population declines, a high prevalence of amphibian malformations and the increasing presence of amphibians on threat or endangered species lists have currently stimulated research investigating amphibian response to both natural and anthropogenic stressors (Andrea et al., 2004). Heavy metals are usually detected in measurable levels in industrial effluents because metallic compounds are common constituents of several raw materials, which serve as feedstocks, catalysts, lubricants and clean up chemicals employed in industrial production processes. The main route by which heavy metals enter the aquatic environment is through the discharge of metal-laden municipal and industrial effluents directly into water bodies or indirectly through drainage and canals (Schumacher et al., 1992). Copper, in trace amounts, is essential for life while in excess is toxic. Its importance in health and disease is well documented (Aaseth and Norseth, 1986; Berman, 1980; Evans, 1973; Linder, 1983; Owen, 1981; Prohaska, 1986; Venugopal and Lucky, 1978). It has also been established in the literature that lead and copper reach drinking water through the dissolution of plumbing materials (Murphy, 1993). A number of metals present in the occupational environment have been reported to be human carcinogens (Anttila et al., 1993). However, there is no direct positive correlation between Cu exposure and cancer (Howell, 1958; Linder, 1983).

In spite of the indiscriminate use of synthetic organic pesticides, a number of inorganic pesticides are still widely used in present day agriculture. Copper sulphate, a broad-spectrum inorganic pesticide, has many-fold uses as fungicide, molluscicide and weedicide. It causes no DNA damage in prokaryotes (Matsui, 1980) and enhances viral transformations (Casto et al., 1979). De Flora et al. (1984) reported the non-mutagenic property of copper sulphate in several strains of Salmonella tphimurium and the negative result in DNA repair tests with several repair-deficient strains of Escherichia coli. The genotoxicity of Cu compounds has been reported in animal cell cultures (Flessel, 1978; Hollstein et al., 1979). Its clastogenic effects have been reported in mice in vivo by Bhunya and Pati (1987) and Agarwal et al. (1990) while Tinwell and Ashby (1990) reported its negative effect in the mouse bone marrow micronucleus assay. The strong clastogenic
potential of copper has been reported in plants (Rosen, 1964). There is limited literature on the clastogenic effect of copper sulphate in frogs in vivo test system. This gives us further impetus to evaluate the clastogenic and genotoxic potential of copper sulphate in the Indian Skipper Frog or Skittering Frog (*Euphlyctis cyanophlyctis*) which is a common frog found in South Asia and is the native frog of Kashmir. They are often seen at the edge of bodies of water with their eyes above the water. *E. cyanophlyctis* was chosen in the present study to evaluate the potential of copper sulphate by using the micronucleus test.

2. MATERIALS AND METHODS

2.1 Test chemical

Copper sulphate, CuSO₄.5H₂O (Fine Chemical, India), Purity (99%) and CAS No. 7758-98-7 was used as test chemical.

2.2 Animals and treatment

Experiments were performed on Indian skittering frog *Euphlyctis cyanophlyctis*. Adult live and apparently healthy specimens of, *E. cyanophlyctis* weighing 30-40gms caught from local unpolluted lentic habitats were acclimatized to laboratory conditions in large well aerated plastic containers for at least 5 days prior to use. Frogs were divided into 1 control and 4 treatment groups. Four frogs were kept in each group. LC₅₀ value was estimated using standard method of Finney (1980) and was found to be 10mg/kg. Based on LC₅₀ value of copper sulphate, four sublethal concentrations were arbitrarily chosen and the frogs were treated with these doses (3.5mg/kg, 5.0mg/kg, 6.5mg/kg and 8.0mg/kg) through an intraperitoneal injection by using 1ml syringe only once in the treatment period. Frogs of all groups were subjected for 24, 48, 72 and 96hrs of exposure periods.

2.3 Micronucleus Test (MNT) Procedure

Fresh blood samples from each frog, experimental as well control, were taken after each duration exposure and smeared onto the clean slides, air-dried for 1-2hrs and then fixed in absolute methanol for 10 min. The slides were stained in Giemsa stain (2%) for 30-35 minutes. Then the animals were anaesthetized and dissected to take out the intestine and kidney. The tissues were hypotonised with 0.50M NaCl solution for 1hour at room temperature. Fixing of the tissues was done in Conroy’s fixative for 45min changing the solution every 15 minutes.
The material was then dabbed on clean slides, air-dried and stained with 2% Giemsa stain for 30-35 minutes.

2.4 Examining of slides
The frequency of MN in erythrocytes as well in intestinal and kidney tissues was established by calculating the number of MN in at least 1500 interphase cells/ specimen (total of 6000 interphase cells from four specimens used for conc. and duration). The micronucleated interphase cells were photomicrographed at 1000X magnification under a binocular research microscope (Olympus CH20iBIMF) using Sony SSC-DC378P camera. Only cells with intact cellular and nuclear membrane were scored.

