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Effects of 4-Nonylphenol exposure on the liver and head kidney histopathology and serum biochemistry in *Channa punctatus* (Bloch)

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Abstract

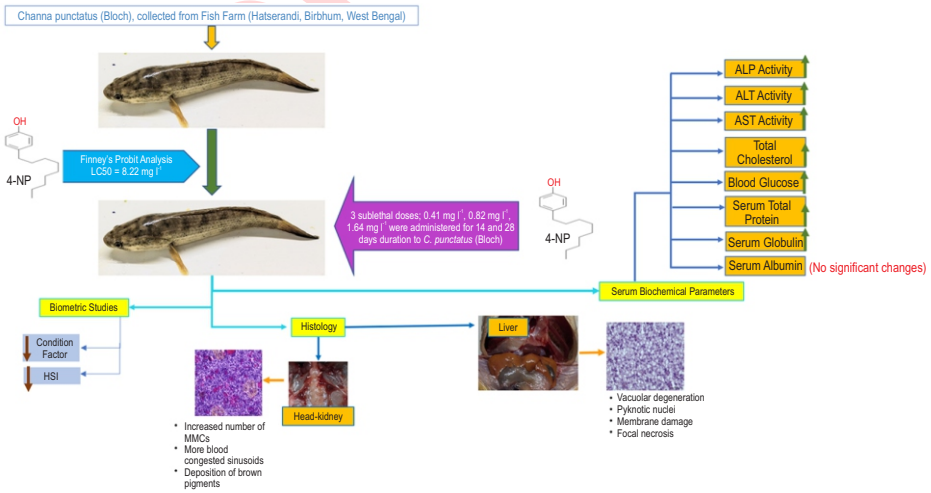
Aim: To assess the toxicological risks of sublethal exposure of 4-NP to fish, *Channa punctatus*.

Methodology: Static bioassay experiment was conducted to estimate 96 hr LC_{50} value of 4-NP for fish, *Channa punctatus* and for sub-chronic toxicity test, fish were subjected to sublethal exposure to 4-NP (0.41 mg l^{-1}, 0.82 mg l^{-1} and 1.64 mg l^{-1} of 4-NP) for 14 days and 28 days. Serum biochemical parameters and histopathology of liver and head kidney of treated and control fish were assessed.

Results: Estimated LC_{50} value of 8.22 mg l^{-1} confirmed that 4-NP is toxic to fish. Condition factor (K) and hepato-somatic indices (HSI) were significantly decreased following 4-NP exposure. The findings showed that dose dependently the treated fish had significantly higher serum levels of alkaline phosphatase, alanine and aspartate transaminase enzymes, total protein, globulin, glucose and cholesterol in serum than control. Exposure to 4-NP resulted in dose dependent changes in the liver histology including fat accumulation, intercellular vacuole formation, necrosis and hepatocyte hypertrophy. Inflammatory cells infiltered in the liver tissue following tissue damage. Whereas, in the head-kidney, 4-NP exposure caused degeneration and vacuole formation in the hematopoietic tissue, blood congestion, and increase in the melano-macrophage centers (MMCs).

Interpretation: A substantial tissue damage in the liver and head kidney followed by consequent alterations in blood biochemical parameters, metabolic disruption and immunomodulation strongly highlight the toxic potential of 4-NP to the fish health and survival fitness.

Key words: 4-Nonylphenol, *Channa punctatus,* Histopathology, Immunomodulation, Serum biochemistry



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Introduction

Nonylphenol is a member of alkylphenol group and it is used for producing Nonylphenol ethoxylates (NPEs). NPEs are extensively used as surfactant and emulsifier in detergents, pharmaceuticals, pesticide formulations, paper, paint, textile and leather processing industries (Won *et al.,* 2014, Noorimotlagh *et al.,* 2020). NPEs contribute approximately 80% of all alkylphenols used (He *et al.,* 2020). It enters the environment through industrial and municipal waste water effluents and gets converted by microorganisms into a stable form, Nonylphenol (Bennett and Metcalfe, 2020). Among various isoforms, 4-Nonylphenol (4-NP) is the most widely utilized commercial form (Giger *et al.,* 1984). Nonylphenol and nonylphenol ethoxylate compounds are estrogenic, endocrine disruptor and toxic compound (Liu *et al.,* 2013). Due to high lipophilicity 4-NP tends to get adsorbed in organic matters and has higher soil adsorption coefficient (Zhu and Zho, 2013). Selvaraj *et al.* (2014) detected the presence of 4-NP in water samples from the rivers of Tamilnadu, India, ranging from non-detectable level to 2200 ng l^{-1}, while, Raju *et al.* (2018) reported 4-NP ranging from 1.22-7.24 µg l^{-1} in water samples and 3.31-30.96 µg kg^{-1} in the sediments of marine ecosystem around Chennai, India. Many European and Asian countries have restricted the use of NP and NPEs, however, NP's persistence has been detected in rivers despite of regulatory measures (Kali *et al.,* 2025).

Toxicity of 4-NP is manifested through both estrogenic and non-estrogenic pathways leading to negative impacts on reproduction, development, growth, immune system, metabolic functions in fish (Bhandari *et al.,* 2021, Lofti *et al.,* 2021, Mukherjee *et al.,* 2022; Ammar *et al.,* 2023, Vega-Lopez *et al.,* 2024). 4-NP mimics 17β-Estradiol, binds to estrogen receptors and disrupts estrogen mediated physiology in animals (Lofti *et al.,* 2021). The other mechanism of nonylphenol-mediated toxicity is alteration in the cellular redox homeostasis (Dwivedi *et al.,* 2022). 4-NP enters the biological systems through adsorption and feeding, bio-accumulates and causes health hazard (Hong *et al.,* 2020). Sharma *et al.* (2018) reported 4-NP induced histopathological alterations in liver, gill and kidney in *Channa punctatus*. Sayed *et al.* (2018) showed 4-NP induced cytotoxicity in erythrocytes of Medaka. Other investigations confirmed 4-NP induced generation of oxidative stress response in different tissues of different fish species including histopathological alterations in gonad of *Heteropneustes fossilis* (Suman et al., 2025), liver of *Clarias gariepinus* (Abd-Elkareem et al., 2023), liver, gills and brain of *Cyprinus carpio* (Ammar *et al.*, 2022). Studies have shown estrogen and xenoestrogen compounds play a crucial role in immunomodulation in fish (Ahmed, 2000; Sinha and Mandal, 2024). However, comprehensive research report on 4-NP induced histopathology in liver and head kidney correlating to blood biochemical alterations, metabolic disruption and immunomodulation is limited. Therefore, the present study is an attempt to evaluate the toxicological risks of 4-NP exposure to *Channa punctatus* by assessing the liver and head kidney histopathology and the consequent alterations in the serum biochemistry.

