Chapter V

Genotoxic evaluation of the herbicide Pyrazonsulfuron Ethyl in *O. mossambicus*

The proper use of herbicides, affecting only restricted terrestrial areas, is a critical issue on forestry and agriculture sustainable management. Nevertheless, following diffuse applications, these agrochemicals frequently reach the aquatic environment by runoff, soil leaching, aerial drift, or inadvertent overspray. The pesticides, especially herbicides, produce deleterious effects on aquatic flora and fauna by affecting various physiological, biochemical and cellular processes (Mullick *et al.,* 2014).The last three decades have witnessed increased interest among the scientific community in detecting the genotoxicity of pesticides used in agriculture. This interest has resulted in identification of a large number of pesticides as a pollutant. Considering this and the fact that several herbicides have already been found to be toxic to aquatic organisms, this type of contamination may pose a severe environmental risk to aquatic ecosystems (Relyea, 2005; Guilherme *et al.,* 2014). Thus, keeping in mind the aquatic organisms and ecosystems health, conducting studies concerning on fish inhabiting contaminated areas must be considered as extremely relevant**.** The aquatic ecosystem as a greater part of the natural environment is faced with the threat of a shrinking genetic base and biodiversity due to indiscriminate use of pesticides (Omitoyin *et al.,* 2006). The majority of these hazardous chemicals are mutagenic in nature (GarajVrhovac and Zeljezic, 2002), either linked to the cancers or might lead to developmental deficits (Leiss and Savitz, 1995; Arbuckle and Server, 1998).

Micronucleus (MN) is regarded as the marker of cytogenetic damage, appearing after the impact of genotoxic compound. Micronuclei are small masses of cytoplasmic chromatin outside the main nucleus of cells, which can originate from a chromosome break or spindle abnormalities (Heddle *et al.,* 1991), i.e.,there are entire or chromosome fragments that were not incorporated inside the nucleus of the daughter cell during cell division and that appear as a small roundish dark structure, identical in appearance to the cell nucleus (Bombail *et al.,* 2001). MN assay provide evidence of DNA breakage, spindle, or other parts of the mitotic apparatus dysfunction caused by clastogens and aneuploidogenic poisons (Heddle *et al.,* 1991). The micronucleus induction assay is well-established method that is useful in the evaluation of genotoxic effects of substantial compounds, in fishes (Al-Sabti and Metcalfe, 1995) and other species (Schmid, 1975; Grisolia *et al.,* 2004). MN analysis has been used as an index of cytogenetic damage for many years (Heddle *et al.,* 1991).MN assay has been used in both laboratory and field studies in vertebrates e.g., fishes (*Cyprinus carpio, Gambusiahol brooki, Poecilia latipinna, Salmotrutta, and Phoxinus phoxinus*) (Sanchez-Galan *et al.,* 1999; Ayllon and Garcia-Vazquez,2000; Buschini *et al.,* 2004; Russo *et al.,* 2004).

Fish are used for the study of the mutagenic and carcinogenic potential of environmental contaminants present in aquatic samples as they can metabolize, concentrate and store pollutants (Al-Sabti, 1991). Fish and shellfish are susceptible to pesticides pollution (Shoaib *et al.,* 2012; Shoaib and Siddiqui, 2015). Fish respond to pollutants similar to higher vertebrates therefore fish (e.g., *Mullus* sp., *Platichthys flesus* L., *Zoarces viviparus*, *Perca* sp.) are used in monitoring programs as sensitive indicators, so-called sentinel organisms (Krishnakumar *et al.,* 1994). Genotoxic chemicals are liable for DNA damage in marine organisms causing malignancies, reduced survival of embryos, larvae and adults. Genotoxicity reduces the 'fitness' (i.e. growth,

fertility and fecundity) in fish populations. Besides, causing mortality, these pollutants can cause genotoxicity in aquatic organisms which can lead to development of tumors in fishes (Folmar *et al.,* 1993).Fishes are used as environmental toxicity bioindicators, because of their sensitiveness and usefulness in the evaluation of ecological risk by chemical contaminants (Solomon *et al.,* 2005; Ballesteros *et al.,* 2009; Lushchak *et al.,* 2009).Many important reasons have contributed to the use of fish as indicator organisms in genotoxicity studies (Szefer *et al.,*1990; Visn-Jeftic *et al.,* 2010). These include their position in the food webs, nutritive value to humans, ability to bioaccumulate toxic chemicals, sensitivity to low concentrations of mutagenic agents and even their aesthetic value.

DNA is a frequent target of pesticides toxicity. According to this statement, it has been shown that the analysis of DNA integrity in aquatic organisms is a highly suitable method for evaluating the impact of environmental genotoxicants, allowing the detection of exposure to low concentrations of contaminants, including pesticides (Scalon *et al.,* 2010). The genotoxic effects of environmental pollutants can be monitored using a broad range of assays, such as the micronucleus test, comet assay and Fluorescence Assisted Cell Sorting (FACS) (Çavas and Könen, 2007).The micronuclei represent acentric chromosome fragments or whole chromosomes lost during cellular anaphase. These structures are easy to visualize in erythrocytes and are therefore often used as a measure of chromosomal aberrations (Rabello-Gay, 1991). Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or from intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Heddle *et al.,*1991, Bolognesi *et al.,* 2006). The formation of morphological nuclear abnormalities (NAs) was first described in fish erythrocytes

by Carrasco et al. (1990). NAs, including lobbed (LB), blebbed (BL), and notched (NT) nuclei and binucleated (BN) cells, have been used by several authors as possible indicators of genotoxicity (Da Silva Souz and Fontanetti, 2006).

Genotoxicity attributes mainly to responses that measures changes in nucleic acids it include the alterations in either DNA or mRNA. Effects may mark as either damage to the genome or some adaptive response in gene expression (Lam and Grey 2003). The evaluation of genotoxic potential in aquatic environment is one of the vital tasks of environmental monitoring to control pollution. There are several reasons behind the biomonitoring of genotoxicity in aquatic organisms like fish. From the ecological perspective, the protection of genetic diversity in natural populations is important for population survival, and avoiding contaminant-induced mutations that skew genetic diversity (Jha *et al.,* 2000, Osman 2014). Second, the detection of carcinogenic effects in aquatic organisms is needed to assess the health of aquatic organisms, as well as to prevent carcinogens from entering the food chain to humans (Flora *et al.,* 1991, Osman 2014).

During the past decade mechanisms underlying cell death have entered into a focus of many researchers in diverse fields of toxicology. These mechanisms include a wide range of signalling cascades that regulate initiation, execution and post-mortem cell disposal mechanisms (Darzynkiewicz *et al.,* 2001 and 2004). There are many morphological and molecular changes occurring during classical caspase-dependent apoptosis *vs.* accidental cell death (necrosis). Alterations in parameters become a foundation to development of many markers for microscopy, cytometry and molecular biology techniques (Wlodkowic *et al.,* 2008). It is important to note, however, that the occurrence of specific apoptotic markers can show a profound divergence.

