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Genotoxicity Testing in Pesticide Safety Evaluation

Gargee Mohanty[1], Jyotirmayee Mohanty[2], Shubha Dipta Jena[1] and S.K. Dutta[3]
[1]*Department of Biotechnology, North Orissa University, Baripada, Odisha,*
[2]*Central Institute of Freshwater Aquaculture Bhubaneswar, Odisha,*
[3]*Department of Zoology, North Orissa University, Baripada, Odisha,*
India

1. Introduction

Genotoxicity is a recently developed branch of toxicology, which deals with the study of deleterious effects of toxic agents present in the environment on the structure and function of DNA. Direct damage to DNA is an increasingly more essential focus on ecotoxicology research for two reasons; firstly, because of the far reaching effects of genotoxins on the health of an organism and the possible future implications if the germline is affected, and secondly, because extremely sensitive methods of detecting DNA damage have been developed, which allowed the improvement of early biomarkers for xenobiotic exposure.

Genotoxicity can result in three types of genetic lesions. Firstly, single-gene mutations, also called point mutations, which include alterations in the nucleotide sequence of DNA, and may involve either the base substitution or frame – shift mutations. Second are the structural chromosomal mutations or genomic mutations which include changes in chromosomal structure, such as breaking of chromosome, or translocation of an arm, commonly called clastogenensis. Third are numerical changes in the genome; aneuploidy and/or polyploidy (Cajaraville et al., 2003).

Induction of DNA damage is one of the primary events in the initiation of carcinogenesis by chemicals. Several chemical pollutants can produce carcinogenic effects through the induction of genetic lesions.

Carcinogens can be divided into two categories; genotoxic and epigenetic. Compounds that react directly or indirectly with DNA are, in most cases genotoxic carcinogens for example; polycyclic aromatic compounds, hydrocarbons, heavy metals, alkylating agents etc. are called as genotoxic compounds.

Epigenetic carcinogens such as pesticides, asbestos, silica, immunosupressors etc. cause carcinogenesis by inducing a multitude of mechanisms that ultimately bring changes in the DNA of the organism (Cajaraville et al., 2003).

Tests for genotoxicity include systems which give an indication of damage to DNA. End points determined are unscheduled DNA synthesis (UDS), sister-chromatid exchange (SCE), single cell gel electrophoresis (SCGE), formation of DNA adducts and mitotic

recombination, chromosomal aberrations studies, micronuclei tests and many modern molecular tools and techniques which will be discussed in the subsequent sections.

1.1 Pesticides

Pesticides are substances used to control pests, including insects and plant diseases. Naturally-occurring pesticides have been in use since centuries, but widespread production and use of modern synthetic pesticides did not begin until 1940s. On a global scale approximately over five billion pounds of conventional pesticides are being in use in different areas like agricultural lands, forests, rangelands management, disease control, domestic use and many more areas annually (EPA,2001). Use of pesticides in India began in 1948 when dichloro diphenyl trichloroethane (DDT) was imported for malaria control and benzene hexachloride (BHC) for locust control (Gupta, 2004). India started pesticide production with manufacturing plant for DDT and BHC in the year 1952. In 1958, India was producing over 5000 metric tonnes of pesticides. Currently, there are approximately 145 pesticides registered for use, and production has increased to approximately 85,000 metric tonnes (Gupta, 2004). However, it is estimated that often less than 0.1 percent of an applied pesticide reaches the target pest, leaving 99.9 percent as an unintended pollutant in the environment, including in soil, air, and water, or on nearby vegetation. Pesticides can also move from the site of application via drift, volatilization, leaching and runoff. In addition to killing insects or weeds, pesticides can be toxic to a host of other non-target organisms including birds, fish, beneficial insects, plants and humans.

Pesticides have the potential to enter aquatic habitats from direct application, terrestrial runoff or wind borne drift. Aquatic toxicology is the study of the effects of environmental contaminants on aquatic organisms, such as the effect of pesticides on the health of fish or other aquatic organisms. A pesticide's capacity to harm fish and aquatic animals is largely a function of its toxicity, exposure time, dose, and persistence in the environment. Exposure of fish and other aquatic animals to a pesticide depends on its biological availability (bioavailability), bioconcentration, biomagnification, and persistence in the environment (Van der Werf, 1996; Louis *et al.*, 1996).

Fish appear to possess the same biochemical pathways to deal with the toxic effects of endogenous and exogenous agents as do mammalian species. Fish species are sensitive to enzymic and hormone disruptors (Grabuski *et al.*, 2004). Chronic exposure to low levels of pesticides may have a more significant effect on fish populations than acute poisoning. Doses of pesticides that are not high enough to kill fish are associated with subtle changes in behavior and physiology that impair both survival and reproduction. Biochemical changes induced by pesticidal stress lead to metabolic disturbances, inhibition of important enzymes, retardation of growth and reduction in the fecundity and longevity of the organism (Murty, 1986; Khan & Law, 2005). Liver, kidney, brain and gills are the most vulnerable organs of a fish exposed to the medium containing any type of toxicant. Fish show restlessness, rapid body movement, convulsions, difficulty in respiration, excess mucous secretion, change in color, and loss of balance when exposed to pesticides. Some agrochemicals can indirectly affect fish by interfering with their food supply or altering the aquatic habitat, even when the concentrations are too low to affect fish directly. It is important to examine the toxic effects of pesticides on fish since they constitute an important link in food chain and their contamination by pesticides imbalances the aquatic system.

Organophosphate (OPs) and the carbamate group of pesticides are the recently developed pesticides used against many pests. The chemicals in these classes kill insects by disrupting

the brain and the nervous system. Some of the commonly used OPs in use are phorate, disulfoton, dimethoate, dichlorvos, diazinon, sevin, chlorpyrifos, etc. These pesticides are esters, amides, or simple derivatives of phosphoric and thiophosphoric acids. Chemically carbamate is a salt of or an ester of carbamic acid. The carbamate compounds include furadan, carbaryl, carbofuran, methomyl, and oxamyl.

Phorate and furadan are two most widely used pesticides in agriculture in India. Phorate is an organophosphate whereas furadan is a carbamate. According to World Health Organisation (WHO), phorate is a Class IA (Extremely Hazardous) pesticide. Phorate 10% CG falls under Class IB (Highly Hazardous). The Food and Agriculture Organisation (FAO) recommends that products that fall under Class IA and Class IB should not be used in developing countries because of safety concerns related to these products. Furadan 3G has been classed as highly toxic in relation to other pesticides (Palmer & Schlinke, 1973). Together with organophosphorus compounds it is held responsible for most of the accidental poisonings in animal agriculture.

Aquaculture has emerged as one of the most promising and fastest growing food producing sectors in the world. It has grown at an annual rate of 10% from 1984 to 1995. India occupies second position in the global aquaculture production. Carp accounts for half of the world inland aquaculture production. India is also regarded as 'Carp country' as carps contributes maximum to the fisheries industry of the country (FAO, 2005) and the major species which contributes to the production are Indian major carps (IMCs) *viz*., *Labeo rohita, Catla catla, Cirrihinus mrigala.* Together these carp species contribute about 23, 00, 000 tonnes per year of total aquaculture production (FAO, 2005). Among all the IMCs rohu is the most preferred species and contribute about 41% of the total carp production (FAO, 2005).

In such a scenario it is important to monitor the activities of the fish in the changing environmental conditions. It is important to focus on the changes in the genetic system as some of the changes very often show lethal effects in the individual organism.

2. Genotoxicity

Genotoxicity is a recently developed branch of toxicology and is a general term that refers to alterations to the gross structure or content of chromosomes (clastogenicity) or base pair sequence (mutagenicity) by exposure to toxic agents. Genotoxicants are very important components to be monitored as they cause mutations that often lead to cancers. Further, understanding the changes at the DNA level of an organism exposed to pollutants is essential to demonstrate an impact at the ecological relevant population or community level (Shugart &Theodorakis, 1996).

An understanding of the processes and mechanisms operating at the genetic level would help to identify the more complex changes at higher levels of organization. DNA molecules bear highly reactive groups and are thus targeted and modified by a range of genotoxic compounds, including reactive oxygen species (ROS), metabolites and organic and inorganic electrophiles, such as heavy metals (Adams, 2001). Contaminants may impact genetic material either directly through interaction with nucleotides or indirectly through impacting natural cellular function such as impeding DNA replication, transcription etc. Direct acting genotoxins include chemical compounds that are electrophilic and hence can potentially react directly with the nucleophilic sites within DNA molecules, and such compounds include carbonium ions, episulfonium ions, free radicals, diazonium ions, strained lactones, sulfonates, halo ethers etc. (Williams & Weisburger, 1991).