Materials and Methods

Fish collection: Healthy adult fish, *Channa punctatus* (average weight of 70 ±10 g) were collected from a local fish farm located at the village, Hatserandi, Birbhum, West Bengal, India. Fish were acclimatized under laboratory conditions by keeping 10 fish each in 50 l glass aquaria for 15 days under continuous aeration. Tap water having DO: 7.57±0.11 mg l^{-1}, total alkalinity: 197.2 mg l^{-1}, total hardness: 142 mg l^{-1}, pH: 7.58±0.2 and temperature: 24ºC-26ºC was used for the experiments. Water parameters were analysed following the methods of APHA (2022). Fish were fed *ad libitum* with dried tubifex worms (crude protein: 58%, crude fat: 8%, crude fibre: 7%, crude ash: 12%, moisture: 5%). Water of the aquaria was partially changed in alternate day to remove uneaten feeds, faecal matter and to maintain water quality.

Chemicals: Test chemical, 4-Nonylphenol (4-NP) was purchased from Himedia (CAS no. 104-40-5). Kits for biochemical assays of Glucose, Alanine transaminase, Aspartate transaminase were procured from the Precision Biomed Pvt. Ltd., India whereas Kits for estimating Alkaline phosphatase, protein, albumin and cholesterol were obtained from the Arkray Healthcare Pvt. Ltd. Gujarat, India.

Experimental Design

Estimation of LC_{50}: In order to estimate the 96 hr LC_{50} value of 4-NP, laboratory acclimatized *C. punctatus* were transferred to 11 aquaria keeping 10 fish in each tank. Of which, fish of 10 aquaria were exposed to a series of 4-NP concentrations ranging from 4 mg l^{-1} to 13 mg l^{-1} and one aquarium was maintained as control. Feeding was stopped 24 hr prior to the experiment, and kept were starved for the entire period of 96 hr experiment. Fish without opercular movement and tactile sensation were considered dead and were removed from the tank to avoid deterioration of the water quality. Fish mortality was separately recorded for each tank every 4 hr interval and cumulative data of mortality over the period of 96 hr experiment were utilized to estimate 96 hr LC_{50} value. The acute toxicity test was conducted following the test guideline no. 203 of OECD (2019). Empirical probit value of the mortality percentages of 96 hours exposure and corresponding log concentrations of 4-NP were plotted in a linear regression curve following Finney (1971) (Fig. 1) and 96 hr LC_{50} value was estimated at 95% confidence limit. Based on the standard toxicological experiment practice, sublethal concentrations were taken as low, 0.41 mg l^{-1} (5% of LC_{50}), medium, 0.82 mg l^{-1} (10% of LC_{50}), and high dose, 1.64 mg l^{-1} (20% of LC_{50}) for the subsequent experiments of the sub-chronic toxicity tests.

Sub-chronic toxicity assessment: Experiments for sub-chronic toxicity assessment were conducted with the laboratory acclimatized fish. Each experimental set was formed by four aquaria with five fish in each; one group was kept as control and rest three groups were subjected to sublethal exposure to 4-NP. Experiments were conducted for 14 and 28 days. Fish were fed *ad libitum* and kept under continuous aeration. Faecal matter and

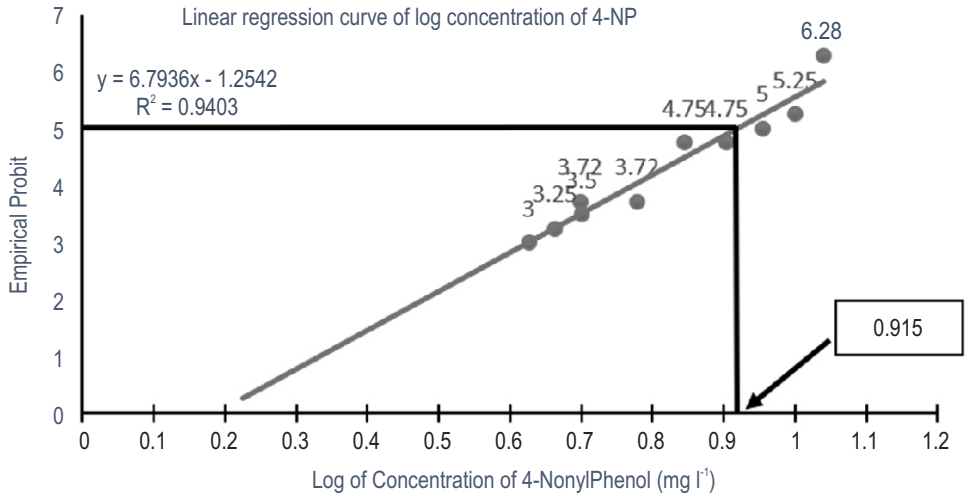

Fig.1: Probit value of the mortality percentage of adult *Channa punctatus* (Bloch) against the log concentrations of 4-Nonylphenol after 96 hours exposure. Arrow indicates the log concentration (0.915) of 4-NP corresponding to Probit value 5 (50% mortality).

uneaten feed debris were removed everyday by siphonic method. Water of aquaria was changed and the corresponding 4-NP concentrations were renewed at alternate day to maintain water quality and consistently same exposure concentrations. Experiments were carried out in triplicate sets. After completion of experiments, fish were anaesthetized with MS-222 (CAS No. 94-09-7), and the blood and tissue samples were collected.

Estimation of Condition factor (K) and Hepato-somatic indices (HSI): Fulton's Condition factor was determined by using the formula $K = \frac{W}{L^3}$, where K is the condition factor, W is the body weight (g), L is the length (cm) of the fish.

$$HSI = \frac{\text{Weight of liver}}{\text{Body weight of fish}} \times 100$$

Serum biochemical assays: Blood samples drawn from caudal vein with a heparinized syringe, was centrifuged at 4000 rpm for 5 min, and the serum samples were stored in -20ºC for biochemical analysis. The serum levels of glucose, total protein, albumin, cholesterol, alanine transaminase, aspartate transaminase, and alkaline phosphatase were estimated spectrophotometrically using a Spectrophotometer (Shimazdu, Model UV 1601) and assay kits following manufacturer's protocol. Serum globulin was estimated by subtracting serum albumin concentration from the total protein.

Histopathological assessment: Tissue samples of the head kidney and liver were procured from the control and treated fish. After proper washing in ice cold fish saline tissue samples were fixed in aqueous Bouin's fluid for 24 hr. Tissues were dehydrated through 30%, 50%, 70%. 90% and absolute alcohol, cleared in xylene, infiltered in paraffin wax (Melting point 58°-60°C) under a vacuum paraffin embedding bath and embedded in paraffin block. Paraffin blocks were cut into 5 µm thin tissue sections using a rotatory microtome (Weswox optic MT-1090A) and affixed on albuminized glass slides. Tissue section were deparaffinised, hydrated gradually through alcohol, stained in Hematoxylin and eosin, dehydrated gradually through ethanol, cleared and mounted with DPX. Stained sections were examined and photographs were recorded under a trinocular research light microscope (Leica DM 3000 LED).

