Induction of genotoxicity after subchronic treatment with 4-nonylphenol in blood cells from gill and kidney and restoration of DNA integrity after recovery by Channa punctatus

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Abstract
The aim of present study was to assess the genotoxic effects of endocrine-disrupting compounds 4-nonylphenol (NP) on Channa punctatus after sub chronic exposure. Blood cells from gill and kidney were used for the study and micronucleated cell (MNC) and aberrant cell (AC) frequencies were used as biological indicators. The exposure was given to C. punctatus for 90 days, and the effect was seen after 30, 60, and 90 days of exposure. Three sublethal concentrations were decided after calculating the safe application rate. Exposure resulted in increased frequency of MNC and AC in both the tissues. Gill tissue was found to be more sensitive to 4-nonylphenol exposure. Highest MNC and AC frequency was found at 90 days of exposure in gill tissue and the values were 4.45±0.28 and 63.71±0.84 respectively. After exposure for 90 days, post-30 days recovery was also studied and we found a significant reduction (p<0.01) in the values of both the parameters showing a great capacity of C. punctatus to restore its DNA integrity.

Keywords: Aberrant cells; Genotoxicity; Micronucleated cells; 4-Nonylphenol; Recovery

INTRODUCTION
In the world market thousands of chemicals are entering annually, and most of them are hazardous to the aquatic environment. Pollution of the aquatic ecosystems is globally recognized as a potential threat to all animals, including humans as well as aquatic organisms that interact with aquatic environment (Biney et al., 1987). In the developing countries, 90-95% of all sewage and 70% of industrial wastes are dumped untreated into surface water (Obiakor et al., 2012). In India, almost all the water bodies are found to be contaminated with one or another industry and ample evidences are available regarding the mismanagement of industrial waste (Jadhav and Singare, 2015; Singare and Dhabarde, 2014).

The history of environmental chemical pollutants is dominated by several chemical compounds of different characteristics such as polycyclic aromatic hydrocarbons, polychlorinated insecticides, polychlorinated biphenyl, synthetic surfactants, and heavy metals, and so on. In the early 1980s, it became evident that alkylphenolic compounds are significant environmental pollutants, these are derived from non ionic surfactants of alkylphenol polyethoxylate (Giger et al., 2009). Subsequently, nonylphenol ethoxylates and their metabolites became a major focus of environmental research. Nonylphenol ethoxylates are cost-effective surfactants used globally in industrial, institutional, commercial, and household applications such as detergents, antistatic agents, emulsifiers, demulsifiers, wetting and dispersing agents, and solubilizers (Fiege et al., 2000; Lorenc et al., 2003; Soares et al., 2008; Vincent and Sneddon, 2009). Furthermore, these chemicals are being used in the products we use in our daily life, such as shampoos, deodorants, skin care products, plastic items, and so on (Cowan-Ellisberry et al., 2014). Due to extensive use of such chemical derivatives substantial quantities of nonylphenol ethoxylates reach sewage treatment works where they are only partially degraded to nonylphenol (NP) due to microbial action (Aheh et al., 1994; Shao et al., 2003; Johnson et al., 2005; Koh et al., 2005; Vazquez-Duhat al., 2005; Nakada et al., 2006; Nguyen et al., 2011) and then via food-chain accumulates in the bodies of biota.

NP that can migrate from food plastic and drinking water is found to be toxic to different biota (Hamlin et al., 2015, Priac et al., 2014). Since its discovery, the production of nonylphenol has increased exponentially by as much as 100-500 million pounds every year and meet the definition of high production volume chemical (EPA, 2010). World-
wide production of NP is approximately 500,000 tons, out of which 60% is finally discharged to water bodies (Ying et al., 2002). The European Union has included NP and their derivatives in the list of priority hazardous substances (Directive 2000/60/EC 2000). Micronucleus test is one used commonly for genotoxicity assessment in fish. Micronucleus assay has advantages over other tests as it is technically easier to use, cheaper, and less time-consuming (Galindo et al., 2014; Bhattacharyya et al., 2016).