In addition to such direct binding chemicals, a large number of chemically inert compounds may be transformed into metabolites with electrophilic and/or nucleophilic properties and so become able to form different changes in the structure and function of DNA (Hodgson & Levi, 1996). Substances such as polycyclic aromatic hydrocarbons, aromatic amines, azo compounds, nitroaryl compounds and nitrosamines are non-polar lipophilic components, which would build up in the organism if they were not actively transformed into water soluble derivatives and excreted out (Sipes and Gandolfi, 1991). This cellular detoxification mechanism produces intermediates, which are more reactive than the parent compound and/or their metabolites, and may therefore act as genotoxins forming DNA adducts. A direct relationship between exposure to polycyclic aromatic compounds and the level of DNA adducts has been shown in several fish species, including English sole (*Pleuronectes vetulus*), winter flounder (*Pseudopleuronectes americanus*), and oyster toadfish (*Opsanis tau*) (Collier *et al.,* 1993).

Apart from forming DNA adducts, genotoxins bring major forms of damage to DNA which include damage to the phosphodiester backbone, changes in ribose sugars and in the purine and pyrimidine bases.

2.1 Genotoxic effects of pesticides in living organisms

Pesticides form an important group of environmental pollutants and the genotoxic effects of several chemical groups of pesticides have been shown by *in vivo* and *in vitro* experiments (Bolognesi, 2003; Abdollahi *et al.,* 2004; Kaushik & Kaushik 2007). However, genotoxicity data for a great majority of pesticides are scanty (Gandhi *et al*., 1995), and where ever exist; the findings from different laboratories are contradictory for many formulations.

Among pesticides, organophosphates and organochlorines are constantly a matter of worry because of their wide use. Both groups of chemicals bear the potentiality to cause genotoxicity and carcinogenicity (Kaushik & Kaushik, 2007). Apart from the OPs and OCs, a new generation of pesticides, the synthetic pyrethroids, once claimed to possess a great safety factor (Kaushik & Kaushik, 2007), is reported to be genotoxic (Bhunya & Pati, 1990). In a survey including halogenated hydrocarbons, organophosphates, carbamates and other classes of pesticides, Borzsonyi *et al*. (1984) found 29 pesticides to be definite or suspected genotoxic agents.

Several studies on the effect of pesticides in different fish species have been carried out recently using different genotoxicological tools (Hai *et al.,* 1997; Das & John, 1999; Pena-Llopis *et al*., 2003). However, genotoxicity studies of pesticides on various indigenous fish species of India are very limited. Banu *et al.* (2001) studied the genotoxic effects of monocrotophos, one of the popular organophosphate pesticides on the fish *Tilapia mossambica* using comet assay and found a dose-related increase and time-related decrease of comet tail length. Pandey *et al*. (2006) evaluated the genotoxic potential of Endosulphan in *Channa punctatus*. They exposed the fish to different doses of pesticides and assessed the DNA damage in gill and kidney tissues by comet assay. The authors found a dose-dependent response in both the tissues.

Oxidative damage is thought to be an important mechanism in the DNA damage caused by organophosphate pesticides (Hodgson & Levi, 1996). More than 100 different oxidative modifications to DNA by OPs have been described (Loft & Poulsen, 2000), and several DNA base oxidation products are known to be mutagenic, including 8-oxo-7, 8-dihydro-2-deoxyguanosine and thymine glycol (Halliwell, 2002). The adverse effects caused due to the generation of ROS and reactive oxygen intermediates (ROI) are lifted off in the organism as

soon as the antioxidant system present in the organisms gets activated in the organism. Wild (1975) focused attention on the electrophilic activity as the fundamental cause of the toxicity of these compounds and considered DNA alkylation as one of the reasons for the production of genotoxicity (Hodgson & Levi, 1996; Yadav & Kaushik, 2002). Oxidative stresses due to the OP pesticides are also well evidenced in fishes like *Cyprinus carpio,* catfish *Ictalurus nebulosus* (Hai *et al.,* 1997) and in the European eel *Anguilla anguilla* (Pena-Llopis *et al.,* 2003).

Carbamates constitute another major group of pesticides and many of them have been reported to show mutagenic properties in various test systems. Zineb, a carbamate fungicide, has been reported to be mutagenic in both somatic and germ-line cells in Drosophila (Tripathy *et al*., 1988). In another report, the same research group has reported that the fungicide ziram is mutagenic in the wing, eye and female germ-line mosaic assays, and in sex linked recessive lethal test in *Drosophila melanogaster* (Tripathy *et al*., 1989). In a more recent study, Franekic *et al*. (1994) reported that ziram, zineb and thiaram are mutagenic in a battery of bacterial test systems. The thiocarbamate pesticide malinate and vernolate have been reported to cause chromosomal changes like SCE and chromosomal aberrations *in vitro* and increased frequency of polychromatic erythrocytes in mouse bone marrow cells (Pinter *et al*., 1989). Studying on the genotoxicity of aldicarb, aldicarbosulfone, aldicarb oxide, carbofuran and propoxur, Canna-Michaelidou & Nicolaou (1996) reported that all the pesticides were 'suspect genotoxic' directly and after S9-actvation in mutatox test. Genotoxicity of carbofuran, carbosulfan and methyl isothiocyanate, a component of the pesticide carbaryl, has also been reported (Chauhan *et al*., 2000; Rencuzogullari and Topaktas, 2000; Kassie *et al*., 2001).

3. Tools in the study of Genotoxicity

In recent years a number of assays have been framed to evaluate the genotoxic effects of chemicals and other potent environmental toxicants in microbes, plants and animals. Emphasis has been given on the effective detection of mutations as it not only provides the basis for biomonitoring, but also serves to identify vulnerable stages in the life history of a species, the nature and dynamics of causal agents and associated phenotypic and population-level effects (Shugart and Theodorakis, 1994).

Several genotoxic effects like, DNA adducts, DNA breakage, chromosomal aberrations and sister chromatid exchanges can be observed in organisms exposed to various pollutants (Venier *et al.,* 1997; Das & John, 1999). Several assays have been successfully employed in the detection of the various genotoxic effects in the living organisms.

Sister chromatid exchange has successfully been used in many fish to assess the toxicity of the test chemical on the DNA. It was used by Kligerman (1979) in *Umbra limi* in the assessment of the affect of some mutagenic agents.

Chromosomal aberration is another tool, which is applied in the field of genotoxicological studies. Different chromosomal aberrations, such as breaks, ring chromosomes and dicentric chromosomes, have been detected in kidney cells after the injection of three fish species (common carp, *Cyprinus carpio*, tench, *Tinca tinca*; grass carp, *Ctenopharyngodon idella*) with aflatoxins B, aroclor 1254, benzidine, benzo [a] pyrene and 20-methylcholanthrene (Al-Sabiti, 1985; Cajaraville *et al.,* 2003). Das & John (1999) evaluated the effect of two organophosphorus pesticides, methyl parathion and phosphamidon on *Etroplus suratensis* using chromosomal aberration as the genetoxicological test tool.

Another less time consuming and authentic way of monitoring the genotoxic effects of pollutants and mutagens is micronuclei formation. There has been an increasing interest in the use of micronucleus test (MNT) as an index of cytogenetic damage in fish and other marine vertebrates and invertebrates (Al-Sabiti, 1994; Venier *et al.*, 1997). Various studies have been shown that the peripheral erythrocytes of fish have a high incidence of micronuclei under laboratory conditions. Among the recent assays, single cell gel electrophoresis or called comet assay has immense use in the detection and evaluation of genotoxic compounds in several test systems (Singh *et al.*, 1988; Collins, 2004; Pandey *et al.*, 2006). Several reviews have been published on the acceptance of comet assay in monitoring the effects of several potent genotoxic agents on the DNA of different animals (Moller *et al.*, 2000; Bolognesi, 2003; Collins, 2004).

Recently, polymerase chain reaction (PCR) has been used in the studies of genotoxicity (Atienzar & Jha, 2006). The detection of unknown mutations involves the identification of heteroduplexes or mismatches between mutated and wild type sequences, based either upon the electrophoretic properties of the sequences or upon the selective chemical modifications of such sequences. The two main types of electrophoretic methods are denaturing gradient gel electrophoresis (DGGE) assay, and the single stranded conformational polymorphisms (SSCP) assay (Cajaraville *et al.,* 2003). The DGGE separates the wild type and mutant DNA heteroduplexes, whereas the SSCP separates single stranded wild type and mutant DNA sequences due to differences in secondary structure (Cajaraville *et al.,* 2003). Although such procedures detect a variety of base substitutions, frame shifts and deletions, the methods fail to detect all mutations present (Cajaraville *et al.,* 2003). Detection of known mutations involves mismatched primer techniques such as the allele-specific oligonucleotide technique, or the allele-specific amplification method. Both of these involve the amplification of mutant and wild type sequences. These approaches are based on the successful amplification of mutant sequences with primers specific to the suspected mutation and therefore require sequence information of the targeted areas (Cajaraville *et al.,* 2003).