Statistical analyses: All experimental data of the respective control and experimental groups were represented as Mean value ± S.D. Statistically significant differences of the serum biochemical parameters between the fish of control and exposed groups of 14 days and 28 days experiments were analysed separately within the same exposure duration by One-way ANOVA followed by Tukey's test analysis at $P<0.05$ and $P<0.01$.

Results and Discussion

4-Nonylphenol is a hazardous chemical and its toxic effects are manifested in both estrogenic and non-estrogenic pathways (Hong *et al.,* 2020, De la Parra-Guerra and Acevedo-Barrios 2023, Liu *et al.,* 2023, Ghafoor *et al.,* 2024). In non-estrogenic pathway 4-NP induces oxidative stress and subsequent lipid peroxidation, histopathology in different organs and disrupts metabolic functions (Won *et al.,* 2014, Yu *et al.,* 2017; Mukherjee *et al.,* 2020). On the other hand, in estrogenic pathway, it affects vitellogenesis, gonad maturation, enzymes involving steroidogenic activities and immune systems (Ruggeri *et al.,* 2008, Lofti *et al.,* 2021, Mukherjee *et al.,* 2022).

96 hr LC_{50} value of 4-NP for *C. punctatus* was estimated to be 8.22 mg l^{-1}. Median lethal concentration of a chemical provides information on its ecotoxicological risk and help in framing safety regulatory measure. The 96 hr LC_{50} value of 4-NP for the fish of

present and previous studies suggests that fish are susceptible to 4-NP contamination. In the present study, 96 hr LC_{50} value of 4-NP for *C. punctatus* was greater than 1.5 mg l^{-1} for *Oreochromis mossambicus* (Midhila and Chitra, 2015), 1.72 µM for *Puntius conchonious* (Bhattacharya *et al.,* 2008) and 1.27mg l^{-1} for *C. punctatus* (Sharma *et al.,* 2015). The LC_{50} value depends on various factors such as physico-chemical properties of test environment, test procedure, different species, and even within same species it may vary due to difference in age, size and health status of the fish. These findings showed that adult *Channa punctatus* was comparatively tolerant to this chemical.

Fish exposed to 4-NP showed a significant (P<0.05) decline in condition factors at the end of 14 and 28 days of experiment (Fig. 2a) Condition factor (K) of a fish depends on its age, sex, reproductive phase, food quality, food intake, biotic and abiotic environmental factors (Pandit *et al.,* 2019). A lower condition factor indicates ill health of the fish. Hence, it is often used to assess the health condition of fish in presence of any hazardous compound (Bhattacharya *et al.,* 2008). Thus, declined Condition factor (K) indicates ill health of 4-NP exposed *Channa punctatus*. Hepatosomatic index (HSI) is also frequently used to evaluate fish health (Adams and McLean, 1985). According to the current study, fish exposed to 4-NP had a significant decline in HSI (Fig. 2b).This result is consistent with the previous studies on the impacts of dimethoate on *Oncorhynchus mykiss* (Dogan and Can, 2011) and nonylphenol on *Oreochromis mossambicus* (Midhila and Chitra 2015). Liver shrinkage and decrease in HSI in the treated fish may occur due to stress induced low food intake, hepatic atrophy and depletion of hepatic energy reserve for maintaining the metabolic cost of detoxication and body homeostasis. Similarly, the energy required to metabolize 4-NP likely divert resources from somatic growth explaining the lower Condition factor.

Fish exposed to 4-NP showed significantly higher blood levels of ALT, AST, ALP, total protein, albumin, globulin, glucose, and cholesterol than control fish (Fig. 3). Upon increasing the exposure concentrations and duration, the blood levels of ALP, ALT and AST, the liver function biomarker enzymes, were significantly (P<0.05) increased in the treated fish (Figs 3a,b,c). Transaminases are found in cytoplasm of hepatocytes. Membrane disintegration or leakage of hepatocyte membrane causes release of transaminases and alkaline phosphatase from hepatocytes into blood stream, which results in elevation of these biomarker enzymes in serum. This study suggests that increased blood levels of these enzymes could be a result of histopathological damage of the liver tissue in treated fish. A similar liver histopathology and an increase in these enzymes in fish blood have been reported by Bhattacharya *et al.* (2008). Elevated blood levels of these enzymes indicate cellular damage as well as impairment of carbohydrate, lipid and protein metabolism (Yu *et al.,* 2018; Ismail and Mahboub, 2016). A similar finding to our current results, the rise in blood levels of ALT, AST and ALP in correlation to liver histopathology has been reported by Ammar *et al.* (2023) in the 4 NP and microplastic treated *Cyprinus carpio*.

Blood glucose, total protein, total cholesterol are important biomarkers for assessing health. Glucose and cholesterol levels were altered significantly (P<0.05) in all treated groups, except in the fish exposed to 0.41mg l^{-1} 4-NP for 14 days. The control fish of 14- and 28 days experiments had the blood glucose levels of 62.72±11.42 mg d l^{-1} and 68.88±7.60 mg d l^{-1} respectively. After 14 days exposure to 0.82 mg l^{-1} and 1.64 mg l^{-1} 4-NP, the glucose levels were elevated to 91.47% and 83.08% incomparision to control. Whereas, in all treated fish in the 28 days experiment, blood glucose level elevated significantly (Fig. 3d). Control fish had the cholesterol levels 52.32±18.96 mgd l^{-1} and 149.66±11.44 mg d l^{-1} after 14 days and 28 days experiments and these levels were elevated significantly in all treated groups (P<0.05), except for fish exposed to highest (1.64 mg l^{-1}) concentration for 28 days (Fig. 3e). Total protein levels in the fish of control groups of 14 and 28 days experiments were recorded to be 2.82±0.12 gd l^{-1} and 2.65±0.22 gd l^{-1}, respectively.

After 14 days of treatment, total protein levels increased significantly (P<0.05) in a dose and duration dependant manner (Fig. 3f). A similar pattern was observed in the globulin level (Fig. 3h). Albumin levels in the control groups of both 14 and 28 days were 1.11±0.18 g d l^{-1} and 1.07±0.26 g d l^{-1}, respectively, and a significant rise (P<0.01) 0.82 mg l^{-1} 4-NP was found in 14 days exposed fish group (Fig. 3g). A similar pattern of alterations in phosphatase and transaminase enzymes, blood glucose and cholesterol levels suggests that these changes are corelated to the hepatic tissue damage and its functions.

A consistent elevation in the serum cholesterol level was noted in all treated groups of 14- and 28-days experiment only with an exception among the fish group exposed to highest NP concentration (1.64 mg l^{-1}) for 28 days, where cholesterol level was found to be decreased significantly compared to control. This represents an exhaustive phase in which the liver's biosynthetic capacity is impaired due to 4-NP induced toxic response, rather than merely reflecting a stress response. A significant increase in total protein and serum globulin, but no change in the albumin levels, suggest an increase in the immunoglobulin synthesis in the treated fish. The results of the current study are consistent with the findings of Ismail and Mahboub (2016) who reported that 4-NP exposure to Nile tilapia increased the blood level of total proteins, globulins, and total cholesterol, and Yu *et al*. (2020) who demonstrated that 4-NP exposure increased the blood cholesterol level among rats.