Gill is the organ for respiration, and it plays a role in osmoregulation and nitrogen excretion processes and is particularly sensitive to adverse environmental conditions. This is the organ that comes into direct contact with the pollutant, it also has a large interface area between external and internal environments of the fish. A number of studies have documented the toxic effect of nonylphenol ethoxylate over fish gill and have concluded that gill is particularly sensitive for the surfactants (Cox, 1996; Sandbacka et al., 2000; Wu et al., 2005). Kidney play an important role in maintaining osmotic homeostasis. It is the main organ of homeostasis and filtration. Because water reabsorption takes place in the distal tubule, so renal tissue is in constant exposure to chemicals and the risk of damage to the tissue is high.

C. punctatus is distributed throughout India. This species is of commercial importance due to its high food value, easy maintenance, and availability throughout the year (Talukdar et al., 2016). Moreover C. punctatus has been used in fundamental research and considered as an excellent model for toxicological studies (Javed et al., 2016; Sharma and Chadha 2017). So keeping all these things in mind, this study aimed at increasing the knowledge and understanding about the toxic effects of 4-NP toward an aquatic vertebrates model Channa punctatus after sub chronic exposure and provide an in-depth view into the efficiency of DNA repair system in C. punctatus.

**MATERIALS AND METHODS**

**Chemical:** 4-nonylphenol used in the present study was obtained from Himedia (India). A stock solution was prepared in ethanol. The stock was prepared so that the concentration of ethanol remains constant in all the treatments.

**Experimental fish and toxicity bioassay:** To determine the 96-hour LC50 value of 4-nonylphenol, acute toxicity bioassay was conducted in a static system in the laboratory the test solution was changed every day to ensure that the chemical level stayed the same throughout the test period. The 96-hours LC50 value of 4-nonylphenol was determined as 1.27 mg/l for C. punctatus (Sharma et al., 2014), following the probit analysis method as described by Finney (1971). Safe application rate (SAR) was calculated using the formula given by Basak and Konar (1977). After calculating the SAR three sublethal concentrations were decided, which were 1/10th (0.15mg/l), 1/15th (0.10mg/l) and 1/20th (0.07 mg/l) of the SAR. SAR = LC0 x LC100 / LC 50

**In-vivo exposure experiment:** The fish specimens (40 no) were exposed to the three aforementioned test concentrations of 4-nonylphenol and in tap water (10 no) and ethanol (10 no) in a static renewal system with the change of test water every day to maintain concentration consistently. The exposure was continued up to 90 days, and blood sample were taken at the intervals of 30, 60, and 90 days at the rate of five different fish per interval. The specimens maintained in tap water were considered as negative control and those in ethanol as a positive control. After 90 days of exposure fish were kept in water without 4-nonylphenol for 30 days and at the end of 30 days recovery was ascertained.

**Micronucleus assay:** For each experimental group as well as for controls drops of blood taken from gill and kidney were smeared onto clean slides. Slides were then fixed in absolute ethanol for 10 min and stained with 10% Giemsa (Palhares and Grisolia, 2002). In each group, 1000 erythrocytes were counted under a binocular microscope (Olympus) using a 100x oil immersion lens. Cells were scored for micronucleated cells (MNC) and aberrant cells (AC). Different nuclear and cytoplasmic abnormalities like nuclear bud, notched nucleus, nuclear and cytoplasmic bridge, vacuolated nucleus and cytoplasm, deformed nuclei, caryolysis and swollen cells were considered as aberrant cells.

**Statistical analysis:** The results are expressed as mean ±S.E. to study the significance of the difference in the frequency of micronucleated and altered erythrocytes among treated and control group one-way analysis of variance (ANOVA) followed by Tukey-HSD test were conducted with the help of SPSS.