Among different types of PCRs, RAPD-PCR and AP-PCR offers a great scope in the detection and comparison of changes between the normal and genotoxicants exposed groups of animals in genotoxicity studies. Despite the problems concerning with the reproducibility and complexity of patterns (Atienzar *et al.,* 1998; Singh & Roy, 1999), these techniques have shown several advantages for the detection of genomic mutations, such as ease, speed and low cost of experiments and the ability to clone aberrant fragments (Navarro & Jorcano, 1999). While these techniques have so far been mainly used for investigation of human cancer tissues, its potential has been shown in the study on Japanese medaka (*Oryzias latipes*) where a correlation between gamma-rays induced genomic damage and embryo malformations was demonstrated (Kubota *et al.,* 1992).

Advances in transgenic approaches that include knockout gene technology and gene silencing have been proven to be powerful assays towards the observation of mutational changes, whereby transgenes are introduced at the zygotic stage of development act as target genes, capable of a phenotypic response to mutational events (Gossen & Vijig, 1993; Bailey *et al.,* 1994). The greatest potential for new biomarkers of early effect lies in toxicogenomics, a field of study that examines how the entire genome responds to toxicants or other hazards (Toraason *et al.,* 2004).

3.1 Comet assays

Comet assay otherwise called single cell gel electrophoresis was first described by Ostling & Johanson (1984) and numerous modifications have been reported to date to allow

detection of various types of DNA damage. The principle of the test is remarkably simple. DNA damage is quantified by the proportion of DNA which migrates out of the nuclei toward the anode when individual cells or isolated nuclei, embedded in a thin agarose layer, are subjected to electrophoresis that results in a "comet-like" shape of nuclei. This enables quantification of DNA in comet tails after staining with an appropriate fluorochrome (e.g. ethidium bromide) or with silver staining protocol (Garcia *et al.,* 2007). The comets can be either classified by visual examination (visual scoring) (or measured from morphological parameters obtained by image analysis and integration of intensity profiles (Collins, 2004).

According to visual scoring, the comets are classified into five different classes, from 0 (no tail) to 4 (almost all DNA in tail), which give sufficient resolution to make distinction among all the five comet classes. The comets are given different class on the basis of the length of the tail, amount of DNA present in the tail. If 100 comets are scored, and each comet assigned a value of 0 to 4 according to its class, the total score for the sample gel will be between 0 and 400 "arbitrary units or damage index"(Collins, 2004, Heaton *et al.,* 2007).

Different types of comet assay for different purposes have been described by Collins (2004). The different modified versions of comet assays were standardized by the employment of various combinations of neutral and alkali pH solutions immediately prior and during electrophoresis. Exposure of DNA to high alkali prior to electrophoresis allows for the preferential detection of DNA single-strand breaks (SSB). This procedure also detects some alkali labile sites depending on the pH of the alkali unwinding solution and the duration of exposure. The majority of alkali labile sites become detectable when electrophoresis is performed in alkaline solution.

Major advantage of comet assay over other techniques is the highly sensitive detection ability for both double and single-strand breaks. Levels of detection have been reported to be as low as one break per chromosome (Mitchelmore & Chipman, 1998) or as few as 200 breaks per cell (Rojas *et al.,* 1999). Double-strand breaks (DSB) cause comet formation even under completely neutral conditions. Treatment of DNA after lysis with specific DNA repair enzymes can be used for the selective detection of abasic sites or pyrimidine dimers (Angelis *et al.,* 2000).

The comet assay being a short - term genotoxicity test has been widely used to reveal a broad spectrum of DNA-damaging agents capable of inducing strand breakage, cross-links and alkali - labile sites (Singh *et al.,* 1988; Fairbairn *et al.,* 1995; Pandey *et al.,* 2006). This technique has been applied in several genotoxicity studies (Pandey *et al.,* 2006), ecotoxicology (Cotelle & Ferard, 1999), biomonitoring (Collins *et al.,* 1997) and clinical radiobiology (Olive, 1999). Its versatility has allowed the investigation of repair mechanisms (Alapetite *et al.,* 1997), in the detection of apoptosis, alkylating, oxidizing and cross-linking agents.

Many reviews details the employment of comet assay in the assessment of the genotoxic potential of many compounds, which notably include metals, pesticides, opiates, nitrosamines and anticancer drugs (Collins, 2004). A significant advantage of the comet assay is its applicability to any eukaryotic organism and nucleated cell type (Mohanty *et al.,* 2009a, 2009b, 2011). This assay can be applied both *in vitro* and *in vivo* conditions after an exposure to different potent genotoxic and mutagenic agents (. This assay is also very useful, as it requires very small cell samples (<10 000 cells) (Shugart, 2000).

In last two decades comet assay has been adapted by many workers to evaluate the genotoxicity potentialities of pesticides in human populations (Garaj-Vrhovac & Zeljezic,

2001). During a study on workers involved in the production of a variety of pesticides, an increase in DNA damage in peripheral blood lymphocytes was found (Garaj-Vrhovac & Zeljezic, 2000). In another experiment on Croatian workers occupationally exposed to a complex mixture of pesticides showed an increase in the values of the comet assay parameters (Garaj-Vrhovac & Zeljezic, 2001) indicating that the pesticides to be the potent genotoxic substances. Grover *et al.* (2003) evaluated the DNA damage in Indian pesticide production workers. Blood leukocytes of a group of 54 pesticide workers and an equal number of control subjects were examined for genotoxicity in this study. The two groups had similar mean ages and smoking prevalence. The mean comet tail length was set as the parameter to measure the extent of DNA damage. The exposed workers had significantly greater mean comet tail lengths than those of control group. The authors put forwarded the possible reasons of comet formation with greater tail length in exposed groups could have happened due to the single-strand breaks in DNA and/or during the repair of DNA strand breaks, DNA adduct formation or DNA-DNA and DNA-protein cross links. Occupational exposure to xenobiotics might have resulted in their covalent binding to DNA, which might lead to chromosome alterations and could be an initial event in the process of chemical carcinogenesis (Fairbairn *et al.,* 1995; Shah *et al.*, 1997).

Comparably fewer studies have been conducted with aquatic invertebrates and these have been restricted to bivalve species such as the marine mussel (*Mytilus edulis*) (Accomando *et al.,* 1999), the zebra mussel (*Dreissena polymorpha*) (Pavlica *et al.,* 2001), Mediterranean mussel (*Mytilus galloprovincialis*) (Frenzilli *et al.,* 2001) and oyster (*Crassostrea americanus*) (Nacci *et al.,* 1996).

This assay has been carried out in fishes such as bullhead (*Ameriurus nebulosus*) Common carp (*Cyprinus carpio*) (Pandrangi *et al.,* 1995), brown trout (*Salmo trutta*) (Belpaeme *et al.,* 1996), flounder (*Pleuronectes americanus*) (Nacci *et al.,* 1996), rainbow trout (*Oncorhynchus mykiss*) (Devaux *et al.,* 1999), butterfish (*Pholis gunnellus*) (Bombail *et al.,* 2001), zebra fish (*Danio rerio*) (Schnurstein and Braunbeak, 2001) tilapia (*Tilapia mossambica*) (Banu *et al.* 2001) and *Channa punctatus* (Pandey *et al.,* 2006) etc.

3.2 RAPD - PCR

The 1993 Nobel Prize for chemistry was awarded to Dr. Kary Mullis, for having invented the polymerase chain reaction (PCR). This remarkable technology has revolutionized the field of molecular biology and has been used in diverse areas of research such as evolution clinical medicine, forensic science, pathogen detection, genotoxicant detection etc. Subsequently, new PCR based methods have been developed. In particular, Williams *et al.* (1990) and Welsh & McClelland (1990) developed the random amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AP-PCR), respectively. The random amplified polymorphic DNA technique uses single primer of arbitrary sequence to amplify the segment of DNA between two inverted priming sites on complementary strands, separated by 150-5000 base pairs. This technique has many advantages over other molecular techniques engaged in the similar type of works. The advantages include the need of minute quantity of template DNA. The technique has the potential to access many loci, thus lead to the identification of loci those were not previously known to be under contaminate-selective pressure within the DNA. Finally, RAPD primers amplify from inverted repeat sites within the DNA. This is important because certain sequences that contain inverted repeats (e.g. transposons) have shown to be responsive to DNA damaging agents (Theodorakis & Shugart, 1997).

Moreover, RAPD is more cost effective and less labour intensive than the similar kinds of molecular techniques like arbitrarily primed-PCR (AP-PCR). This technique does not require any previous knowledge of the species genome sequence. It also avoids the use of radioisotopes. In the field of ecotoxicology, most RAPD studies describe the RAPD changes such as differences in band intensity as well as gain/loss of stable RAPD bands (Atienzer & Jha, 2006).