2.5 Statistical analysis
The frequency of micronuclei obtained from the experimental as well as controlled group were expressed as mean ±SD. Statistical analysis of the data was carried out using the non-parametric Kruskal-Wallis test and the computer software called ‘PRIMERS- 4.0’ was used for it. Difference between means are regarded as significant if p<0.05.

3. RESULTS

3.1 Effect of copper sulphate treatment in Intestine
Frequencies of micronuclei determined in the different treatments are summarized in Table 1 and Figure 1 & 4. Frogs exposed different concentrations and durations of copper showed a dose and time dependent increase in the incidence of micronuclei which were significantly higher than that of controls (at 24hrs, p<0.05; at 48, 72 and 96hrs, p<0.01). In frog treated in vivo with 3.5mg/kg of copper, frequency of MN was recorded to be 0.97±0.865(at24hrs) with a slight decrease to 0.95±0.404 (at 48hrs), followed by an increase in value at 72hrs (1.12±0.670) and at 96hrs (1.13±0.942). An elevated response was observed in the frequency of MN (exposed to 5.0mg/kg) from 24 to 96hrs of exposure (1.14±0.834 after 24hrs, 1.97±0.531 after 48hrs, 2.15±0.3 after 72hrs and 3.47±1.114 after 96hrs). Specimens exposed to 6.5mg/kg showed significantly elevated incidence of MN at 24hrs (1.47±0.35), at48hrs (2.12±0.618), at 72hrs (3.97±1.102) and at 96hrs (3.97±1.717). Values of MN frequency were same at 72 and 96hrs of exposure. Treatment with 8.0mg/kg showed a highly significant increase of micronuclei in relation to the respective controls at all the exposure periods. The values recorded were
3.32±0.942 (at 24hrs), 5.47±1.117 (at 48hrs), 8.12±0.618 (at 72hrs) and 11.62±0.865 (at 96hrs).

Figure 1: Shows the micronuclei frequency in Intestinal cell.

3.2 Effect of copper sulphate treatment in RBCs

Values of MN in the peripheral erythrocytes after different treatments and exposure periods have been shown in Table 1 and Figure 2 & 5. Frog treated in vivo with 3.5mg/kg of copper revealed minimum value (0.8±0.627) of MN frequency at 24hrs and maximum value (1.47±0.35) of MN frequency at 72hrs. 0.62±0.531 and 1.12±0.670 are the values of MN recorded at 48hrs and 96hrs respectively. An elevated response was observed during treatment with 5.0mg/kg wherein the values obtained were 1.32±0.942, 1.62 ±0.865,1.8±1.0 and 3.3 ±1.205 after 24, 48, 72 and 96hrs of exposure respectively. Exposure to 6.5mg/kg induced 0.97±0.865, 1.97±0.531, 3.47±0.35 and 4.8±1.232, MN frequency after 24, 48, 72 and 96hrs respectively. Frogs exposed to sublethal concentration of 8.0mg/kg revealed significant induction of MN frequency after all the exposure periods in comparison to control. Minimum frequency (2.97±0.865) and maximum frequency (3.32±0.942) and maximum frequency (11.8 ±0.848) was recorded after 24 and 96hrs of exposure through 5.62 ±0.865 and 7.47±2.232 at 48 and 72hrs of exposure respectively. Data was found to be statistically significant in all treatment doses versus respective controls (at 24hrs, p<0.05; at 48, 72 and at 96hrs, p<0.01) as shown in Table 1.
3.3 Effect of copper sulphate treatment in Kidney cells

Frequencies of MN recorded in the kidney tissue after different treatments and exposure periods have been shown in Table 1 and Figure 3 & 6. MN frequencies observed in frog treated in vivo with 3.5mg/kg increased from 0.62±0.531 at 24hrs to 1.47±0.67 at 96hrs through 0.95±0.404 at 48hrs and 1.47±0.35 at 72hrs, the value being same at 72 and 96hrs of exposure. Increased levels of MN were observed after all the exposure periods in experimental frog treated with 5.0mg/kg. Maximum frequency of MN was obtained after exposure period of 96hrs (3.45±1.350) and minimum frequency was obtained after exposure of 24hrs (1.62±0.865). Significant difference (at 24hrs, p<0.05, at 48 and 72hrs, p<0.01 and at 96hrs, p<0.001) in the incidence of MN between experimental and control values were also observed after all the exposure periods in treatment with 6.5mg/kg and 8.0mg/kg. Maximum value recorded was 4.65±1.223 and 9.8±1.840 after 96hrs of exposure in treatment with the respective concentrations. Similarly, minimum values (0.95±0.404 and 2.15±0.834 for 6.5 and 8.0mg/kg treatment respectively) were recorded after 24hrs of exposure.