Liver is the primary detoxification organ and plays a crucial role in metabolizing and elimination of toxicants from the body. Nonylphenol exposure may cause hepatotoxicity leading to histopathology and metabolic disorder in fish. Previous studies have elucidated that (Schwaiger *et al.*, 2000; Midhila and Chitra, 2015; Ismail and Mahboub, 2016; Kazemi *et al.*, 2016). The current findings showed that hepatic cords were arranged in two layers with thin sinusoids between them, and hepatocytes possessed central nucleus and granulated cytoplasm in the control fish, *Channa punctatus* (Figs. 4a-b; 5a-b). Whereas,

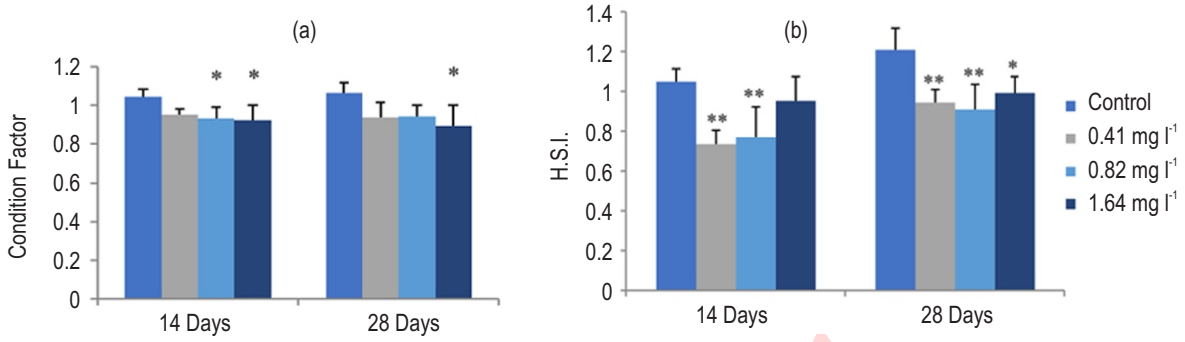

Fig. 2: Condition Factor (K) and Hepatosomatic indices of *C. punctatus* after exposure to different concentrations of 4-Nonylphenol for 14- and 28-days.Each column representing Mean ± SD (n=5) a.- Condition factor; b.- Hepatosomatic indices. *Indicate significant differences ($p< 0.05$) and ** indicates significant difference ($p<0.01$) from the respective control.

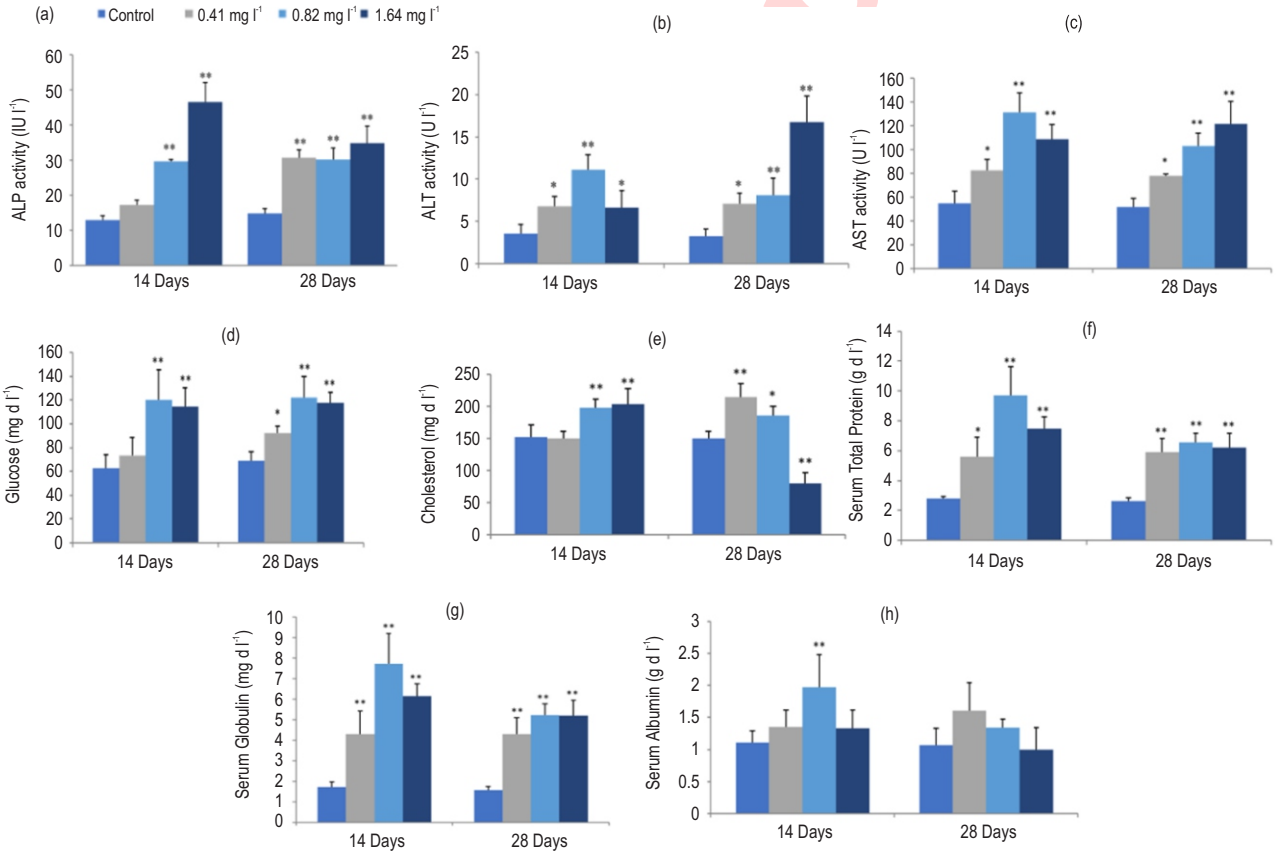

Fig. 3: Effects of exposure to three sublethal concentrationsof 4-Nonylphenol on serum biochemical parameters in *C. punctatus* after 14- and 28-days exposure. Each column representing Mean ± SD (n=3) of the respective parameter.a. Alkaline Phosphatase (ALP); b. Alanine transaminase (ALT); c. Aspartate transaminase (AST); d. Glucose; e. Cholesterol; f. Serum total protein; g. Serum globulin; h. Serum albumin. One way ANOVA followed by Tukey test was conducted to find significant differences. * Indicate significant differences at $p<0.05$ and ** indicates significant differences at $p<0.01$ from the respective control within same exposure duration.