Moreover, burgeoning data demonstrate that elimination of many cells may rely on alternative mechanisms (i.e. caspase-independent apoptosis-like PCD [programmed cell death], cornification, autophagy, necrosis-like PCD, mitotic catastrophe, etc) with critical connotations in both physiological and pathological processes (Leist and Jaattela and Kroemer and Martin, 2005). The colloquial term "apoptosis" should be, therefore, restricted only to the demise program featuring all "hallmarks of apoptotic cell death", namely: (i) activation of caspases as an absolute marker of cell death ; (ii) tight (geometric) compaction of chromatin; (iii) activation of endonucleases(s) causing inter nucleosomal DNA cleavage and extensive DNA fragmentation; (iv) appearance of distinctive cellular morphology with preservation of organelles, (v) cell shrinkage, (vi) plasma membrane blebbing and (vii) nuclear fragmentation followed by formation of apoptotic bodies (Blagosklonny 2000 and Zhivotovsky, 2001).

In this context, a gross majority of classical apoptotic attributes can be quantitatively examined by flow cytometry, the preferred platform for rapid assessment of multiple cellular attributes at a single cell level (Telford *et al.,* 2004). The major advantages of flow cytometry include the possibility of multiparameter measurements (correlation of different cellular events at a time), single cell analysis (avoidance of bulk analysis), and rapid analysis time (thousand of cells per second). Flow cytometry overcomes, thus, sensitivity problems of traditional bulk techniques such as fluorimetry, spectrophotometry or gel techniques (Wlodkowic *et al.,* 2009).

The possibility of using changes in DNA integrity to the genetic material as markers of exposure and effect of genotoxicants has been previously investigated (Osman 2014). The presence of DNA adducts has been taken as a confirmation of exposure to specific genotoxicants (Shugart 1999). These tests rely on the premise that any changes to DNA may have long lasting and

profound consequences (Lam and Grey 2003). Sister chromatid test, chromosome aberrations, comet assay, and micronucleus test are currently the most widely employed methods to detect DNA lesions in ecotoxicology. Sister chromatid test and chromosome aberrations are time consuming, resource intensive, require proliferating cell population (Rigolin *et al.,* 2012), and not very effective due to the relatively large number of small chromosomes of many aquatic animal species. Theses disadvantages have stimulated the development and the use of comet assay and micronucleus test as simpler, more sensitive and time efficiency genotoxicity tests.

Over the years fish erythrocytes are vital biological cell for investigating the genotoxicity of contaminants present in the environment (Ergene *et al.,* 2007; Ali *et al.,* 2014). Besides MN, fish erythrocytes are conveniently used for studying DNA damage, apoptosis (Ateeq *et al.,* 2005). The overall mechanism of genotoxicity of environmental pollutants can be evaluated using a broad range of both *in vitro* and *in vivo* biomarker assays (Ali *et al.,* 2014).

Nonetheless, the genotoxic potential of PE as well as the mechanisms behind its possible DNA damaging action, remain completely unknown. Bearing in mind the knowledge gaps identified, the main goal of the present work was to assess the genotoxicity of the herbicide PE on *Oreochromis mossambicus* found in tropical and subtropical habitats, live in rivers, lagoons, creeks and streams. *O. mossambicus* is very steady, euryhaline fish, have a wide range of salinity and temperature tolerance (Moyle, 1976). Hence, we hypothesized, after observing the detrimental effects of PE on overall physiology (describe in Chapter 1, 2, 3 and 4), the aim of the present study is to investigate the genotoxic potential of PE with the help of MN assay and cell death using FACS analysis.

Materials and Methods:

Fishes were exposed to three different concentrations of PE (LD, MD, HD) for7 and 14 days, and 3 males and 3 females (a total of 6) from control and each treatment group were sacrificed. Blood was collected using tail ablation method with the help of micro syringes pre-rinsed with heparin (anticoagulant). The collected blood from the control and experimental groups was expelled on clean glass slides and thin smears were prepared. The slides were kept on ice cold condition for 2 hrs, fixed in methanol for 10 minutes, and stained in 10% Giemsa (v/v-Sigma#G5637). To detect the presence of micronuclei in erythrocytes, the slides were analyzed using 100 X oil-immersion lens. 1000 cells were scored from each slide for the presence or absence of micronuclei in their cytoplasm in every selected patch. Micronuclei were identified as small (diameter less than one-third of the main nucleus) non-refractive, circular or ovoid chromatin bodies separated from the main nucleus and have similar staining as the main nucleus. The whole process for done for 3 replicates for each group.

FACS analysis

Staining Procedure:

Cells (treated and untreated) were washed twice with cold PBS and then was resuspend in 100 µL of 1X binding buffer (cells numbers was$1x10^6$ in a 5ml FACS tube). Further, 5 µL of FITC Annexin V and 5 µL PI (or 1-2 µg/ml) was added to 100 µL cells. The cells were vortexed and were incubate for 15 mint at RT (25ºC) in the dark.200 µL of 1x binding buffer (1 M Hepes/NaOH (pH 7.4), 1.4 MNaCl, 25mM $CaCl_2$) was added to each tube, and was analyzed by flow cytometry within 1hr. Unstained Cells, cells stained with Annexin V (no PI) (compensation

control) and cells stained with PI (no FITC Annexin V) (compensation control) was used as control with three replicates of each.

Data Analysis:

Data obtained from MN assay and FACS were subjected to two-way analysis of variance (ANOVA) to determine the effect of treatment as well as period of treatment. Furthermore, Dunnet multiple comparison test was conducted to test the significant difference between control and treated animals and p values < 0.05 were considered statistically significant. All statistical analyses were performed by using Graphpad prism 5 software.

Result:

The micronucleus induction observed per 1000 cell in an area of observed field was found to be progressively increasing in a range of0.2 ± 0.06 to $0.8 \pm 0.04$for 7 days and 0.9 ± 0.04 to1.4 ± 0.01 for 14 days respectively, in dose and time dependent manner as summarized in Table 5.1 and 5.2. Each erythrocyte was representing 2-3 micronuclei per cell and was observed closer to the main nucleus of varied size and shape. Moreover, all treated groups showed a direct proportion of erythrocytes with higher frequency of micronucleus. It was statistically significant ($p<0.05$) atsublethal MD and HD of 7 and 14 days. However, sub lethal LD treatment regime showed annon-significant increase. Furthermore, the number of nuclear abnormalities was higher with HD on7^{th} and14^{th} days (Fig. 5.1) where lobed, notched, blabbed and bi-nucleated nucleus was clearly visible and was statistically ($p<0.05$) higher at HD of 14^{th} compared to 7^{th}days and control. The assay revealed that, the frequency of other nuclear abnormalities was higher compared to micronuclei in the observed erythrocytes.

Data of FACs analysis revealed that, there was a dose and time dependent changes in the cell proliferation and cell death of the erythrocytes. The cells were found to be primarily in G1 phase of cell cycle on7th and 14thday in all the exposed fishes. However, a specific trend of increase or decrease was not observed among the exposed groups in time dependent context. The 7 days exposure showed a significant ($p<0.05$) decrease at LD, MD, and HD exposed fishes compared to control. However, there was a non-significant increase in LD exposed fishes with respect to MD and HD. Conversely, 14thday exposure was having a non-significant increase in MD exposed fishes compare to LD and HD.