RAPD-PCR has been used to detect not only DNA damage and mutations but also changes in genetic diversity and gene frequencies. The first study measuring genotoxic effects using the RAPD assay was performed by Savva *et al.* (1994). In the study, the RAPD profiles generated from rats exposed to benzo [a] pyrene (B [a] P) revealed the appearance and disappearance of bands in comparison to control patterns. These changes observed in the fingerprints of exposed animals were supposed to be produced due to the presence of DNA adducts; mutations or DNA strand breaks. In genotoxicity studies, the RAPD approach adopts comparison of RAPD profiles obtained from control and treated population at a defined time. Krane *et al.* (1999) suggested that RAPD based measures of genetic diversity may be suitable for development as a sensitive means of directly assessing the impact of environmental contaminants upon ecosystems. Theodorakis *et al.* (1999) indicated that the probability of survival and degree of DNA strand breakage in radionuclide-exposed mosquitofish were dependent on RAPD genotype, and were consistent with the hypothesis that the contaminant-indicative RAPD bands were markers of loci which imparted a selective advantage in radionuclide-contaminated environments.

RAPD-PCR has also been successfully utilized in *in vitro* genotoxicity test. Becerril *et al.* (1999) studied the effect of the well known carcinogen, mitomycin C in RTG-2 fish cell line by RAPD-PCR. The bands obtained were analyzed to show a difference in the banding pattern of control with that of the exposed groups.

4. Application of comet assay and RAPD – PCR in the assessment of genotoxic effects of two pesticides on *Labeo rohita* fingerlings

4.1 Comet assay

A study was carried out by Mohanty *et al.*, (2009a, 2009b and 2011) with an objective to study the genotoxic effects of two pesticides, phorate, an organophosphate and furadan, a carbamate on rohu (*Labeo rohita*) fingerlings.

To evaluate the DNA damage, rohu fingerlings were exposed to control, 0.001, 0.002 and 0.01ppm of phorate control, 0.002, 0.004 and 0.02ppm of furadan for a total time period of 96h. Samplings were carried out at 24, 48, 72 and 96h, and six numbers of fishes were sampled at each sampling hour for each dose of pesticide. Three tissue samples such as blood, liver and gill were selected for the study. DNA damage in these tissue samples of pesticide treated fish was carried out by alkaline comet assay and the comet slides were stained with silver stain for visualization. Two slides per fish were prepared and 100 randomly selected non-overlapping cells were scored for comets. The comets were visually assigned a score on an arbitrary scale of 0–4 (i.e., ranging from 0 – undamaged to 4 - maximum DNA damage) based on perceived comet tail length migration and relative proportion of DNA in the comet tail. The mean percent of overall DNA damaged cells was calculated by adding the number of cells scored under comet classes 1, 2, 3 and 4, and was termed as total percentage of damaged cells. The extent of DNA damage score in terms of arbitrary units (AU) for each slide was derived by multiplying the number of cells assigned

to each class of damage by the numeric value of that class and summing the overall values {may vary within a range of 0 (all cells undamaged - 0×100) to 400 (all cells damaged at class 4 – 4 X 100)} (Fig.1). The statistical analyses were carried out with Microsoft excel 2007 and SPSS statistical package version 10. The test of significance was determined by nonparametric Mann-Whitney U test at 5% level.

Results obtained from alkaline comet assay in the blood, liver and gill cells of rohu fingerlings exposed phorate and furadan showed that the baseline damage was minimal in blood cells compared to the liver and gill cells, whereas the DNA damage was maximum in liver cells followed by the gill and blood cells. The fishes treated with 0.001ppm phorate dose did not show much difference in DNA damage compared to the control groups and hence was concluded that the dose was low to cause genotoxic effects in rohu fingerlings. However, the significant differences in DNA damage observed at 0.002 and 0.01ppm dose levels proved phorate to possess potent genotoxic effects on rohu DNA (Fig 2 and Table 1). Similarly, results obtained from furadan treated fishes revealed that furadan at 0.002ppm dose or more was capable to produce sufficient DNA damage and hence, was also highly genotoxic to rohu (Fig 3; Table 4).

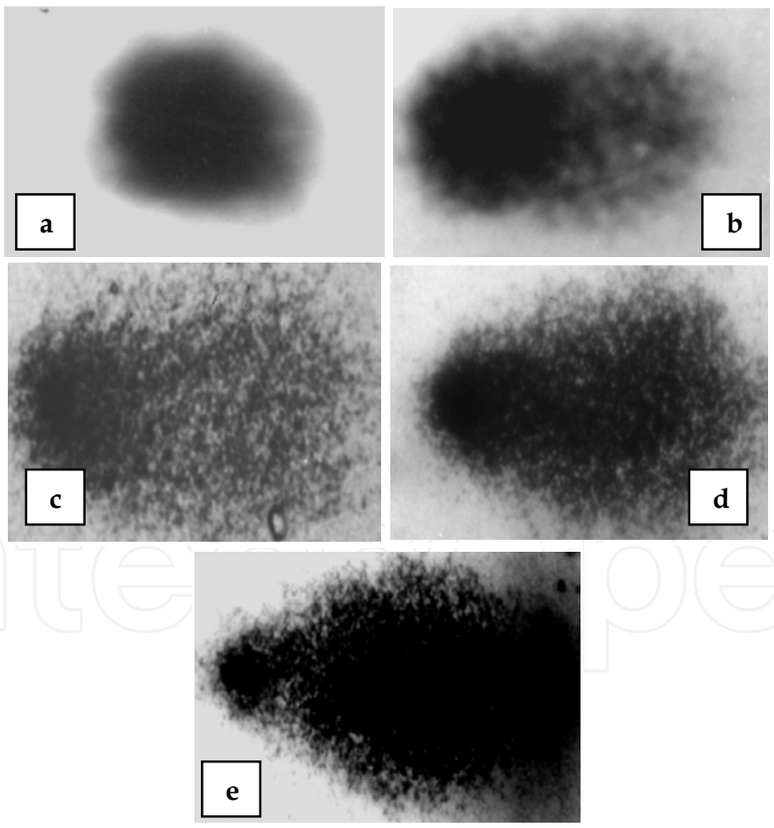
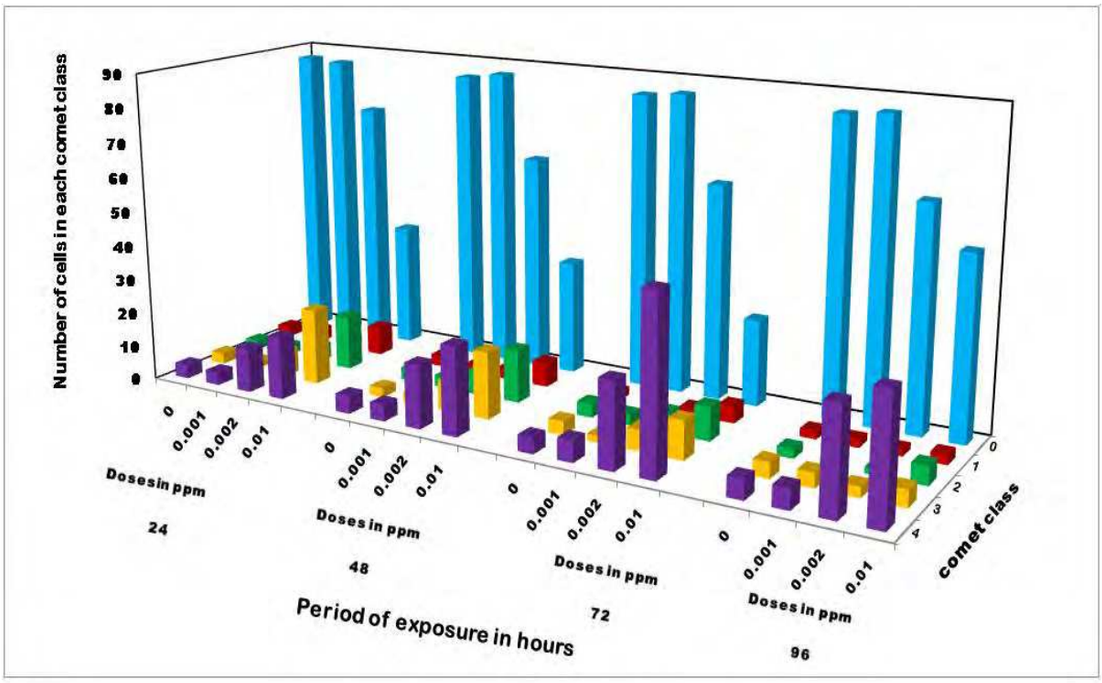
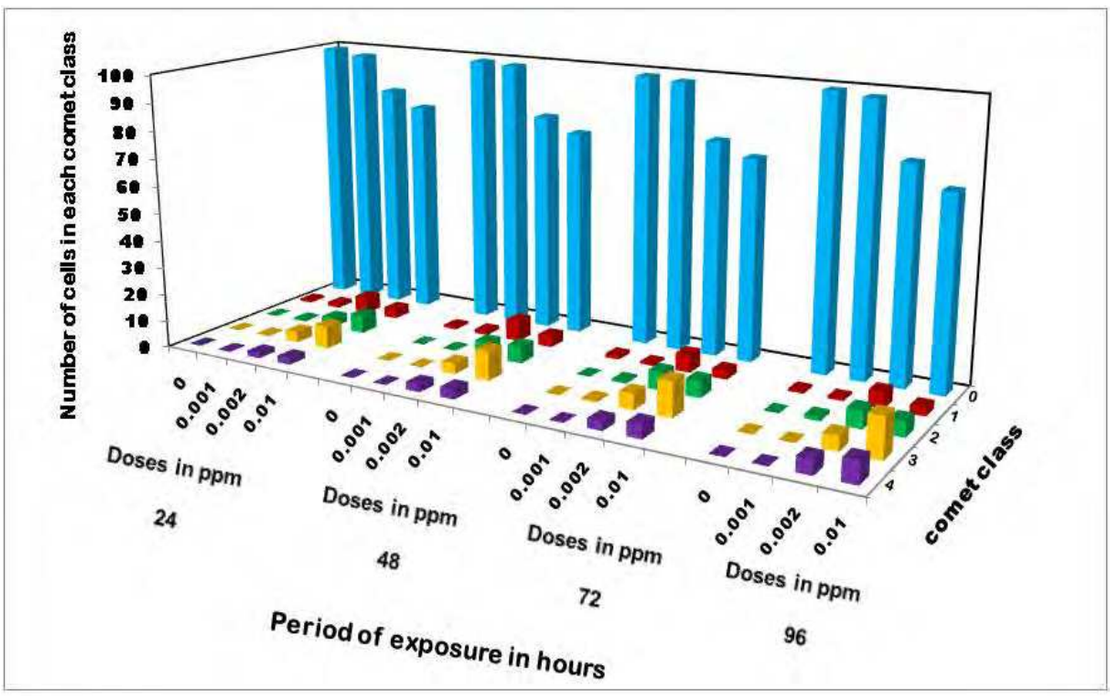