histopathological abnormalities were prominent in the liver tissues of fish exposed to 4-NP (Figs. 4c-h; 5c-h). After 14-days exposure to 0.41 mg l^{-1} 4-NP, liver sections showed hepatocytes with less granulated and vacuolated cytoplasm (Figs. 4c,d). Other prominent changes were membrane damage in hepatocytes, focal necrosis, dilation of blood vessels and infiltration of inflammatory cells. No blood congestion was noticed in sinusoids. Fish treated with 0.82 mg l^{-1} 4-NP showed a moderate histological alteration in their liver sections (Figs. 4e,f). The main alterations included hepatocytes with less granular and vacuolated

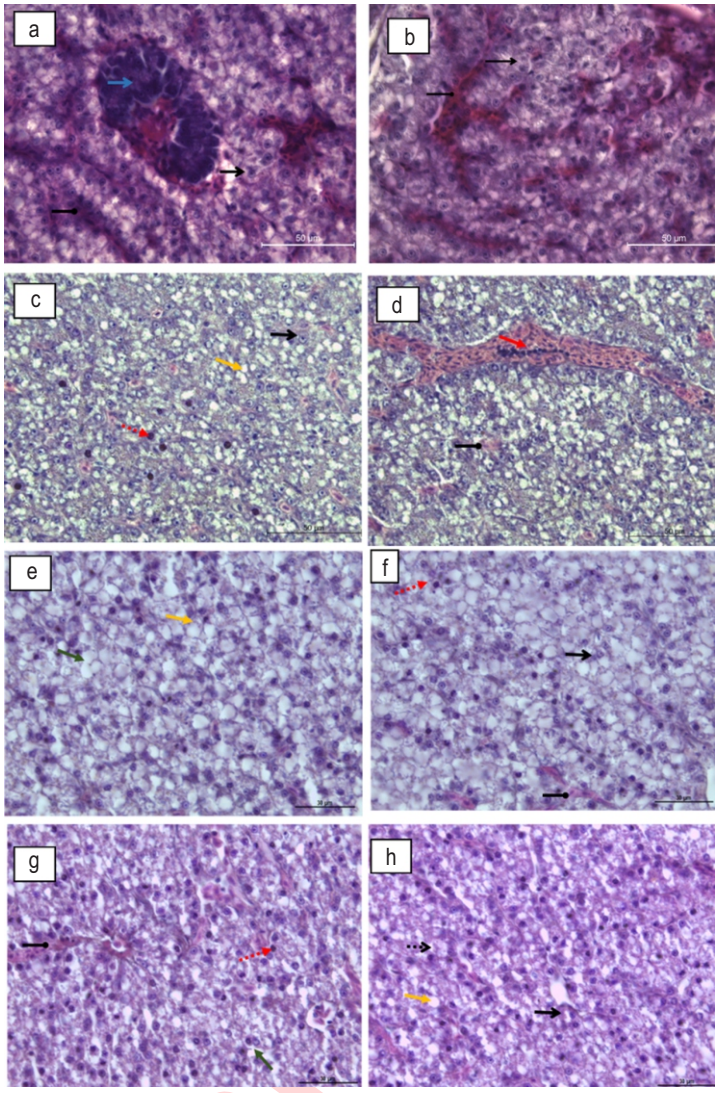

Fig. 4: Histological features of liver tissue of control and 4-Nonylphenol exposed *Channa punctatus* after 14 days experiment .H&E staining(a & b) Control showing polygonal hepatocytes (black arrow) interspaced with thin sinusoids (black oval arrow). Hepatopancreas (blue arrow) and blood vessels (BV). (c-h) Histopathology in the liver tissue of the fish exposed to different sublethal concentrations of 4-NP(c & d) 0.41 mg l^{-1} 4-NP (scale bar 50 µm); (e & f) 0.82 mg l^{-1} 4-NP (scale bar) and (g & h) 1.64 mg l^{-1} 4-NP (scale bar 30µm) showing hepatocytes (black arrow), intracellular vacuolization (yellow arrow), sinusoids (black oval arrow) in all exposed groups, pyknotic nuclei (red broken arrow), infiltration of inflammatory cells (red arrow), dilated blood vessel (BV), membrane damage (green arrow), focal necrosis (red asterisk); intercellular spaces (red oval arrow), swelled hepatocytes (black broken arrow) observed in 0.82 and 1.64 mg l^{-1} 4-NP exposed groups.

cytoplasm, intercellular vacuolization, hepatocyte enlargement, pyknosis, membrane damage and localized necrosis. However, the liver of the fish groups exposed to 1.64 mg l^{-1} of 4-NP displayed severe liver damage which showed hepatocytes with vacuolated cytoplasm, pyknosis and hyperplasia (Figs. 4 g,h). Hepatocyte degeneration and extensive intercellular vacuolization altered the usual structure of the hepatic cords. Following a 28-day experiment, the liver tissue of the control fish (Figs. 5a,b) had normal histological organization, however, fish treated with 0.41 mg l^{-1} 4-NP (Figs. 5c,d), liver tissue had necrosis and enlarged, degenerating hepatocytes with intracellular vacuoles. The hepatic cord's usual configuration was disturbed by the extreme intercellular vacuolization. Hepatocytes in the 0.82 mg l^{-1} 4-NP exposed group displayed vacuolated degeneration, swelling and brown pigment deposition (Figs. 5e, f). In certain places, necrotic and damaged hepatocytes were seen. Liver histology in fish treated with 1.64 mg l^{-1} 4-NP (Figs. 5g, h) revealed hypertrophied hepatocytes with brown pigment deposition, localized necrosis and severe intercellular vacuolization.