The analysis of synthesis phase (S phase)illustrates a significant ($p<0.05$) increased in cells of S phase of cell cycle at LD, MD, and HD exposed erythrocytes compared to untreated cells on 7th day only. Among the treated groups, the 7 days treatment expresses that LD and MD displayed a significant increase in S phase cells compare to HD. In contrast, similar non-significant increase was seen at14th day of exposure among the treated groups. The G2-M phase erythrocytes, was found to be significantly ($p<0.05$) lower in dose and time dependent fashion. However, MD exposed cells at 7 days were found to be increased (non-significant) in this phase compare to LD and HD exposed cells. Similar trend was observed at 14 days of exposure, where there was a significant decreased in all the treated groups compared to control (Fig. 5.2 and 5.3)

Apart from cell cycle analysis, apoptosis was also analyzed among treated groups. The apoptosis was found to be significantly ($p<0.05$) increasing in 7 and 14 days exposure in all the treatment groups compare to control (Fig. 5.8 and 5.9). The highest percentage was found in HD exposed cells of both the duration compare to LD and MD.

Table: 5.1 Frequency of Micronuclei and nuclear abnormalities counted in the species *Oreochromis mossambicus* exposed to PE at 7th days

Nuclear Abnormalities	***Oreochromis mossambicus***			
	Control	**1/20th LC_{50}**	**1/10th LC_{50}**	**1/5th LC_{50}**
Micronucleus	0.1± 0.02	0.2± 0.06	0.5± 0.09	0.8 ± 0.04*
Binucleated cells	1.3±0.22	1.5±0.30	3.2±0.59	8.6± 0.59**
Blebbed cells	1.2±0.29	1.4±0.34	3.9±0.61	6.4±0.34*
Lobed cells	4.9±0.53	5.1±0.41	7.4±0.19	10.5± 0.92**
Notched cells	2.7± 0.62	2.9± 0.57	5.7± 0.94	7.8± 0.64**
Total Abnormality	10.2±2.1	11.1±2.7	20.7±3.4	34.1± 4.3**

Table 5.2: Frequency of Micronuclei and nuclear abnormalities counted in the species *Oreochromis mossambicus* exposed to PE at 14th days

Nuclear Abnormalities	***Oreochromis mossambicus***			
	Control	**1/20th LC_{50}**	**1/10th LC_{50}**	**1/5th LC_{50}**
Micronucleus	0.1± 0.02	0.9 ± 0.04	1.2 ± 0.09	1.4±0.01**
Binucleated cells	1.3±0.22	8.9± 0.72	10.2± 0.59	12.3±0.67***
Blebbed cells	1.2±0.29	6.9±0.96	8.1±0.82	9.4± 0.52**
Lobed cells	4.9±0.53	10.8± 0.92	14.5± 0.92	19.7±0.24***
Notched cells	2.7± 0.62	8.6± 0.19	10.4± 0.37	13.2±0.42***
Total Abnormality	10.2±2.1	36.1± 1.9	44.4± 4.9	56.0± 7.5***

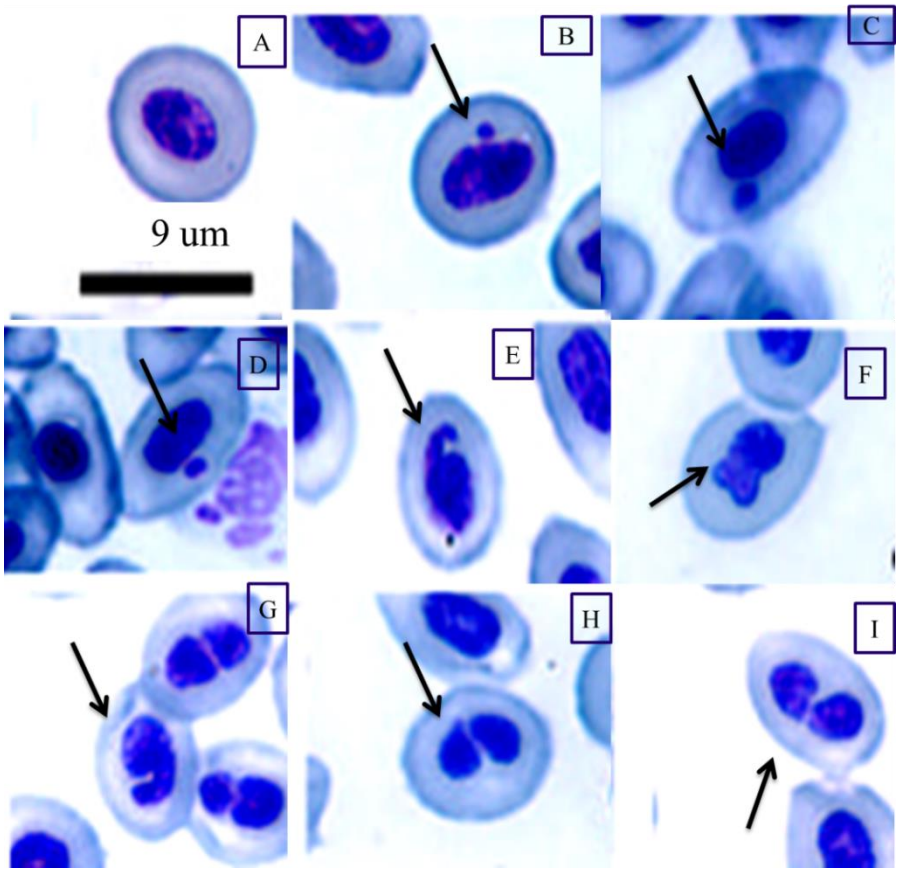
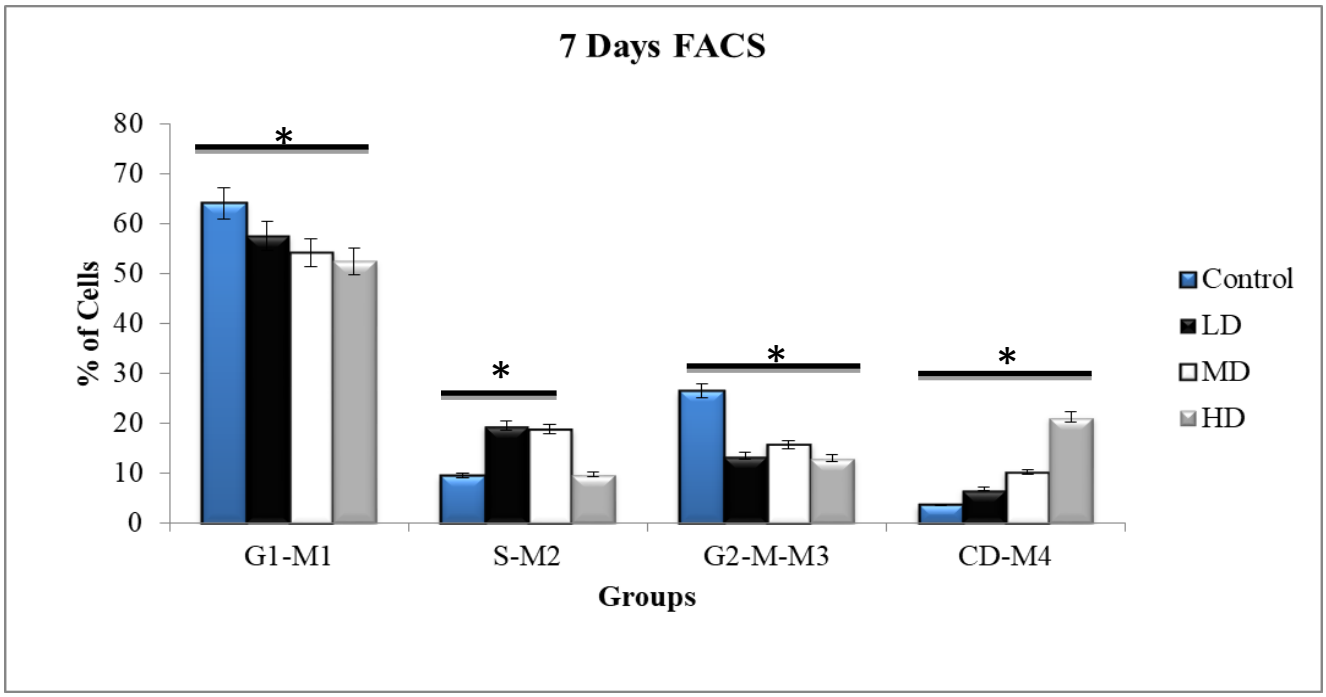