**RESULTS**

**MNC and AC frequency in blood cells from the gill and kidney:** MNC and AC frequencies in blood cells from gill and kidney tissue of C. punctatus are presented in Table 1 and 2 respectively. All the concentrations at their respective durations evoked significant DNA damage in gill tissue when compared with control groups (Table 1). After 90 days exposure to 4-NP, the highest genotoxicity (4.45%) in gill erythrocytes was found in the group treated with 4-NP at 0.15 mg/l concentration. The time-dependent increase was observed at all concentrations and the highest effect was seen at the highest duration of exposure. In blood cells from gill tissue, AC frequency increased from 4.38 % in the control group to 63.71% (highest effect) in fish.
Table 1. Frequency of percent MNC and AC in blood cells from gill of fish C. punctatus after exposure to different concentrations of 4-NP for 30, 60 and 90 days.

<table>
<thead>
<tr>
<th></th>
<th>30 days</th>
<th>60 days</th>
<th>90 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.03±0.002</td>
<td>0.03±0.01</td>
<td>0.04±0.002</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.08±0.01</td>
<td>0.07±0.003</td>
<td>0.10±0.008</td>
</tr>
<tr>
<td>0.07 mg/l</td>
<td>0.65±0.08</td>
<td>0.75±0.12</td>
<td>1.42±0.05</td>
</tr>
<tr>
<td>0.10 mg/l</td>
<td>1.36±0.08</td>
<td>1.94±0.29</td>
<td>3.37±0.09</td>
</tr>
<tr>
<td>0.15mg/l</td>
<td>1.85±0.20</td>
<td>3.26±0.26</td>
<td>4.45±0.28</td>
</tr>
<tr>
<td>AC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.46±0.11</td>
<td>4.35±0.08</td>
<td>4.38±0.06</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5.12±0.06</td>
<td>5.37±0.014</td>
<td>5.39±0.003</td>
</tr>
<tr>
<td>0.07 mg/l</td>
<td>13.35±1.13</td>
<td>26.39±0.49</td>
<td>28.8±0.89</td>
</tr>
<tr>
<td>0.10 mg/l</td>
<td>35.80±1.75</td>
<td>31.80±0.89</td>
<td>50.31±2.05</td>
</tr>
<tr>
<td>0.15mg/l</td>
<td>38.1±0.86</td>
<td>50.53±1.44</td>
<td>63.71±0.84</td>
</tr>
</tbody>
</table>

The values given as mean ±standard error. Different letters (a, b, c) between the columns are significantly different (Tukey’s test, p≤0.01) and signify the effect of duration of exposure at each concentration.

Table 2 Frequency of percent MNC and AC in blood cells from kidney of fish C. punctatus after exposure to different concentrations of 4-NP for 30, 60 and 90 days.

<table>
<thead>
<tr>
<th></th>
<th>30 days</th>
<th>60 days</th>
<th>90 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.22±0.005</td>
<td>0.21±0.005</td>
<td>0.21±0.008</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.22±0.012</td>
<td>0.24±0.006</td>
<td>0.23±0.005</td>
</tr>
<tr>
<td>0.07 mg/l</td>
<td>0.57±0.03</td>
<td>0.86±0.06</td>
<td>1.26±0.02</td>
</tr>
<tr>
<td>0.10 mg/l</td>
<td>0.98±0.003</td>
<td>1.77±0.19</td>
<td>2.14±0.19</td>
</tr>
<tr>
<td>0.15 mg/l</td>
<td>1.30±0.16</td>
<td>1.75±0.11</td>
<td>2.60±0.22</td>
</tr>
<tr>
<td>AC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.95±0.13</td>
<td>7.99±0.02</td>
<td>7.95±0.04</td>
</tr>
<tr>
<td>Ethanol</td>
<td>7.96±0.13</td>
<td>7.99±0.02</td>
<td>7.95±0.04</td>
</tr>
<tr>
<td>0.07 mg/l</td>
<td>23.71±0.90</td>
<td>30.87±4.97</td>
<td>38.87±0.57</td>
</tr>
<tr>
<td>0.10 mg/l</td>
<td>29.26±0.46</td>
<td>38.26±0.56</td>
<td>55.32±0.54</td>
</tr>
<tr>
<td>0.15mg/l</td>
<td>33.7±2.27</td>
<td>46.68±0.63</td>
<td>53.38±1.76</td>
</tr>
</tbody>
</table>