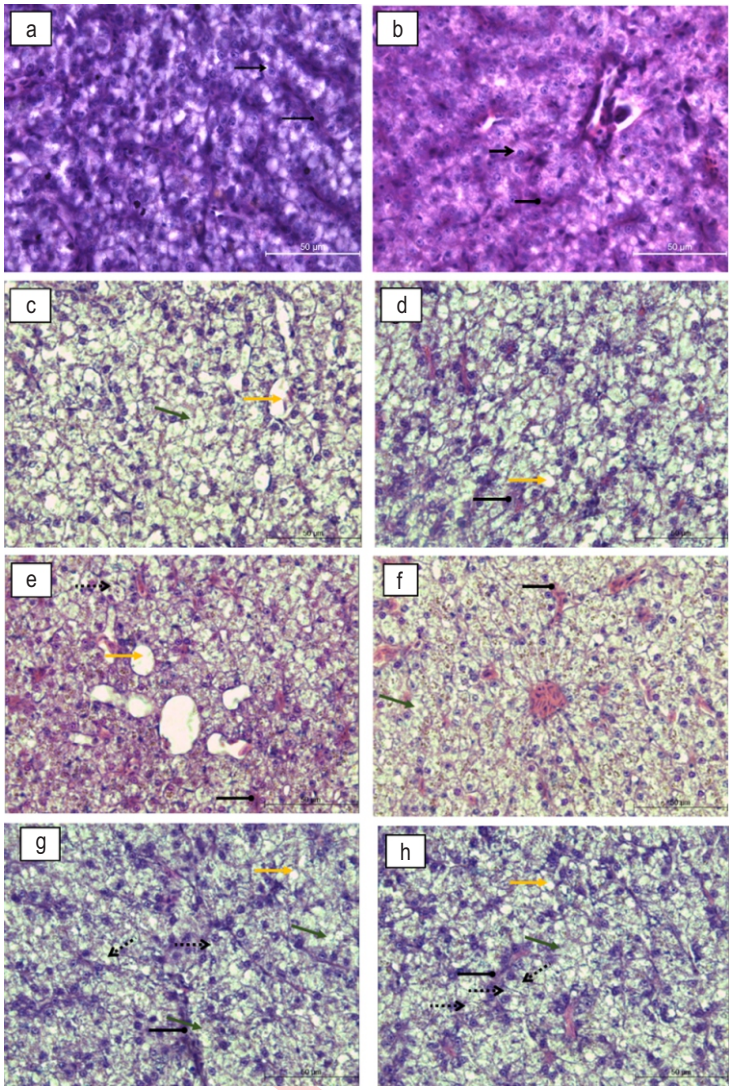

Fig. 5: Histological features of liver tissue of control and 4-Nonylphenol exposed *Channa punctatus* after 28 days experiment. H&E staining; scale bar 50 µm (a & b) control showing normal arrangement of hepatocytes (arrows) (c to h) Histopathology in the liver tissue of the fish exposed to different sublethal concentrations of 4-NP (c & d) 0.41 mg l^{-1} 4-NP; (e & f) 0.82 mg l^{-1} 4-NP (g & h) 1.64 mg l^{-1} 4-NP; showing intracellular vacuolization (yellow arrow), intercellular vacuolization (red oval arrow), membrane damage (green arrow), focal necrosis (red asterisk), hypertrophied hepatocytes (black broken arrow);sinusoids (black oval arrow), Hepatocytes containing brown pigments (brown broken arrow) and central vein (CV).

According to the current study, abnormal metabolic activities are correlated to the liver histopathology. *Labeo rohita* treated to BPA also showed metabolic disruption corresponding to liver histopathology (Mukherjee *et al.,* 2020). Inflammatory cells infiltrated liver as an immunological reaction to the tissue injury. The possible reasons behind cell enlargement and cytoplasmic vacuole formation may be due to accumulation of lipids, high metabolic activity and depletion of energy reserves in the presence of stressors (Yu *et al.,* 2018). The presence of cells with pyknotic nuclei indicates induction of apoptosis. According to Kaptaner and Unal (2011), 4-NP exposure enhances apoptosis of hepatocytes. Accumulation of lipofuscin or ceroid in the intracellular lysosome as brown pigments also describe the pathological condition of the liver. The head kidney contained haematopoietic cells, stromal or reticular cells, steroidogenic interrenal and chromaffin cells, macrophages, granulocytes and an extensive network of blood-filled sinusoids. Chromaffin cells were dispersed throughout the haematopoietic tissue, while interrenal cells were distributed around the cardinal vein.

The haematopoietic tissue (Ht) consisting of erythropoietic and lymphopoietic cells was supported by a network of stromal cells. Melano-macrophages and melano-macrophage centres (MMCs) were distributed throughout the

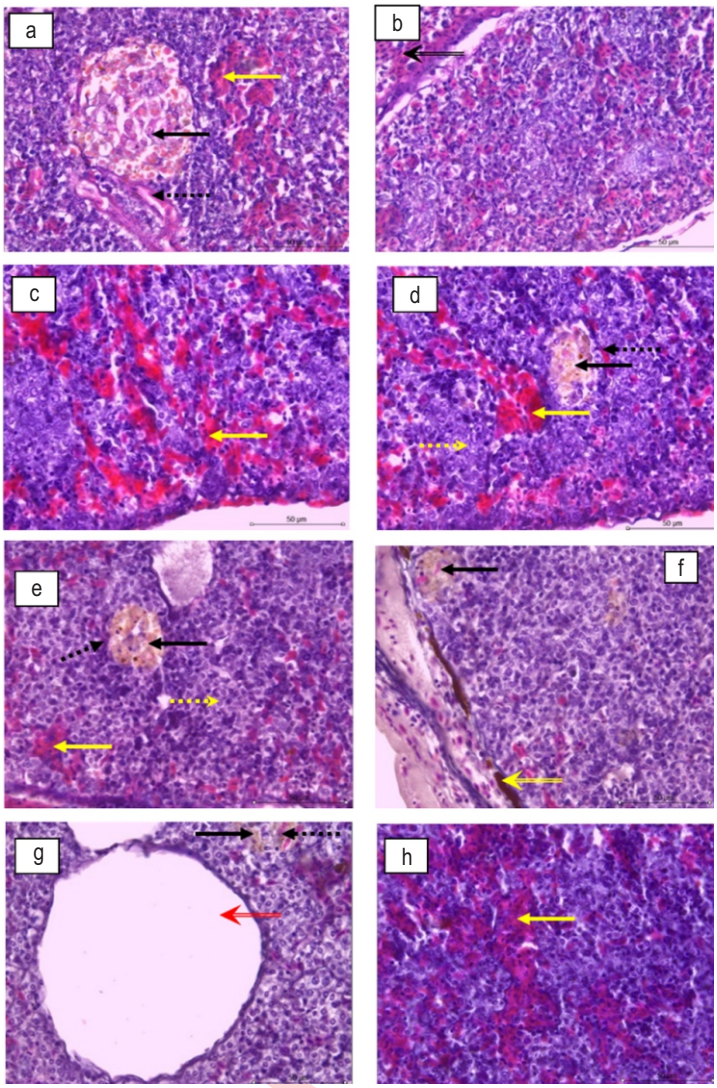

Fig. 6: Histological features of head kidney of control and 4-Nonylphenol exposed *Channa punctatus* after 14 days experiment. H&E staining; scale bar 50 µm (a & b) Control showing the normal arrangement of hematopoietic tissue (Ht), a Melano-macrophage Centre (black arrow), associated blood capillary (black broken arrow), blood vessels and sinusoids (yellow arrow) (c-h)Histopathology in the head kidney of the fish exposed to different sublethal concentrations of 4-NP (c & d) 0.41 mg l^{-1}; (e-f) 0.82 mg l^{-1} and (g-h) 1.64 mg l^{-1} showing sinusoids (yellow arrow), blood vessels (black double lined arrow), MMC (black arrow), MMC associated blood capillary (black broken arrow), hematopoietic tissue (Ht); melanin deposition within MMCs (green double lined arrow), brown pigment deposition (yellow double lined arrow), vacuoles (red double lined arrow).

haematopoietic tissues in control fish (Figs. 6 a-b; Figs. 7a-b). After 14-days experiment, no histopathological changes were found in the head kidney of fish exposed to 0.41 mg l^{-1} 4-NP, except blood congested sinusoids (Fig. 6 c-d). However, in 0.82 mg l^{-1} 4-NP treated fish, hematopoietic tissue was found to decrease along with the increase of vacuoles and melano-macrophages centres (MMCs). Brown pigment depositions were increased in the stromal tissue (Figs. 6e-f). The lipid rich protein lipofuscin and ceroid are formed and deposited as brown pigments in the intracellular lysosome as a function of incomplete metabolism and oxidative stress.