Figure 5.1: Shows 100X Microscopic images of erythrocytes of *O. mossambicus* exposed to PE. A-control, B and C- Micronucleus in 7 days exposure, D- Micronucleus in 14 days exposure, E- Blebbed in 14 days exposure, F- Lobed in 14 days exposure, G-Notched in 14 days exposure, H and I- Binucleated in 14 days exposure.



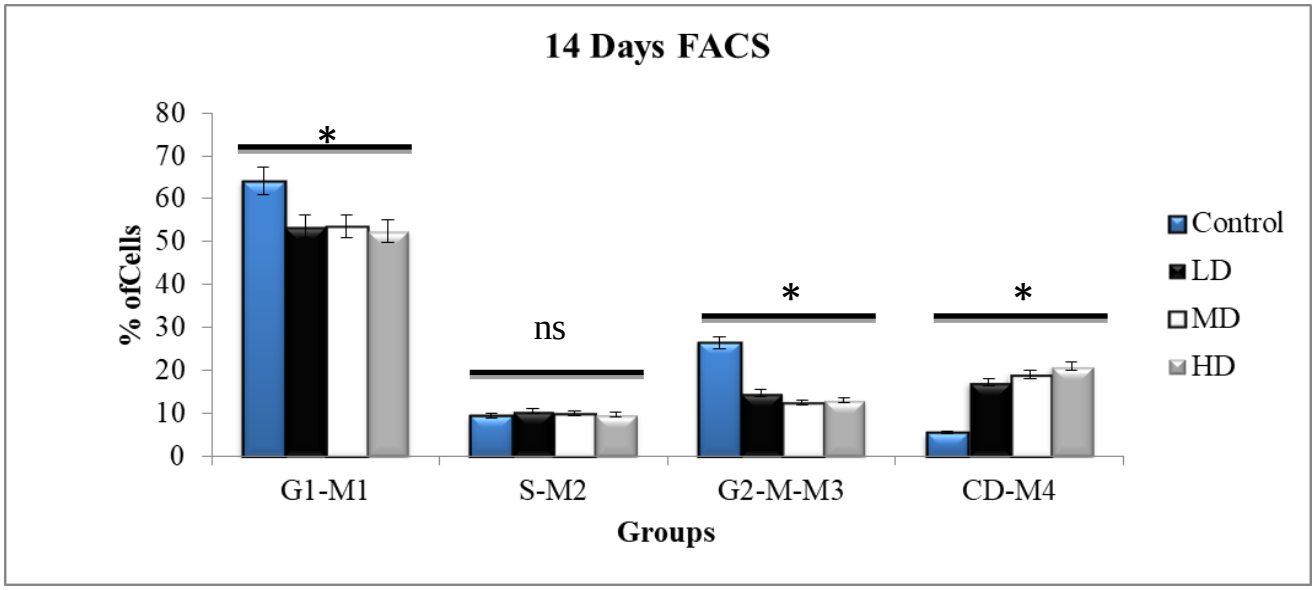
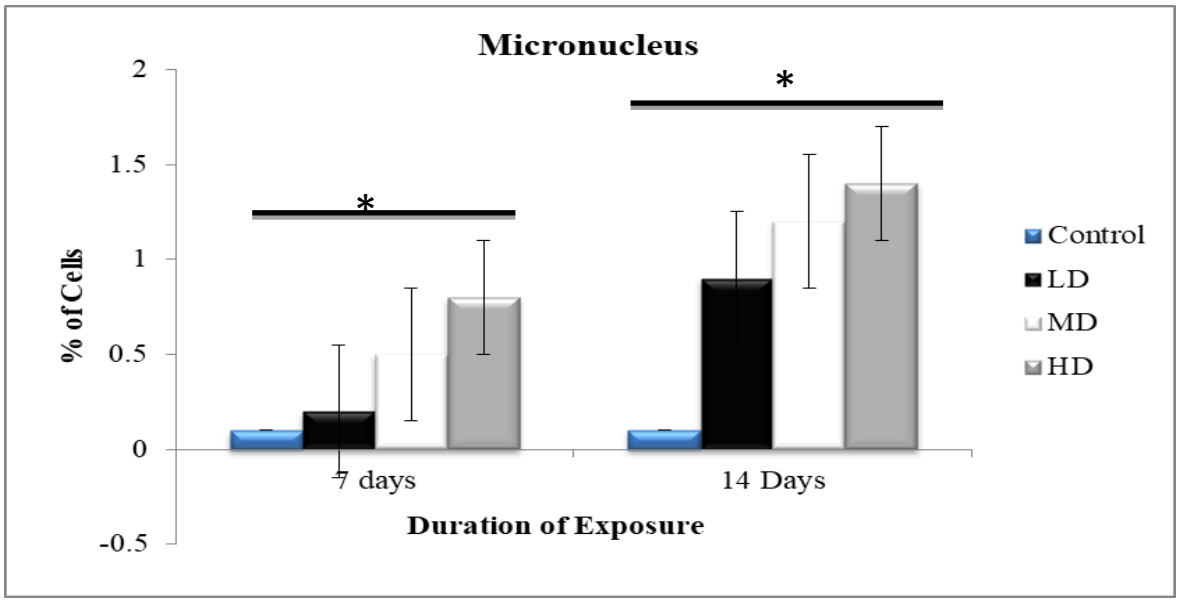


Fig 5.2: FACS Analysis in peripheral blood erythrocytes of *O.mossambicus* exposed to PE for 7 and 14 days