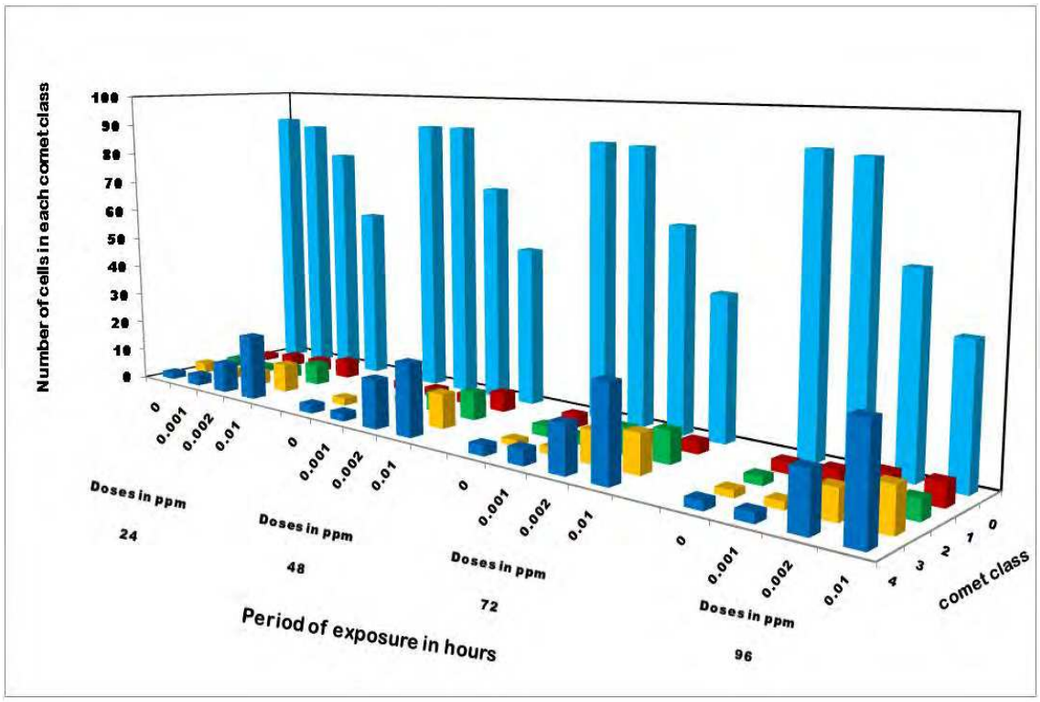
Fig. 1. Representative comet images of nuclei from cells of *Labeo rohita*. a. class 0 (undamaged), b. class 1, c. class 2, d. class 3, e. class 4 (maximum damage).


Number of cells in each comet class
90
80
70
60
50
40
30
20
10
0
0
0.001
0.002
0.01
Dosesin ppm
24
Doses in ppm
48
Doses in ppm
72
Doses in ppm
96
Period of exposure in hours
comet class
0
1
2
3
4

(a)

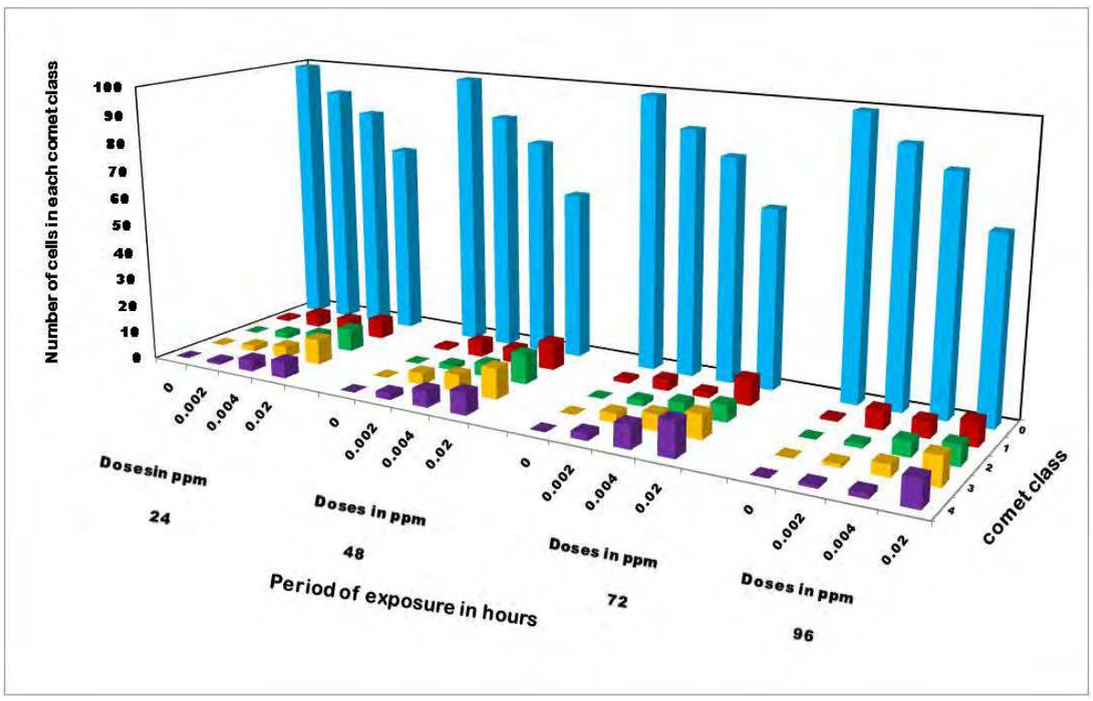

Number of cells in each comet class
100
90
80
70
60
50
40
30
20
10
0
0
0.001
0.002
0.01
Doses in ppm
24
Doses in ppm
48
Doses in ppm
72
Doses in ppm
96
Period of exposure in hours
comet class
0
1
2
3
4

(b)