Table 1: Frequencies of micronuclei (%) in different tissues of *Euphlyctis cyanophlyctis* exposed to Copper Sulphate.
<table>
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<tr>
<th>Concentration (mg/kg)</th>
<th>Duration (Hrs)</th>
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<td>4</td>
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</table>

RBCs

<p>| | | | | |
|                      |               |                  |                               |                                 |
|                      |               |                  |                               | 0.97±0.346                      |
|                      |               |                  |                               | 0.45±0.3                        |
|                      |               |                  |                               | 0.3±0.346                       |
|                      |               |                  |                               | 0.47±0.618                       |
|                      |               |                  |                               | 0.9±0.627&lt;sup&gt;a&lt;/sup&gt;           |
|                      |               |                  |                               | 0.62±0.531&lt;sup&gt;a&lt;/sup&gt;           |
|                      |               |                  |                               | 0.95±0.404&lt;sup&gt;b&lt;/sup&gt;           |
|                      |               |                  |                               | 1.47±0.35&lt;sup&gt;b&lt;/sup&gt;           |
|                      |               |                  |                               | 1.47±0.67&lt;sup&gt;c&lt;/sup&gt;           |
|                      |               |                  |                               | 1.32±0.942&lt;sup&gt;a&lt;/sup&gt;           |
|                      |               |                  |                               | 1.62±0.865&lt;sup&gt;a&lt;/sup&gt;           |
|                      |               |                  |                               | 1.62±0.865&lt;sup&gt;a&lt;/sup&gt;           |
|                      |               |                  |                               | 2.47±0.618&lt;sup&gt;b&lt;/sup&gt;           |
|                      |               |                  |                               | 2.97±1.164&lt;sup&gt;b&lt;/sup&gt;           |
|                      |               |                  |                               | 3.3±1.205&lt;sup&gt;b&lt;/sup&gt;           |
|                      |               |                  |                               | 3.45±1.350&lt;sup&gt;c&lt;/sup&gt;           |
|                      |               |                  |                               | 0.97±0.865&lt;sup&gt;a&lt;/sup&gt;           |
|                      |               |                  |                               | 1.97±0.531&lt;sup&gt;b&lt;/sup&gt;           |
|                      |               |                  |                               | 1.97±0.531&lt;sup&gt;b&lt;/sup&gt;           |
|                      |               |                  |                               | 4.15±0.834&lt;sup&gt;b&lt;/sup&gt;           |
|                      |               |                  |                               | 4.65±1.223&lt;sup&gt;c&lt;/sup&gt;           |
|                      |               |                  |                               | 2.97±0.865                       |
|                      |               |                  |                               | 2.15±0.834                       |</p>
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<td>6000</td>
<td>8.12(\pm 0.618^b)</td>
<td>7.47(\pm 2.232)</td>
<td>5.95(\pm 2.087)</td>
</tr>
<tr>
<td>72</td>
<td>4</td>
<td></td>
<td>6000</td>
<td>11.62(\pm 0.865)</td>
<td>11.8(\pm 0.848)</td>
<td>9.8(\pm 1.840^c)</td>
</tr>
</tbody>
</table>

Figure 4: Frequencies of micronuclei in Intestine after treatment with Copper.

Figure 5: Frequencies of micronuclei in RBC after treatment with Copper.
Figure 6: Frequencies of micronuclei in Kidney after treatment with Copper