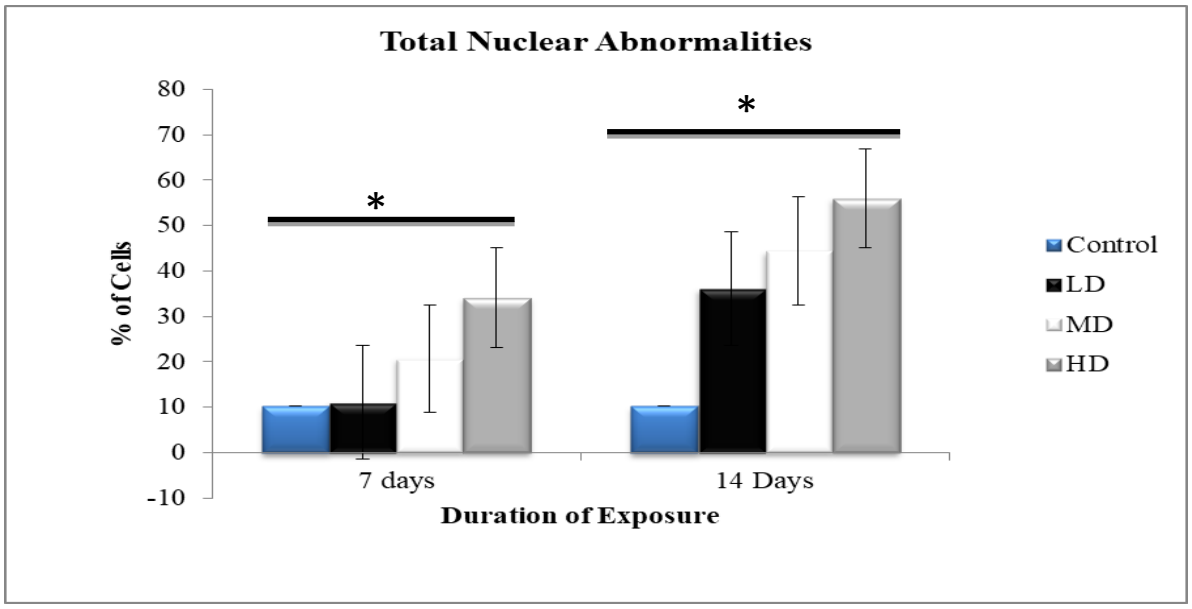
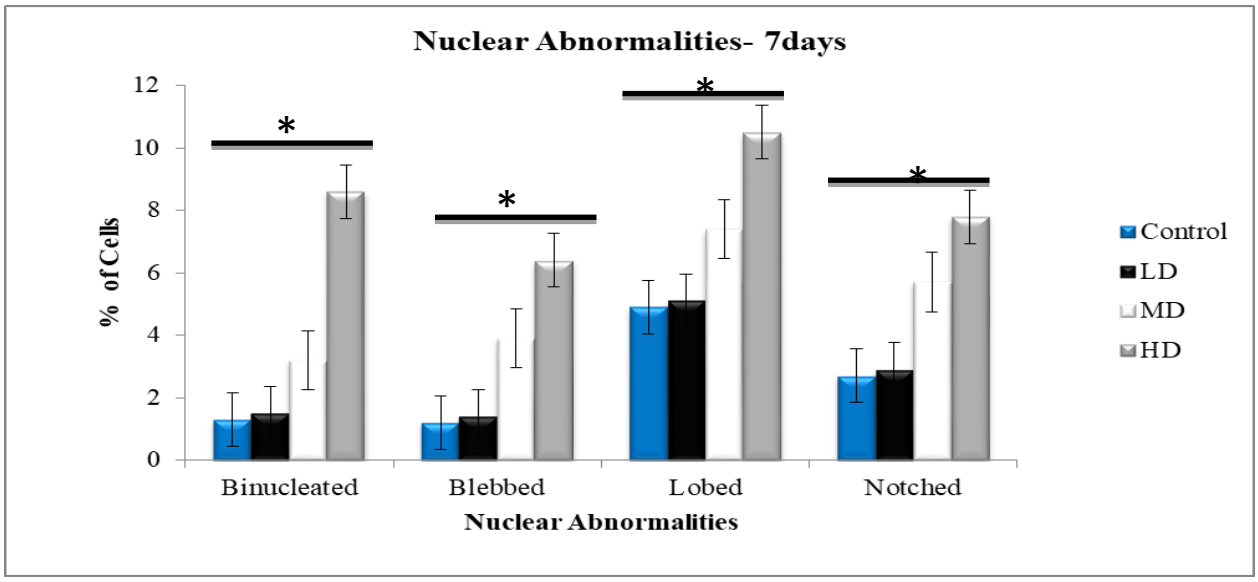


Fig 5.3: Micronucleus frequencies in peripheral blood erythrocytes of *O.mossambicus* exposed to PE for 7 and 14 days



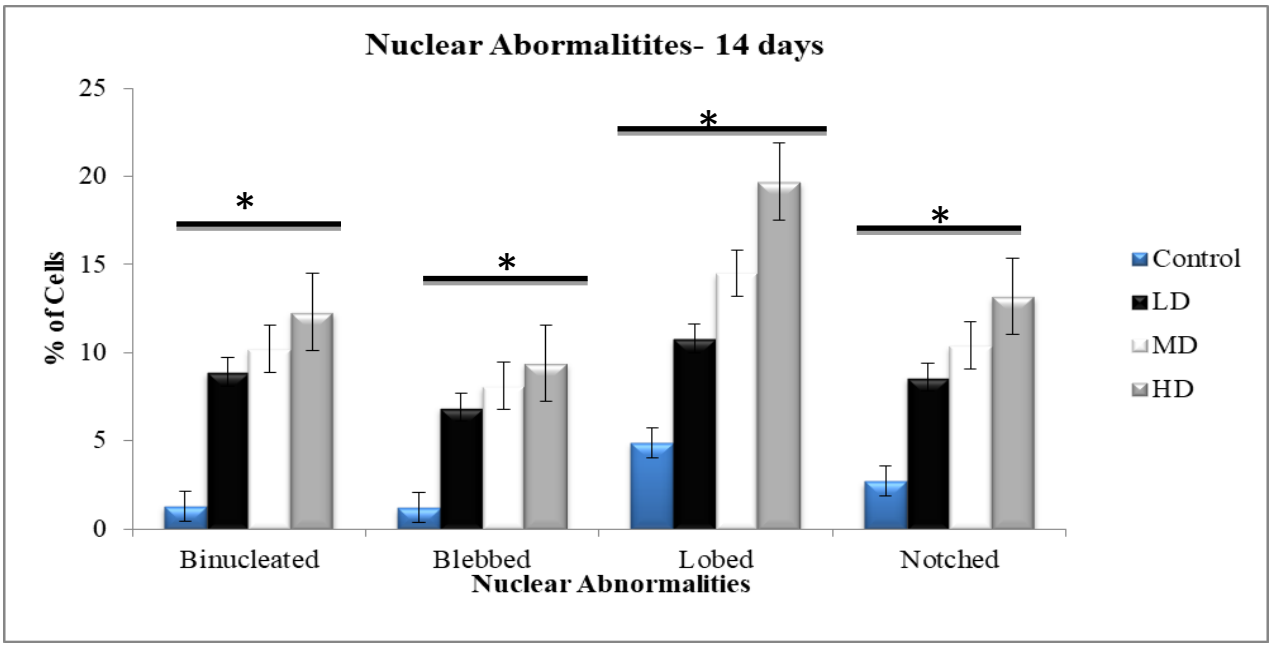


Fig 5.4: Nuclear Abnormalities frequencies in peripheral blood erythrocytes of *O. mossambicus* exposed to PE for 7 and14 days.