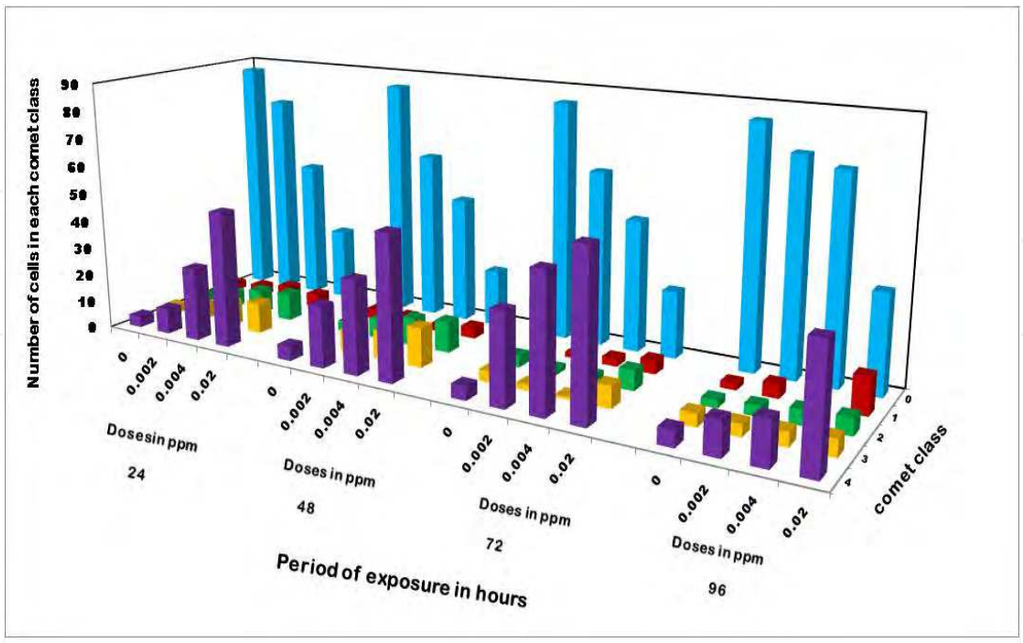


(c)

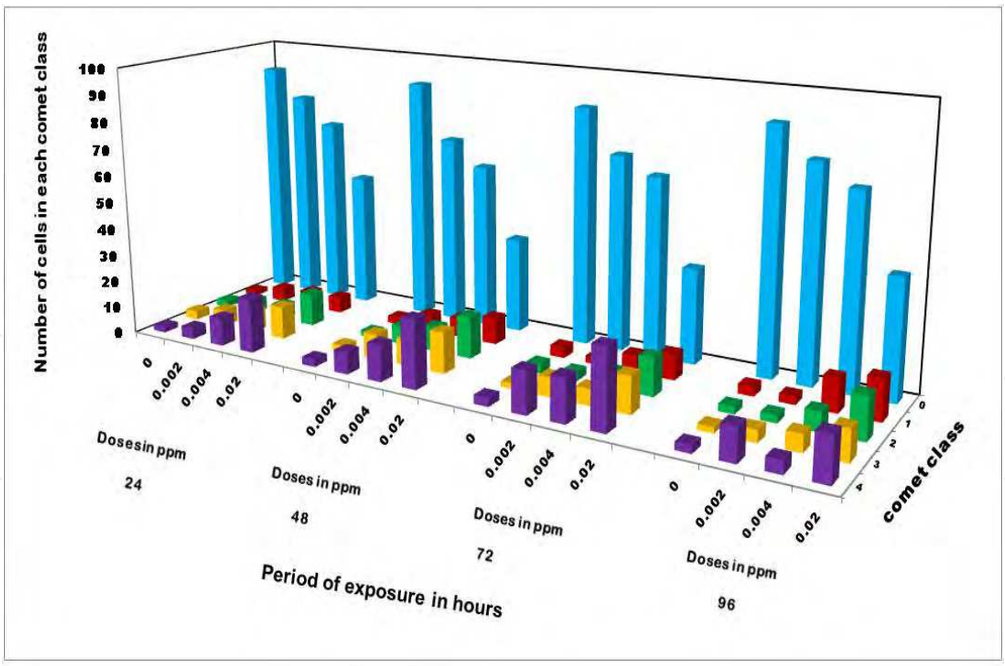
Fig. 2. Frequency of cells in each comet class (%) measured by alkaline comet assay in (a) blood (b) liver (c) gill cells of *Labeo rohita* following exposure to different doses of phorate for various time periods.



(a)



(b)



(c)

Fig. 3. Frequency of cells in each comet class (%) measured by alkaline comet assay in (a) blood (b) liver (c) gill cells of *Labeo rohita* following exposure to different doses of furadan for various time periods.

Hours of exposure	Phorate doses (ppm)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)
		Blood Cells		Liver Cells		Gill Cells	
24	0.00	0.83± 0.30[a]	0.83 ±0.30[a]	13.17 ± 0.79[a]	34.33 ± 3.21[a]	9.67 ± 0 .71[a]	25.02 ± 1.80[a]
	0.001	2.67± 0.42[b]	4.51± 1.05[a]	13.83 ± 0.60[a]	34.33 ± 2.40[a]	12.00 ± 0.36[a]	30.33 ± 1.47[b]
	0.002	15.5± 0.56[c]	32.66±1 .56[b]	27.83 ± 0.47[b]	85.83 ± 1.92[a]	22.00 ±1.06[b]	63.84 ± 3.28[c]
	0.01	21.01± 0.57[d]	51.52±1 .45[c]	64,00 ± 0.57[c]	177.5 ± 1.95[a]	43.00 ± 0.57[c]	131.34 ± 1.83[d]
48	0.00	1.00± 0.36[a]	1.00±0. 36[a]	14.84 ± 0.60[a]	39.36 ± 2.76[a]	10.00 ± 0.57[a]	25.32 ± 2.33[a]
	0.001	1.67± 0.49[a]	1.84±0. 60[a]	13.16 ± 0.60[a]	36.64 ± 2.29[b]	10.00 ± 0.51[a]	24.84 ± 1.01[a]
	0.002	19.66± 0.61[b]	41.16±1 .47[b]	37.34 ± 0.33[b]	116.36 ± 1.52[c]	29.84 ± 0.94[b]	92.68 ± 2.55[b]
	0.01	24.68± 0.42[c]	63.16±1 .77[c]	67,00 ± 0.36[c]	196.66 ± 1.89[d]	48.83 ± 0.30[c]	148.66 ± 2.88[c]
72	0.00	1.33± 0.21[a]	1.33±0. 21[a]	15.17 ± 0.60[a]	41.18 ± 1.57[a]	12.49 ± 0.61[a]	29.47 ± 1.20[a]
	0.001	2.32± 0.42[a]	4.47±1. 11[a]	13.99 ± 0.57[a]	39.49 ± 1.91[b]	13.00 ± 0.68[a]	35.17 ± 2.18[b]
	0.002	22,00± 0.44[b]	49.83±1 .49[b]	38.16 ± 0.47[b]	128.98 ± 2.51[c]	36.00 ± 0.36[b]	109.00 ±1.61[c]
	0.01	26.67± 0.66[c]	72.67±1 .66[c]	75.17 ± 0.60[c]	255.34 ± 2.51[d]	55.00 ± 0.36[c]	176.34 ± 1.25[d]
96	0.00	0.83± 0.30[a]	0.83±0. 30[a]	15.17 ± 0.65[a]	43.85 ± 1.70[a]	11.17 ± 0.65[a]	26.67 ± 1.87[a]
	0.001	2.16± 0.30[b]	3.49±0. 42[a]	14.00 ± 0.25[a]	41.67 ± 1.22[b]	12.49 ± 0.56[a]	28.98 ± 2.11[a]
	0.002	22.34± 0.42[c]	54.52±1 .38[b]	36.00 ± 0.68[b]	131.83 ± 2.74[c]	41.17 ± 0.47[b]	119.35 ± 3.19[b]
	0.01	30.34± 0.76[d]	87.19±2 .98[c]	47.83 ± 0.30[c]	167.3 ± 2.27[d]	58.17 ± 0.65[c]	186.68 ± 1.58[c]

* indicates significant difference from respective control (0.00ppm phorate) at $p < 0.05$ by Mann-Whitney U nonparametric test.
Different alphabets in damage cell percentage within each time period indicate significant difference at $p < 0.05$ by Mann-Whitney U nonparametric test.

Table 1. DNA damage measured by alkaline comet assay {DNA damaged cells (%) and DNA damage scores in arbitrary units (AU)} in different cells of *Labeo rohita* following exposure to different doses of phorate for various time periods.