4. DISCUSSION

As part of the global loss of species, amphibian populations are declining throughout world (Wake, 1991; Alford and Richards, 1999; Houlanhan et al., 2000; Blaustein and Kiesecker, 2002; Stuart et al., 2004). Concern about amphibians is due in part to their value as indicators of environmental stress (Blaustein and Wake, 1995). Many are in close contact with water as larvae, and most have some contact with land as adults. Therefore they experience both aquatic and terrestrial stressors. Since 1989 amphibians have been widely advocated as excellent biological indicators or sensitive indicators of environmental health (Blaustein and Wake, 1990; Vitt et al., 1990; Wyman, 1990; Wake, 1991). Amphibians have moist, permeable skin and unshelled eggs that are directly exposed to soil, water and sunlight, and that can readily absorb toxic substances such as heavy metals and pesticides. Although environmental pollution may interfere with amphibian growth and development, the induction of genetic damage after chronic exposure to low doses of chemicals especially heavy metals is perhaps the most important biological effect. Pesticides also effect frogs and other amphibians in different ways since they destroy their natural biotic balance in fields, induce the growth of extra legs and eyes (Kegley et al., 1997). Some pesticides are cholinesterase inhibitors. This is indication that field application of these pesticides and chemicals may be deleterious to amphibians (Jolly et al., 1978; Thybaud, 1990; Berril et al., 1993; Materna et
Moreover, amphibians are important components of many ecosystems, acting as prey, predators or herbivores. Because of their contribution to trophic dynamics, loss of amphibians will probably affect other organisms (Blaustein et al., 1994; Blaustein and Kiesecker, 2002). Khan et al. (2003a, b) and Khan and Yasmeen (2009) studied the induced effects of various chemicals on *Rana cyanophlyctis* and *R. tigrina* and reported that amphibians in general are sensitive but *R. cyanophlyctis* is more sensitive to the chemicals used by them than *R. tigrina*. Jaylet et al. (1986) first adapted the MN test to amphibians. Many MN tests on amphibians have been proven to be suitable for evaluating mutagens and genotoxic agents (Van Hummelen et al., 1989; Chen and Xia, 1993; Zoll-Moreux and Ferrier, 1999). It has been used as a measure of genotoxicity in amphibians (Ferrier et al., 1998; Compana et al., 2003) and has shown potential for *in situ* monitoring of water quality (Gauthier, 1996). MN derivative from chromosomal fragments or whole chromosomes which are not incorporated into main nucleus during cell division as a consequence of DNA fragmentation (clastogenic origin) or of alteration of the mitotic apparatus (an eugenic origin) (Schmid, 1975; Heddle et al., 1991; Norppa and Flack, 2003).

In the present study of the genotoxic effect of copper sulphate on different tissues of frog, *Euphlyctis cyanophlyctis*, the data revealed a time dependent increase in micronucleus frequencies with the increase in doses of copper in intestine, RBCs and kidney. With respect to the dose, maximum effect was found to be induced at the highest dose of exposure i.e. 8.0mg/kg while prominent effect with respect to the duration of exposure was induced after the maximum period of exposure i.e. 96hrs. Comparison between the micronucleus frequencies induced in the various tissues revealed highest MN frequencies in RBCs, followed by a narrow margin in intestine and least in kidney. In intestine, the value of MN frequency recorded ranged from 0.97±0.865 (at 24hrs with a dose of 3.5mg/kg) to 11.62±0.865 (at 96hrs with a dose of 8.0mg/kg) showing a significant increase in value of micronuclei with the increase in dose and duration of exposure excepting for 48hrs (at3.5mg/kg) treatment dose, where a fall in value of MN frequency was observed whereas at treatment dose of 6.5mg/kg, the MN frequency was almost same at 72 and 96hrs of duration. The incidence of MN frequencies observed in kidney tissue ranged from 0.62±0.531
(at 24 hrs with a dose of 3.5 mg/kg) to 9.8 ± 1.840 (at 96 hrs with a dose of 8.0 mg/kg). However, the value of MN frequencies recorded at 72 and 96 hrs of duration (with a dose of 3.5 mg/kg) were almost the same.

In general, while comparing the effect of different concentrations on the intestine, RBCs and kidney, at concentration of 3.5 mg/kg, minimum MN frequency was observed in kidney and RBCs (at 24 and 48 hrs respectively) and maximum was observed in the kidney only at 96 hrs of duration. With treatment dose of 5.0 mg/kg, minimum and maximum MN frequency value was observed in the intestine only (at 24 hrs and 96 hrs of duration respectively). At treatment dose of 6.5 mg/kg, minimum value of MN frequency was observed in kidney and maximum in the RBCs while at treatment dose of 8.0 mg/kg, minimum value was recorded in kidney (at 24 hrs) and maximum value recorded in RBCs (at 96 hrs). Thus, a positive relationship between metal concentrations and micronuclei frequencies were recorded for all the treatment groups with respect to their dose and the exposure periods compared to the control groups. The frog treated with different doses of the lead acetate (3.5 mg/kg, 5.0 mg/kg, 6.5 mg/kg, 8.0 mg/kg) at 24 hrs of duration showed significant results (p<0.05) in all the studied tissues but with increase in duration of the doses, the result obtained were highly significant, (p<0.01) in intestine and RBCs and (p<0.001) in kidney.

5. CONCLUSION
The present work investigated the cytotoxicity and genotoxicity of copper sulphate (CuSO₄) on erythrocytes and other tissues (intestine and kidney) of the Indian skittering frog, *Euphlyctis cyanophlyctis* using micronucleus test in controlled laboratory conditions with different sub-lethal doses to a maximum of 96 hrs. The results and the data evaluated indicates that CuSO₄, which is used as a fungicide, molluscicide and weedicide can be genotoxic at higher concentrations, therefore if inefficiently used in aquatic and agricultural systems, might reach levels that pose genotoxicity to tadpoles and frogs making it a potential threat to water ecosystems and to human health.

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7. REFERENCES