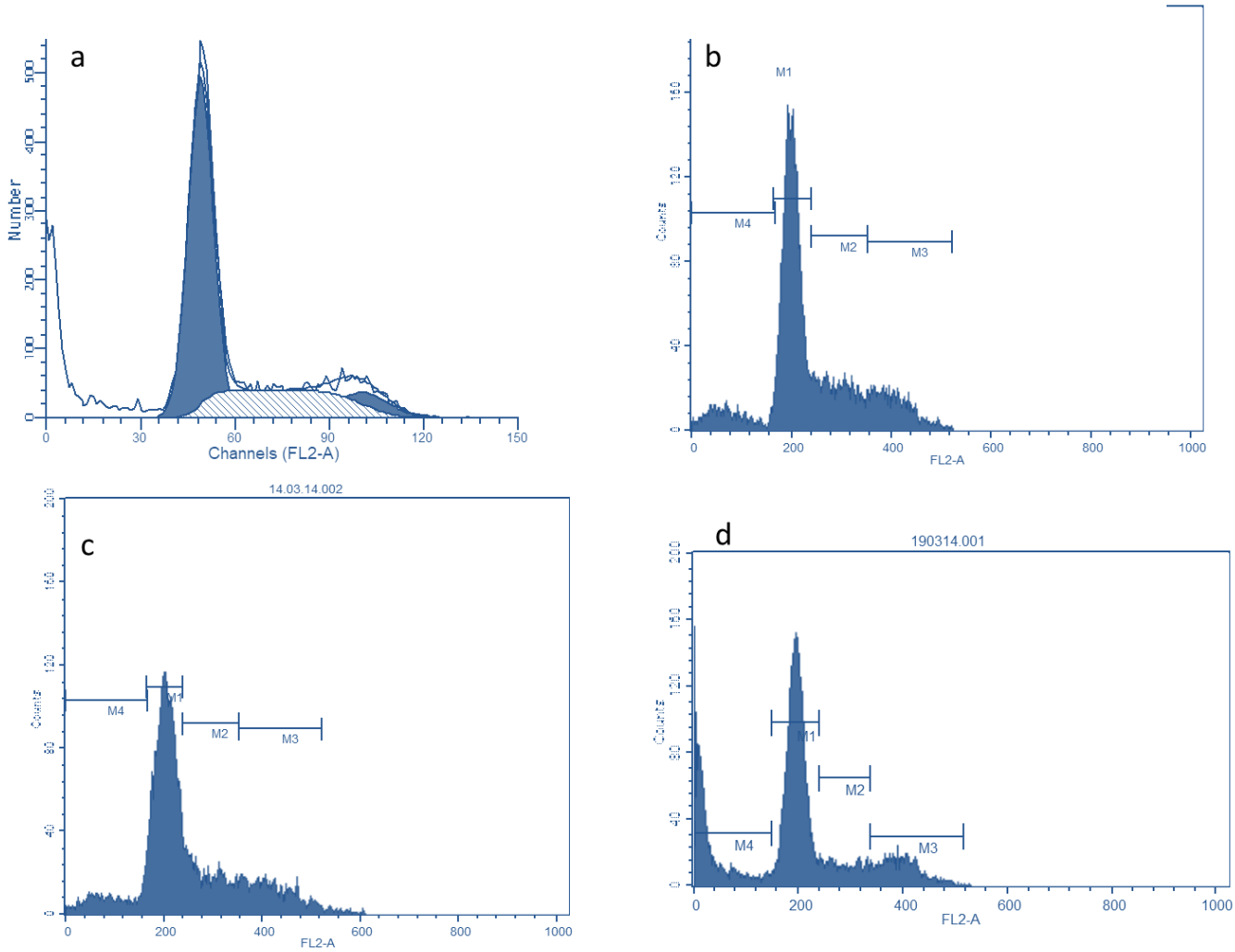


Fig 5.5: FACS graph Analysis in peripheral blood erythrocytes of *O.mossambicus* exposed to PE for 7 days

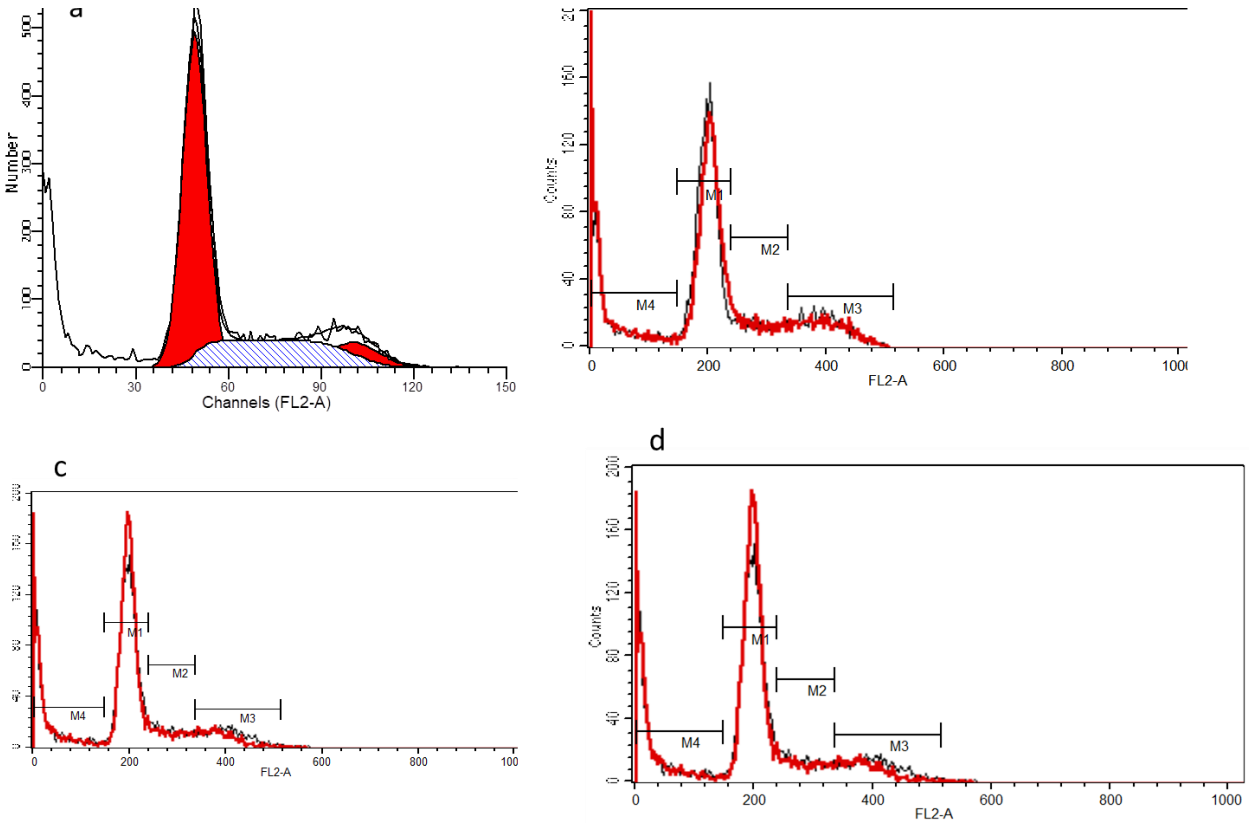


Fig 5.6: FACS graph Analysis in peripheral blood erythrocytes of *O. mossambicus* exposed to PE for 14 days