Hours of exposure	Furadan doses (ppm)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)	DNA damaged cells (%) (mean ± SE)	AU (mean ± SE)
		Blood Cells		Liver Cells		Gill Cells	
24	0.00	0.83 ± 0.30[a]	0.83 ± 0.30[a]	13.17 ± 0.79[a]	34.33 ± 3.21[a]	9.67 ± 0.71[a]	25.02 ± 1.80[a]
	0.001	10.17 ± 0.30[b]	19.34 ± 0.76[b]	25.17 ± 0.30[b]	70.17 ± 1.30[b]	20.17 ± 0.30[b]	49.68 ± 0.98[b]
	0.002	17.17 ± 0.30[c]	41.68 ± 1.62[c]	49.83 ± 0.40[c]	154.83 ± 1.42[c]	29.82 ± 0.47[c]	82.80 ± 0.49[c]
	0.01	30.34 ± 0.42[d]	74.85 ± 1.53[d]	74.16 ± 0.30[d]	250.98 ± 1.63[d]	50.00 ± 0.36[d]	146.00 ± 1.21[d]
48	0.00	1.00 ± 0.36[a]	1.00 ± 0.36[a]	14.84 ± 0.60[a]	39.36 ± 2.76[a]	10.00 ± 0.57[a]	25.32 ± 2.33[a]
	0.001	14.00 ± 0.57[b]	31.16 ± 1.51[b]	40.00 ± 0.25[b]	128.01 ± 1.46[b]	30.17 ± 0.30[b]	79.34 ± 0.66[b]
	0.002	22.17 ± 0.30[c]	57.18 ± 1.85[c]	54.83 ± 0.47[c]	183.66 ± 1.62[c]	39.00 ± 0.51[c]	107.33 ± 2.12[c]
	0.01	39.83 ± 0.40[d]	96.66 ± 1.28[d]	79.83 ± 0.16[d]	271.32 ± 1.33[d]	64.99 ± 0.51[d]	184.81 ± 2.86[d]
72	0.00	1.33 ± 0.21[a]	1.33 ± 0.21[a]	15.17 ± 0.60[a]	41.18 ± 1.52[a]	12.66 ± 0.61[a]	29.64 ± 1.30[a]
	0.001	11.83 ± 0.47[b]	27.99 ± 1.75[b]	38.00 ± 0.25[b]	140.34 ± 1.94[b]	28.17 ± 0.30[b]	91.33 ± 1.89[b]
	0.002	20.00 ± 0.57[c]	60.17 ± 2.12[c]	53.33 ± 0.42[c]	199.17 ± 1.42[c]	34.16 ± 0.30[c]	99.65 ± 1.66[c]
	0.01	36.33 ± 0.33[d]	96.66 ± 1.35[d]	76.33 ± 0.42[d]	269.16 ± 2.76[d]	65.32 ± 0.33[d]	192.30 ± 1.49[d]
96	0.00	0.83 ± 0.30[a]	0.83 ± 0.30[a]	15.17 ± 0.65[a]	43.85 ± 1.70[a]	11.17 ± 0.65[a]	26.67 ± 1.87[a]
	0.001	10.17 ± 0.30[b]	16.84 ± 0.30[b]	24.67 ± 0.56[b]	72.17 ± 2.67[b]	22.17 ± 0.40[b]	69.51 ± 1.30[b]
	0.002	17.33 ± 0.33[c]	36.49 ± 0.61[c]	28.00 ± 0.25[c]	87.83 ± 1.19[c]	30.00 ± 0.16[c]	63.83 ± 0.98[c]
	0.01	35.32 ± 0.42[d]	90.98 ± 1.77[d]	65.32 ± 0.42[d]	204.3 ± 1.81[d]	56.83 ± 0.30[d]	141.49 ± 3.15[d]

*indicates significant difference from respective control (0.00ppm furadan) at $p < 0.05$ by Mann-Whitney U nonparametric test.

Different alphabets in damage cell percentage within each time period indicate significant difference at $p < 0.05$ by Mann-Whitney U nonparametric test.

Table 2. DNA damage measured by alkaline comet assay {DNA damaged cells (%) and DNA damage scores in arbitrary units (AU)} in different cells of *Labeo rohita* following exposure to different doses of furadan for various time periods.

4.2 RAPD-PCR

RAPD-PCR was carried out with DNA samples of blood from 0.01ppm phorate-treated and 0.02ppm furadan-treated fishes. The fishes were exposed to pesticides for a period of 96h and samplings were carried out at 24, 48, 72 and 96h. A total of six 10 mer oligonucleotide primers were selected for the study on the basis of their capability to amplify rohu DNA to generate multiple bands. Three individual samples of each of the four sampling hours were run in 1.8% agarose gel along with the controls for each primer. Three control samples out of 12 samples collected at four different time periods were run in individual gel as all 12 samples for each primer showed similar banding pattern. Each change observed in RAPD profiles i.e. disappearances and appearance of bands in comparison to the control RAPD profiles, was given the arbitrary score of +1. The average was then calculated for each experimental group exposed to the pesticides for varying time periods. The template genomic stability (%) was calculated as '100 - (100a/n)' where 'a' is the average number of changes in DNA profiles and 'n' the number of bands selected in control DNA profiles. The

Name of the Primer	Change in the RAPD profile	24h		48h		72h		96h	
		control	treated	control	treated	control	treated	control	treated
OPC 08	No of bands disappeared	0	6	0	4	0	2	0	3
	No of bands appeared	0	0	0	0	0	0	0	0
OPC 11	No of bands disappeared	0	0	0	6	0	0	0	0
	No of bands appeared	0	0	0	0	0	0	0	0
OPC 19	No of bands disappeared	0	1	0	1	0	5	0	4
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 04	No of bands disappeared	0	2	0	2	0	2	0	0
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 13	No of bands disappeared	0	1	0	1	0	5	0	3
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 19	No of bands disappeared	0	5	0	5	0	6	0	7
	No of bands appeared	0	0	0	0	0	0	0	0

Table 3. Frequency of appearance and disappearance of bands in the RAPD profiles of genomic DNA from blood samples of *Labeo rohita* following exposure to 0.01ppm of phorate for various time periods.

template genomic stability for blood DNA samples of phorate treated fishes showed significant changes compared to the control group only at 72h (Fig. 4, Table 3). In furadan treated fishes the blood DNA samples failed to show any significant change in the template stability at any time period (Fig. 5, Table 4).

Name of the Primer	Change in the RAPD profile	24h		48h		72h		96h	
		control	treated	control	treated	control	treated	control	treated
OPC 08	No of bands disappeared	0	8	0	2	0	9	0	5
	No of bands appeared	0	0	0	0	0	0	0	0
OPC 11	No of bands disappeared	0	7	0	1	0	3	0	2
	No of bands appeared	0	0	0	0	0	0	0	0
OPC 19	No of bands disappeared	0	0	0	0	0	2	0	3
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 04	No of bands disappeared	0	2	0	1	0	1	0	1
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 13	No of bands disappeared	0	0	0	0	0	0	0	0
	No of bands appeared	0	0	0	0	0	0	0	0
OPY 19	No of bands disappeared	0	4	0	1	0	2	0	4
	No of bands appeared	0	0	0	0	0	2	0	2

Table 4. Frequency of appearance and disappearance of bands in the RAPD profiles of genomic DNA from blood samples of *Labeo rohita* following exposure to 0.02ppm of furadan for various time periods.