The values given as mean ±standard error. Different letters (a, b, c) between the columns are significantly different (Tukey’s test, p≤0.01) and signify the effect of duration of exposure at each concentration.

Table 3. Frequency of percent micronucleated cell and aberrant cells in blood cells from gill and kidney tissue of fish C. punctatus after exposure to different concentrations of 4-NP for 90 days and after 30 days recovery.

<table>
<thead>
<tr>
<th>Cells from gill tissue</th>
<th>Parameters</th>
<th>Groups</th>
<th>0.07mg/l</th>
<th>0.10mg/l</th>
<th>0.15mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>%MNC</td>
<td>Exposed gp</td>
<td>1.42±0.05</td>
<td>3.37±0.09</td>
<td>4.45±0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery gp</td>
<td>0.47±0.05</td>
<td>0.68±0.01</td>
<td>1.2±0.05</td>
<td></td>
</tr>
<tr>
<td>%AC</td>
<td>Exposed gp</td>
<td>28.8±0.89</td>
<td>50.31±2.05</td>
<td>63.71±0.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery gp</td>
<td>15.07±0.35</td>
<td>19.48±0.38</td>
<td>23.57±0.29</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cells from kidney tissue</th>
<th>Parameters</th>
<th>Groups</th>
<th>0.07mg/l</th>
<th>0.10mg/l</th>
<th>0.15mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>%MNC</td>
<td>Exposed gp</td>
<td>0.41±0.02</td>
<td>0.69±0.04</td>
<td>1.01±0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Recovery gp</td>
<td>38.87±0.57</td>
<td>55.32±0.54</td>
<td>53.38±1.76</td>
<td></td>
</tr>
<tr>
<td>%AC</td>
<td>Exposed gp</td>
<td>12.94±0.42</td>
<td>14.64±0.42</td>
<td>18.79±0.22</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as mean ±standard error. Different letters (a, b) are significantly different (Tukey’s test, p≤0.01) and signify the difference between exposed and recovery group.

treated with 4-NP at 0.15 mg/l concentration after 90 days of exposure.

Erythrocytes from kidney also showed higher mean values for MNC frequency after sub chronic exposure of 4-NP as compared to the MNC frequency in the control group. Among treated groups a 12.47 fold (highest value, 2.6 ± 0.2) increase was noted in MNC frequency as compared to the control group (0.21 ± 0.008; Table 2). AC frequency in kidney erythrocytes is found to be higher in groups treated with 4-NP as compared to both the control groups. Highest rate of AC frequency found in the kidney was 55.32 %, which was 6.95 folds higher compared to the control group. Highest effect was seen after treatment with a chemical concentration of 0.10 mg/l after 90 days exposure. Among both tissues, blood cells from gill showed more DNA damage as values of both parameters were found to be high in gill tissue.

MNC and AC frequencies in blood cells from gill and kidney of recovery period: Recovery
pattern in MNC and AC frequencies in blood cells from gill and kidney of fish after 30 days is given in Table 3. At all the concentrations a significant reduction in the value of both the parameters, that is, MNC and AC frequencies (ANOVA and Tuk- eys’s test) was observed.