Discussion

Assessing toxicity is important in determining how sensitive animals are to toxic agents, and can be used to measure the extent of damage to target organs and the resultant behavioural, biochemical and physiological alterations (Nwani *et al.*, 2011). A number of literature studies of insecticides are available where they have proved the formation of micronucleus in the erythrocyte of teleost (Ventura *et al.*, 2008, Katsumiti *et al.*, 2009,Vryzas *et al.*, 2011, Piancini *et al.*, 2015). However, studies are at its basal level, when the effect(s) of herbicide are taken into consideration. Hence, a less studied herbicide PE was selected for the present study to assess the genotoxicity. As shown in the results, the DNA damage was triggered off in erythrocytes of *O. mossambicus* due to PE exposure at different concentrations suggesting its potential genotoxic and mutagenic properties. The control fishes had their DNAs intact, thus DNA damage can be said to be a result of the clastogenic action of the PE. Environmental mutagen has been reported to increase both micronuclei and DNA migration in fish (Russo *et al.*, 2004).

Promoting DNA damage has been reported to be the first mechanism of action of genotoxic agents, which can possibly result in three outcomes: the damage can be repaired, the damage can become irreversible, or the damage may lead to cell death (Vicari *et al.*, 2012). According to Akcha *et al.*, (2003), the absorption and biotransformation of genotoxic environmental pollutants could lead to the formation of DNA strand breaks in erythrocytes.

An essential strategy for realizing better insight into the ability of organisms to repair damaged DNA and other protective mechanisms for excreting the toxic chemicals may belong-term genotoxicity study. Because MN is usually given off along with the main nucleus, their presence would suggest their origin at a cell cycle that is more recent (Chandra and Khuda-Bukhsh, 2004;

Graziela *et al.*, 2010, Srivastava *et al.*, 2006). According to Jerbi *et al.*, (2011), the formation of micronucleated cells may be an indication of aneugenic and/or clastogenic actions, because the presence of MN can be related to entire chromosomes, caused by a malfunctioning of the spindle, or with chromosome fragments, derived from chromosome breakage. This study showed progressive increase in the number of micronucleated erythrocytes and other abnormalities till day 14.

Maximum frequency of micronuclei was also observed on day 14^{th} thus, demonstrating highest effect of PE on chromosome breakage, apparently confirming that this can root genotoxic damage in erythrocytes of *O. mossambicus*. The study postulates that a long time of exposure may lead to decrease in intensity of formation of MN as due to formation of other nuclear abnormalities associated with severe chromosomal aberrations (Karaismailoglu, 2015, Yadav *et al.*, 2015, Bhatnagar *et al.*, 2016). To overcome the toxicant stress and to stabilize the micronuclei frequency fish have the tendency to reduce the frequency of the micronuclei and thereby promoting a defensive mechanism (Grisolia *et al.*, (2009); Malla *et al.*, 2011; Da Rocha *et al.*, 2011, Bhatnagar *et al.*, 2016 and Braham *et al.*, 2017). Various studies on erythrocyte nuclear abnormalities have been proved to a signal of cytogenetic damage in fish species (Strunjak-Perovic *et al.*, 2009; KousarandJaved, 2015). In the present study, we also reported a significantly increasing trend of different types of nuclear abnormalities during the exposure time. Among them, the maximum frequency was reported for lobed nucleus. PE belongs to the sulphonyl urea group and may induce mutation by the virtue of its alkylating activity (Anbumani and Mohankumar 2011). Thus, this unique property of alkylation may probably lead to cause DNA damage resulting in the formation of micronuclei and other nuclear

abnormalities observed (Nwani *et al.,* 2013; Vasanth *et al.,* 2017and Ayanda *et al.,* 2018).Our results are consistent with the results obtained from Barshiene *et al.,* (2007), who have discovered a significant increase in the frequency scale MN in erythrocytes, to fish trout California (*Oncorchynchus mykiss*) after treatment of herbicide for 14 days, with the blend of heavy metals. Further, our results are also consistent with results obtained by Bushra *et al.,* (2002), who have detected increase in scale significantly the frequency of MN in erythrocytes, the fish (*Clarias batrachus*) after treatment for 48, 72 and 96 hours, two herbicides: 2,4 dichlorophenoxyacetic acid and butachlor. Further, Konen *et al.,* (2008) also have reported increased in scale significantly the frequency of MN in erythrocytes, the fish (*O. niloticus*), after treatment for 3, 6 and 9 days herbicides Treflan Trifluralin and by Cavalcante *et al.,* (2008) in peripheral blood erythrocytes of fish (*Prochilodus lineatus*), after treatment for 6 and 96 hours, with the herbicide Roundup.

The toxicological effect of PE has probably originated from its oxidizing action. Once entered in to the cell, in the presence of cellular reductants, it can induces DNA fragmentation or DNA strands breakage, which is considered to be premutagenic change. The DNA fragmentation or DNA strand breaks are also considered to be a kind of lesion that is potentially premutagenic; the production of breaks in the DNA strands being related to mutagenic and carcinogenic properties of chemicals (Frenzilli *et al.,* 2000, Kammann *et al.,* 2001). Hence, our results are in accordance with the results of Nwani *et al.,* 2011, Bhatnagar *et al.,* 2016, Zeqiraj *et al.,* 2015 which clearly revealed genotoxicity even at all sub-lethal concentrations.

Although the MN test is a sensitive assay to evaluate genotoxic compounds in fish under controlled conditions as an index of cumulative exposure (Yin *et al.,* 2009, Bhatnagar *et al.,*

2016), however, it might suffer variations according to tests organisms (Nwani *et al.,* 2010). Further, as the preexisting mature (and non-dividing) erythrocytes would predominate in the blood, the detection of induced MN in mature blood cells will be at a low frequency in the beginning of the exposure. On the other hand, with the progression of the experiment, a greater number of the dividing cells (polychromatic erythrocytes) would be expected to predominate in the blood and, therefore, a latency period is normally required between treatment and subsequent MN peak (Nwani *et al.*, 2010, Bhatnagar *et al.*, 2016).

So, due to above constraint, cell cycle analysis and apoptosis rate was performed with the help of FACS. This study stands at par with other conventional studies as it analyses the effect of PE on erythrocytes using flow cytometry. The erythrocytes were significantly ($p<0.05$) higher in case of G1 phase in treated groups, however, it was significantly ($p<0.05$) less compared to control. This may be an adaptive mechanism of formulating stress proteins to combat the stress response of PE. The decrease in G1 phase also attributes a decrease in the overall doubling time of the cell cycle. The PE may in all probability act on the cyclin dependent kinases (CDK 4/6) and its cyclins (Cyclin D) which results in quick transformation of G1 to S phase. The possible trend obtained was in both the duration and was inversely proportional to the dose exposed. As, studies are scarce *w.r.t* to G1 phase of cell cycle analysis and effect of herbicide on tilapia, it is difficult to comment on the exact mechanism of cell cycle at this juncture. As far as, S phase is concerned, there was a significant rise in the LD and MD exposure at 7 days PE exposure. The prominent steep may be due to burden caused by the pesticides while the synthesis of DNA and time taken to repair so as to maintain and conserve the sequence. Moreover, it may have induced the expression of Cyclin A and CDK 2, or in gap may have decreased the expression of cyclin E.