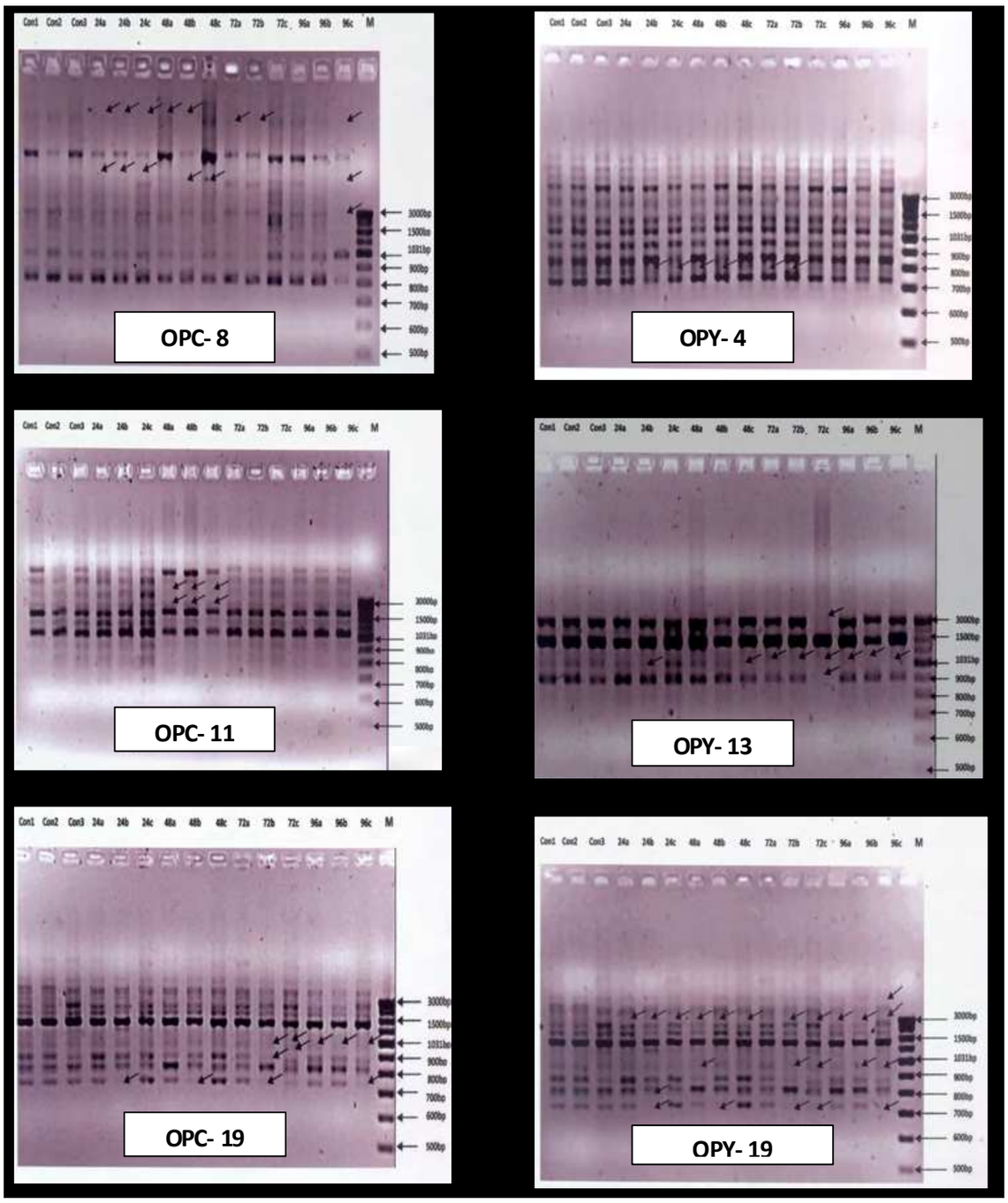


Fig. 4. RAPD profiles of genomic DNA from blood samples of *Labeo rohita* following exposure to 0.01ppm phorate for various time periods. The primers used to amplify are indicated below each photograph. White arrows show appearance of bands and black arrows show disappearance of bands in comparison to the controls. Lanes: con 1. con 2 and con 3 – three control fishes; 24 a. 24 b. 24 c – three fishes with 24 h exposure to phorate; 48 a. 48 b. 48 c – three fishes with 48 h exposure to phorate; 72 a. 72 b. 72 c – three fishes with 72 h exposure to phorate; 96 a. 96 b. 96 c – three fishes with 96 h exposure to phorate; M – molecular size marker.

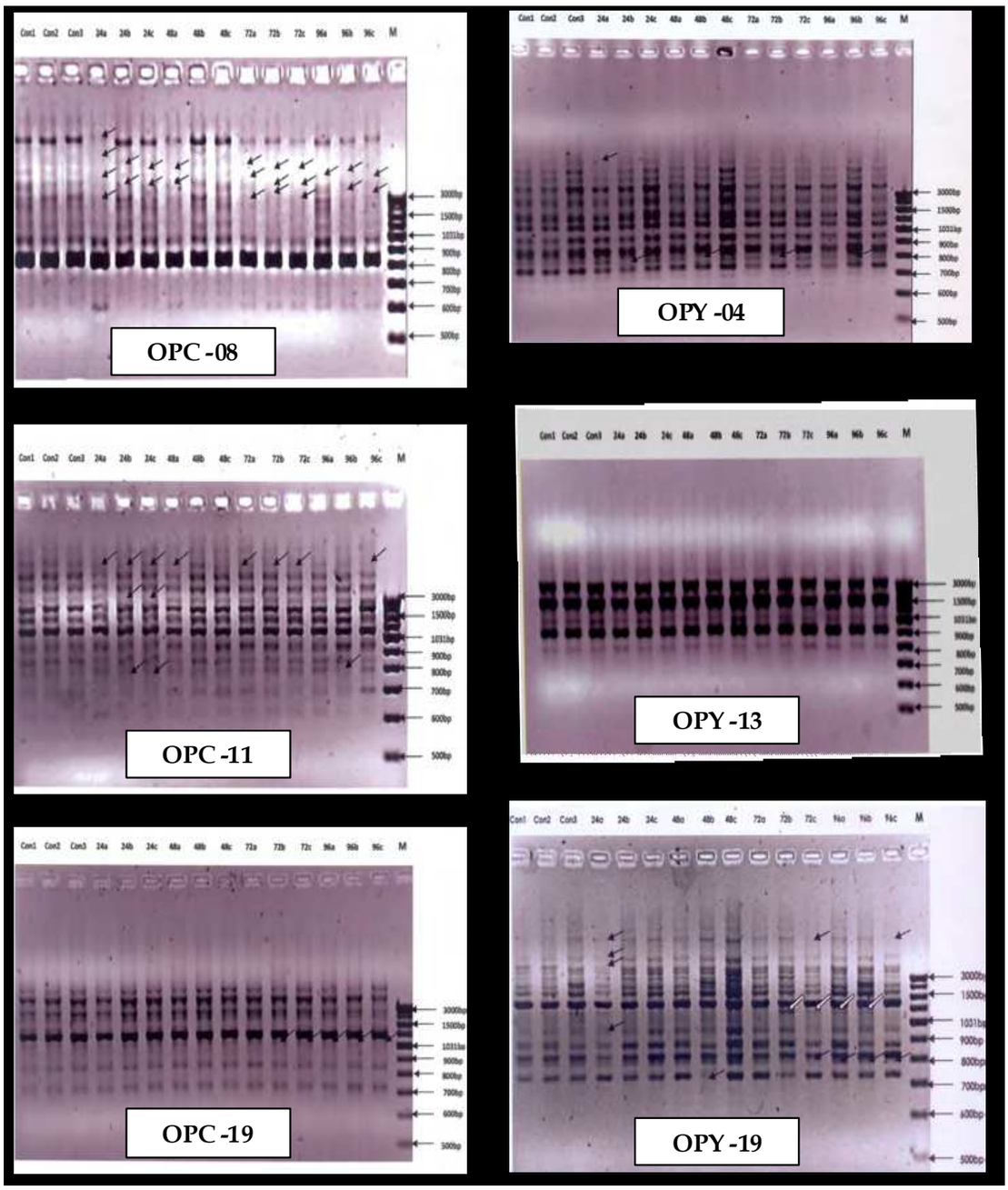


Fig. 5. RAPD profiles of genomic DNA from blood samples of *Labeo rohita* following exposure to 0.02ppm furadan for various time periods. The primers used to amplify are indicated below each photograph. White arrows show appearance of bands and black arrows show disappearance of bands in comparison to the controls. Lanes: con 1. con 2 and con 3 – three control fishes; 24 a. 24 b. 24 c – three fishes with 24 h exposure to phorate; 48 a. 48 b. 48 c – three fishes with 48 h exposure to phorate; 72 a. 72 b. 72 c – three fishes with 72 h exposure to phorate; 96 a. 96 b. 96 c – three fishes with 96 h exposure to phorate; M – molecular size marker.

5. Conclusion

From the entire study it could be generated that rohu (*Labeo rohita*) may serve as a model organism in genotoxicity studies of potential genotoxins. Blood, liver and gill cells were found to be suitable tissues system in aquatic organisms for the genotoxic studies. Alkaline Comet assay and RAPD - PCR were found to be appropriate tools in measurement of DNA damage. The two pesticides phorate and furadan used in the study were found to possess genotoxic potentials to *Labeo rohita.* RAPD-PCR employed in our study showed as a promising tool in the analysis of DNA damage due to pesticide effects.

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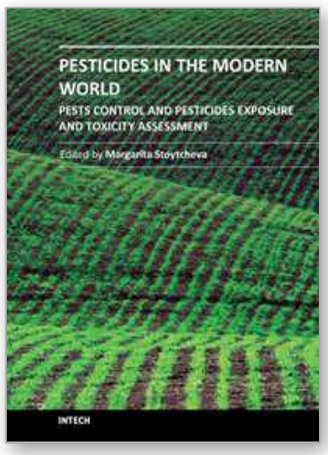

Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment

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The present book is a collection of selected original research articles and reviews providing adequate and up-to-date information related to pesticides control, assessment, and toxicity. The first section covers a large spectrum of issues associated with the ecological, molecular, and biotechnological approaches to the understanding of the biological control, the mechanism of the biocontrol agents action, and the related effects. Second section provides recent information on biomarkers currently used to evaluate pesticide exposure, effects, and genetic susceptibility of a number of organisms. Some antioxidant enzymes and vitamins as biochemical markers for pesticide toxicity are examined. The inhibition of the cholinesterases as a specific biomarker for organophosphate and carbamate pesticides is commented, too. The third book section addresses to a variety of pesticides toxic effects and related issues including: the molecular mechanisms involved in pesticides-induced toxicity, fish histopathological, physiological, and DNA changes provoked by pesticides exposure, anticoagulant rodenticides mode of action, the potential of the cholinesterase inhibiting organophosphorus and carbamate pesticides, the effects of pesticides on bumblebee, spiders and scorpions, the metabolic fate of the pesticide-derived aromatic amines, etc.

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Phone: +385 (51) 770 447
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