**DISCUSSION**

Various genotoxics form strong covalent bonds with DNA, which result in mutation, and DNA ad- duct formation and prevent accurate DNA replica- tion (Hartwell et al., 2000). Genotoxins affecting the germ cells (sperm and egg cells) can pass the genetic changes down to descendants (Hartwell et al., 2000). Studies pertaining to DNA damaging effects of xenoeestrogens are important, because the changes may be the precursors of some of developmental abnormalities, and infertility (Atiezer et al., 2002). Micronuclei are induced by aneugenic agents, but it is also hypothesized that they may be induced by a clastogenic process (Anitha et al., 2000). The micronucleus assay is a cell cycle dependent assay that determines stable aberrations.

Micronucleus test has been used by a number of researchers for assessing genetic damage (Nwani et al., 2011; Kumar et al., 2013; Galindo et al., 2014). Several researchers have also identified other nuclear abnormalities, including nuclear bud, fragmented nucleus, vacuolated nucleus and binucleated cells as an indicator of genotoxicity (Ayllon and Garcia-Vazquez, 2000; Cavas and Ergene-Gozuvara, 2003; Cavas et al., 2005; Talapatra and Banerjee 2007; Muranli and Guner, 2011). Although the mechanism of their formation has not yet been fully explained, according to Osman et al., (2014) nuclear abnormalities can be the precursor of MN and may be a process to eliminate amplified genes from the nucleus. These abnormalities are considered an indicator of geno- toxic damage and therefore, may compliment the scoring of MN in routine genotoxicity surveys (Muranli and Guner, 2011; Serrano-Garcia and Montero-Montoya, 2001).

The different nuclear abnormalities may be the result of gene amplification and problem in chromo- some attachment which may cumulatively lead to micronucleus., binucleus, budding, notched or lobed nuclei (Bolognesi et al., 2006; Ergene et al., 2007). A number of vacolated cells were observed in the present study and the reason may be unequal distribution of hemoglobin and one of the common alterations observed in our study was swollen cells which may be due to the necrosis of cellular outline. Similar results were obtained by Mekkawy et al., (2011) after treating the fish C. galipinus with 4-NP. Six types of nuclear lesions were detected along with MN by Osman et al., (2011) who used Nile tilapia (O. niloticus) and African catfish (C. gariepinus) collected from the course of River Nile.

Jiraungkoorskul et al., (2007) and Tsangaris et al., (2011) reported that MN study using erythrocytes is most sensitive as compared to information provided by gill, liver and fin cells. Palhares and Gri- solia (2002) suggested that early erythrocytes show a higher sensitivity than the peripheral eryth- rocytes. On the other hand, several authors sup- ported the use of renal and gill erythrocytes of fish (Ayllon and Garica-Vazquez 2000; Serrano- Garcia and Montero-Montoya 2001; Cavas et al., 2005). Talapatra and Banerjee (2007) used gill and kidney erythrocytes of Labeo bata from sewage fed fish farm. Fagri et al., (2008) suggested that gill is more sensitive than a hemopoietic tis- sue to the stressors for MN induction. Thus in the present study comparison has been done for the induction of MN and other aberrant cells using blood cells from gill and blood cells from the kid- ney. We observed a higher frequency of MN and a higher percentage of aberrant cells in blood cells from the kidney and gills of fish subjected to sub- chronic exposure as compared to controls. The effect of dose and duration of exposure was also found to be significant. Sharma and Chadha., (2016a) observed a significant increase in nuclear and cytoplasmic alteration in blood cells after acute exposure in C. punctatus after treatment with 4-nonylphenol. Similarly 4-nonylphenol is found to be immune-supresser (Sharma et al., 2018).