(Yabu *et al.*,2001; Liu *et al.*, 2017and Corlu and Loyer, 2012). However, this was not observed in case of 14th day exposure, suggesting the adaptation or more precisely sequence alteration (mutation) might be induced consequently normalizing the S phase duration that was found to be similar to control. The mutation(s) which may be induced by the exposure of PE can be deletions/additions/ insertions/point signifying the activity of alkylting agent (Kondo *et al.*, 2010; ZhiyiandHaowen, 2004; Rocco *et al.*, 2010 and 2011).

Further, the study proves that there is a reduction in G2 to M phase under the 7th and 14th day exposure groups. This reduction attributes the operation of following mechanisms: Firstly, PE may affect the activity of cylins (Cyclin A and Cyclin B) and Cyclin (CDK 1) dependent kinases which are the guardians (checkpoints) from G2 to M phase. Secondly, the mutations caused in S phase may lead to mis-folding of stress proteins and deactivation of chaperones thus, resulting in reducing the duration of the phases. These probable postulations explain the alarming condition of tilapia erythrocytes under PE exposure.

The MN studies and FACS results thus postulated that erythrocytes have entered the apoptosis; together these data show that PE exposure is potentially hazardous to *O. mossambicus*, even at sub-lethal levels as evidenced by the cell death study which was found to be significantly increasing in a dose and time dependent manner. At 7 day exposure, there was linearity in the increase in the apoptotic population of erythrocytes i.e. from LD to HD. While, this linearity was also observed for 14 days exposure period. The highest cell death was accounted for HD of both the durations suggesting cells were under stressed which lead them to undergo apoptosis at sub lethal exposure of PE. Hence, the study suggest, in field, even the sub-lethal concentration can cause toxicity to fresh water fish and results in altering the aquatic ecosystem.

In conclusion, the present study is the first one to establish the cytotoxic and genotoxic potential of PE in *O. mossambicus* among the group of sulhponylurea. It also further proves the suitability of the MN and FACS as tools for evaluating toxicity. The increase in the intensity of MN and other nuclear abnormalities w.r.t to cell death found suggest the severity of PE on erythrocytes of tilapia. However, the exact mechanism behind the toxicity can be validated with the help of expression studies, immuno-histochemical localization and sequencing of cell cycle markers. The toxicity of the herbicide to *O. mossambicus* provides a basis to project the potential harm that may be caused to other inhabitants of the aquatic ecosystem and those who depend on them. Therefore, it may be imperative to ensure careful, efficient use of this herbicide so as to prevent adverse effects not only to the genetic component of aquatic environment but also the human health.

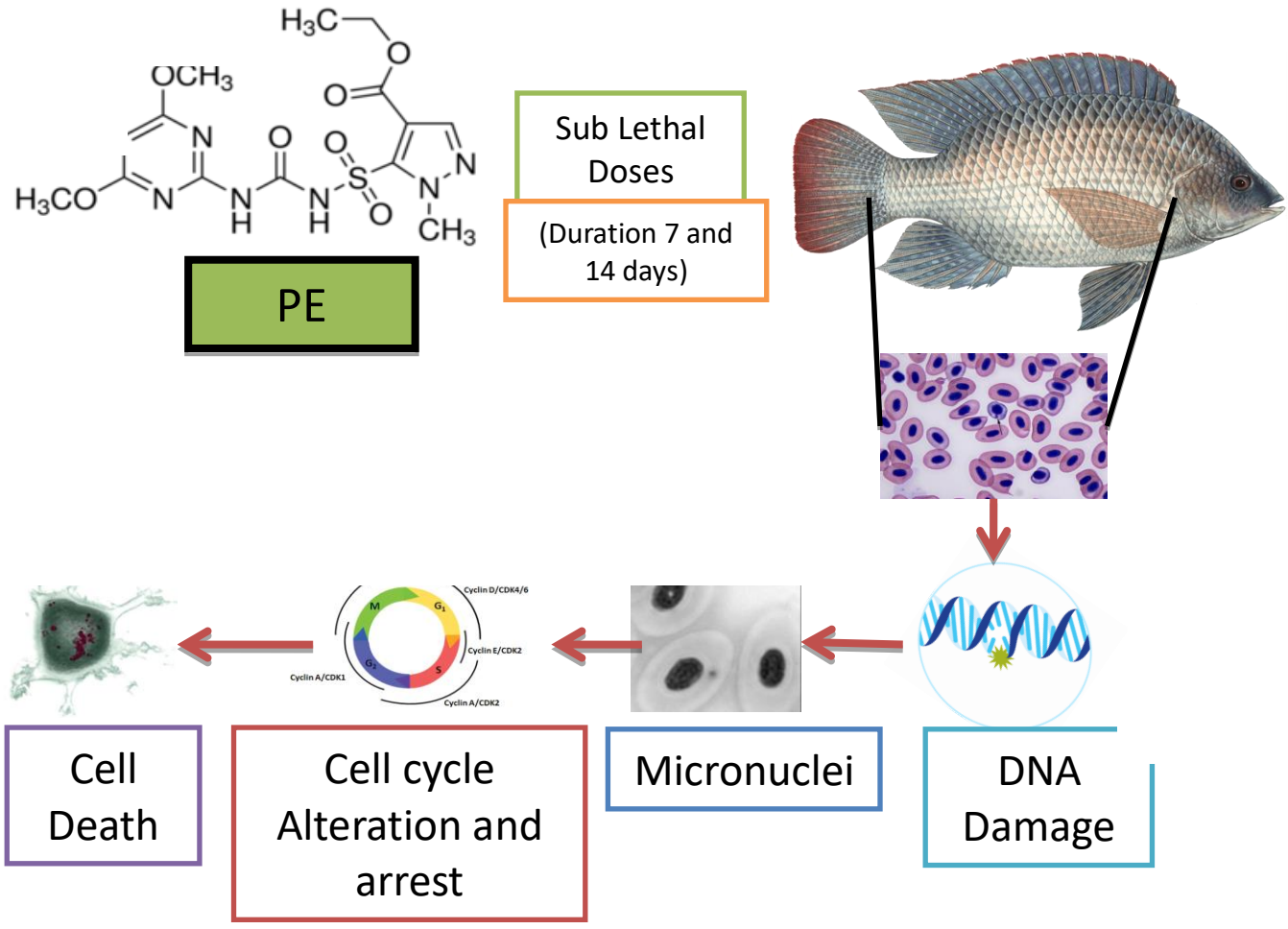


Fig 5.7: Flow chat Diagrammatic presentation of the MN assay and Cell cycle analysis exposed by PE