Fish treated with 1.64 mg l^{-1} of 4-NP showed notable histological alterations in the head kidney, including decreased hematopoietic tissue, large empty vacuoles, dilated sinusoids with increased blood infiltration, and more MMCs (Fig. 6 g-h). After 28-days treatment with 0.41 mg l^{-1} 4-NP, head kidney showed increased blood congestion, melanin deposition in stromal tissue, small vacuoles and enlarged MMCs (Figs. 7c-d), whereas fish treated with 0.82 mg l^{-1} 4-NP (Figs. 7e-f) and 1.64 mg l^{-1} 4-NP (Fig. 7 g-h) head kidney showed reduced haematopoietic tissue, enlarged vacuoles, dilated sinusoids, brown pigment deposits in stroma and enlarged MMCs.

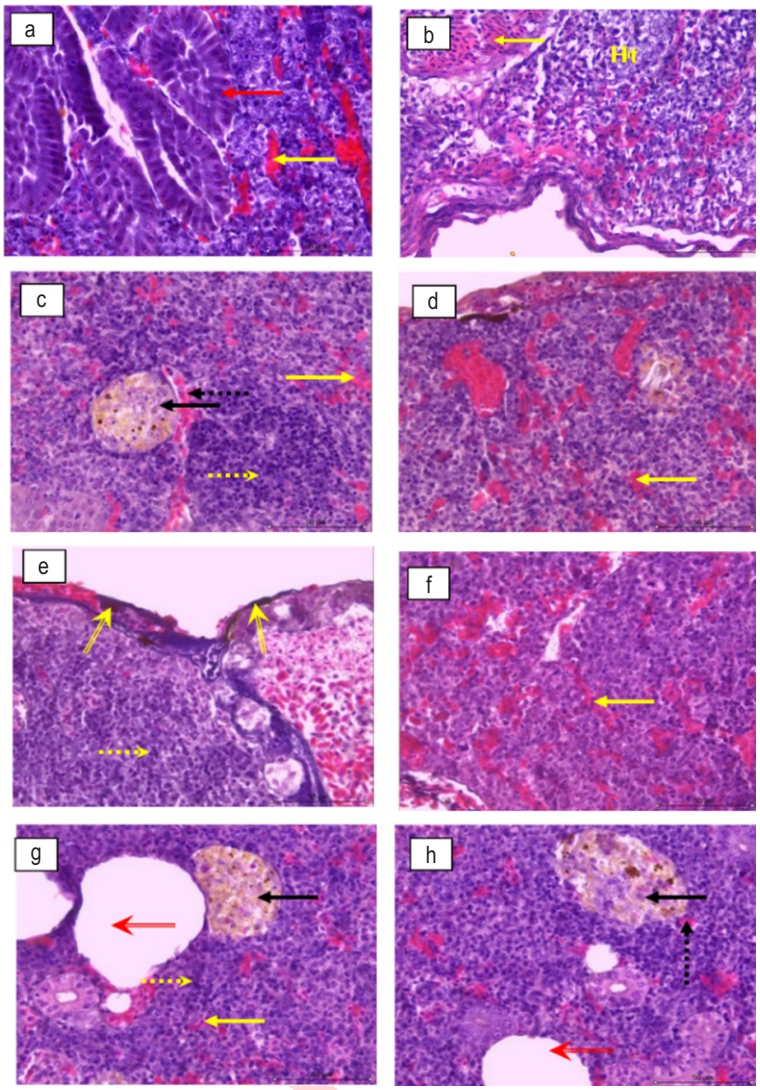

Fig. 7: Histological features of head kidney of control and 4-Nonylphenol exposed *Channa punctatus* after 28 days experiment. H&E staining; scale bar 50µm (a & b) Control showing normal distribution of hematopoietic tissue (Ht), Columnar interrenal cell (Red arrow), Blood vessels and sinusoids.(c-h) Histopathology in the head kidney of the fish exposed to different sublethal concentrations of 4-NP(c-d) 0.41 mg l^{-1}; (e-f) 0.82 mg l^{-1} and (g-h) 1.64 mg l^{-1} showing increased MMCs (black arrow) and its associated capillary (black broken arrow), degeneration of hematopoietic tissue (Ht), melanin deposition within MMCs (green double lined arrow), brown pigment deposition (yellow double lined arrow), large vacuoles (red double lined arrow).

Fish head kidney serves as haematopoietic as well as endocrine organ (Rocha *et al.,* 2001; Santos *et al.,* 2011), and it also acts as a secondary lymphoid organ (Press and Evensen, 1999). In addition to provide the structural support, stroma contributes in generation of non-specific immunity and elimination of cellular debris (Meseguer *et al*., 1994; Press and Evensen, 1999). Haematopoietic tissue of the head kidney possess different stages of erythropoiesis, granulopoiesis, lymphopoiesis and thrombopoiesis cells (Abdel-Aziz *et al.,* 2010), and the composition may vary depending on the presence of environmental pollutants (Witeska *et al.,* 2023). Melano-macrophage centres (MMCs) are characteristically found in the haematopoietic tissues like head kidney, spleen, andliver of fish (Tsujii and Seno, 1990, Steinel and Bolnick, 2017).

MMCs consist of large aggregates of macrophages filled with phagocytized cellular debris, melanin pigments, lipofuscin, hemosiderin granules etc. (Agius, 1985). MMCs perform diversified functions ranging from removal of cellular debris, foreign elements, pathogens, and particulate matters from circulation; scavenging of degraded products and cells from damaged tissue to antigen trapping and their processing for generation of immune responses (Agius, 1985; Herraez and Zapata, 1986). Agius and Roberts (2003) showed that tissue

damage and persistent inflammation lead to the development of MMCs. Another study reported that polluted water of Elbe River induced pathological condition in *Gymnocephalus cernuus* and abundance of liver MMCs (Kranz and Peters, 1984). Thus, it can be stated that increased cell or tissue damages or development of inflammatory foci trigger increased phagocytic activity leading to increase MMCs in fish immune organs, however, the newly developed and enlarged MMCs occupy the haematopoietic tissue and interfere with the haematopoietic activities in the head kidney (Fishelson, 2006). The present investigation showed increased MMCs in the head kidney in 4-NP exposed fish, which shows higher phagocytic activity which may occur as a result of increased cell and tissue damage. High phagocytic activity in the presence of environmental xenobiotic components is an indicative biomarker of immunotoxicity response (Fournier *et al.,* 2000).

These, the findings of this study documents that 4-Nonylphenol exposure can pose a risk to the fish health and survival. The histopathology followed by altered blood parameters and increased MMCs may be used as biomarkers for the toxicity assessment. Further, detailed study is recommended to investigate on the management and amelioration options to reduce the 4-NP induced toxic impacts in fish.