The present study highlighted, inter tissue differ- ences regarding the induction of MN. Gill tissue was found to show more effected as the values of both the parameters MNC and AC are found to be higher in gill tissue.. Gill is the organ that comes into contact with contaminants prior to other or- gans and are also exposed constantly. So this might be the reason for a more severe damage in gill tissue. Various investigatios were conducted where gill was found to be more sensitive than erythrocytes and kidney blood cells (Ali et al., 2009). A group of researchers had confirmed that gill tissue had a much higher sensitivity than other tissues (Ali et al., 2008, 2009; Sharma et al., 2007; Pandey et al., 2006; Nwani et al., 2010). The highest level of damage in gill and liver tis- sues of Zebra fish due to exposure to detergent was observed by Figueroa (2013). A large number of clastogenic and aneugenic compounds have been reported to cause nuclear lesions in kidney erythrocytes of fish. Barsiene et al., (2006) found significant induction of MN in peripheral blood and kidney erythrocytes. Furthermore, Rodriguez-Cea et al., (2003) tested the renal erythrocytes in brown trout inhabiting polluted area and found induction of micronuclei. These findings are fur- ther supported by Rybakovas et al., (2009) who tested peripheral blood and cephalic kidney eryth- rocytes for MN, nuclear bud and fragmented
apoptotic cells in Flounder (*Platichthys flesus*), Dab (*Limanda limanda*) and Cod (*Gadus morhua*), collected from 12 offshore sites from the Baltic Sea and 11 sites in the North. Fag et al., (2008) reported a higher MN frequency in peripheral blood as compared to kidney erythrocytes when several fish were exposed to environmental stress in Egypt. In contrast, Palhares and Grisolia, (2002) did not find a significant difference between the micronucleated cells of the kidney and the gill erythrocytes. Similarly, Manna and Sudhu-kar (1986) found a nonsignificant increase in the frequency of micronuclei in gill and kidney blood cell after irradiation in two fish species. Furthermore Cavalcanto et al., (2008) reported a nonsignificant level of induction of MN in erythrocytes of fish *P. lineatus* after its exposure to herbicide roundup® for 96 hours. The possible reason for DNA damage in the present study may be its microtubular disrupting activity or due to formation of reactive intermediates. Furthermore Vazquez-Duhalt et al., 2005 reported that nonylphenol induce DNA adduct formation and mutation or genomic rearrangements. Moreover increased DNA damage may also lead to apoptosis and apoptosis was observed in fish sertoli cells after nonylphenol exposure (Weber et al., 2002; Miura et al., 2005; Yi et al., 2009). Mekkawy et al., (2011) observed that 4-NP increased percentage of apoptotic cells in *C. gallinipes*. The bio-transformation of 4-nonylphenol may result in the production of intermediates which are highly reactive, toxic and can cause DNA breakage both directly and indirectly. A significant decrease in DNA damage was found in all the tissues after the 30 day recovery period. Damage drop to control value suggested the possibility of a complete turnover of fish erythrocytes and other cells. The life span of erythrocytes in fish is between 1 and 3 months (Udroiu 2006). Sharma and Chadha, (2016 and 2018) found that fish *C. punctatus* retained its DNA integrity and hematological parameters to control value in blood cells from peripheral circulation after the 30 days recovery period when treated with 4-nonylphenol. Similar results were found in Trout, s response (Bony et al., 2008) to vineyard pesticide, as well as in Carp and Bullheads (Pandrangi et al., 1995) and in Chub (Devaux et al., 1998). Guilherme et al., (2014) reported that blood cells of *A. anguilla* showed evident recovery at 14 days after exposure to herbicides based pesticide Round-up®. Furthermore Marques et al., (2014) found that DNA integrity returned to the control level on the first day after cessation of exposure.

**Conclusion**

Both the tissues (gill and kidney) of fish *C. punctatus* tested showed DNA damage in time and dose dependent manner after sub chronic exposure. Blood cells from gill were found to show higher damage as compared to blood cells from the kidney as the value of both the parameters MNC and AC were found higher in gill blood cells. The various regions of DNA damage, induced by NP may be due to its microtubule disrupting activity, DNA adduct formation, mutation and genomic rearrangement or may be due to the formation of toxic intermediates that may lead to DNA damage. A significant recovery from DNA damage found after 30 days suggest the strong capacity of *C. punctatus* to retain its DNA integrity.

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