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References

Abd-Elkareem, M., A. E.-D. H. Sayed, N. S. A. Khalil and M. H. Kotob: *Nigella sativa* seeds mitigate the hepatic histo-architectural and ultrastructural changes induced by 4-nonylphenol in *Clarias gariepinus*. *Sci. Rep*., **13**, 4109 (2023).

Abdel-Aziz, E.S.H., S.B.S. Abdu, T.E.S. Ali and H.F. Fouad: Haemopoiesis in the head kidney of tilapia, *Oreochromis niloticus* (Teleostei: Cichlidae): a morphological (optical and ultrastructural) study. *Fish Physiol. Biochem.,* **36**, 323-336 (2010).

Adams S.M. and R.B. McLean: Estimation of largemouth bass, *Micropterus salmoides* Lacépède, growth using the liver somatic index and physiological variables. *J. Fish Biol.,* **26**, 111-126 (1985).

Agius, C. and R.J. Roberts: Melano - macrophage centres and their role in fish pathology. *J. Fish Dis.,* **26**, 499-509 (2003).

Agius, C.: The melano-macrophage centres of fish: a review. In: Fish Immunology. (Eds.: M.J. Manning and M.F. Tanter). Academic Press, London, pp. 85-105 (1985).

Ahmed, A.S.: The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. *Toxicology,* **150**, 191-206 (2000).

Ammar, E., M. Hamed, M.S. Mohamed and A.E.D.H. Sayed: The synergetic effects of 4-nonylphenol and polyethylene microplastics in *Cyprinus carpio* juveniles using blood biomarkers. *Sci. Rep.,* **13**, 11635 (2023).

Ammar, E., M.S. Mohamed and A.E.D.H. Sayed: Polyethylene microplastics increases the tissue damage caused by 4-nonylphenol in the common carp (*Cyprinus carpio*) juvenile. *Front. Mar. Sci.*, **9**, 1041003 (2022).

APHA: Standard Methods for the Examination of Water and Wastewater. (W. C. Lipps, E. B. Braun-Howland, & T. E. Baxter, Eds.; 24th Edn.,). American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC USA. APHA Press. 1516 pages (2022).

Bennett, E.R. and C.D. Metcalfe: Distribution of degradation products of alkylphenol ethoxylates near sewage treatment plants in the lower Great Lakes, North America. *Environ. Toxicol. Chem.,* **19**, 784-792 (2000).

Bhandari, G, A.R. Bagheri, P. Bhatt and M. Bilal: Occurrence, potential ecological risks, and degradation of endocrine disrupter, nonylphenol, from the aqueous environment. *Chemosphere,* **275**, 130013 (2021).

Bhattacharya, H., Q. Xiao and L. Lun: Toxicity studies of nonylphenol on rosy barb (*Puntius conchonious*): A biochemical and histopathological evaluation. *Tissue Cell,* **40**, 243-249 (2008).

De la Parra - Guerra, A.C. and R. Acevedo - Barrios: Studies of endocrine disruptors: nonylphenol and isomers in biological models. *Environ. Toxicol. Chem.,* **42**, 1439-1450 (2023).

Dogan, D. and C. Can: Endocrine disruption and altered biochemical indices in male *Oncorhynchus mykiss* in response to dimethoate. *Pestic. Biochem. Physiol.,* **99**, 157-161 (2011).

Dwivedi, S., L.C. D'Souza, N.G. Shetty, S.V. Raghu and A. Sharma: Hsp27, a potential EcR target, protects nonylphenol-induced cellular and organismal toxicity in *Drosophila melanogaster*. *Environ. Pollut*., **293**, 118484 (2022).

Finney, D.J.: Probit Analysis. 3rd Edn., Cambridge University Press,

Cambridge, 333 pages (1971).
Fishelson, L.: Cytomorphological alterations of the thymus, spleen, head - kidney, and liver in cardinal fish (Apogonidae, Teleostei) as bioindicators of stress. *J. Morphol.,* **267**, 57-69 (2006)
Fournier, M., D. Cyr, B. Blakley, H. Boermans and P. Brousseau: Phagocytosis as a biomarker of immunotoxicity in wildlife species exposed to environmental xenobiotics. *Am. J. Zool.,* **40**, 412-420 (2000).
Ghafoor, N., N. Ehsan, M.F. Hayat, R. Azmat, M. Ahmed and A. Ishtiaq: Aucubin mitigates nonylphenol-induced renal damage by attenuating apoptosis, oxidative stress and histopathological profile. *J. King Saud. Univ. Sci.,* **36**,103044 (2024).
Giger, W., P.H. Brunner and C. Schaffner: 4-Nonylphenol in sewage sludge: accumulation of toxic metabolites from non-ionic surfactants. *Science,* **225**, 623–625 (1984).
He, X., Z. Qi, J. Gao, K. Huang, M. Li, D. Springeal and X. Zhang: Nonylphenol ethoxylates biodegradation increases estrogenicity of textile wastewater in biological treatment systems. *Water Res.,* **184**, 116137 (2020).
Herraez, M.P. and A.G. Zapata: Structure and function of the melano-macrophage centres of the goldfish *Carassius auratus. Vet. Immunol. Immunopathol.,* **12**, 117-126(1986).
Hong, Y., C. Feng, Z. Yan, Y. Wang, D. Liu, W. Liao and Y. Bai: Nonylphenol occurrence, distribution, toxicity and analytical methods in freshwater. *Environ. Chem. Lett.,* **18**, 2095-2106 (2020).
Ismail, H.T.H. and H.H.H. Mahboub: Effect of acute exposure to nonylphenol on biochemical, hormonal, and haematological parameters and muscle tissues residues of Nile tilapia; *Oreochromis niloticus. Vet. World,* **9**, 616-625 (2016).
Kali, S.E., H. Österlund, M. Viklander and G. Blecken: Occurrence, concentration and distribution of 50 organic contaminants in water and bottom sediment from urban streams affected by stormwater discharges. *Water Res.,* **283**, 123847 (2025).
Kaptaner, B. and G. Ünal: Effects of 17α-ethynylestradiol and nonylphenol on liver and gonadal apoptosis and histopathology in *Chalcalburnus tarichi. Environ. Toxicol.,* **26**, 610-622 (2011).
Kazemi, S., S.N. Mousavi-Kani, M. Ghasemi-Kasman, F. Aghapour, H. Khorasani and A.A. Moghadamnia: Nonylphenol induces liver toxicity and oxidative stress in rat. *Biochem. Biophys. Res. Commun.,* **479**, 17-21 (2016).
Kranz, H. and N. Peters: Melano-macrophage centres in liver and spleen of ruffe (*Gymnocephalus cernua*) from the Elbe Estuary. *Helgol. Meeresunters.*, **37**, 415–424 (1984).
Liu, F., X. Cao and L. Zhou: Lipid metabolism analysis providing insights into nonylphenol multi-toxicity mechanism. *iScience,* **26**, 108417 (2023).
Liu, Y., X. Dai and J. Wei: Toxicity of the xenoestrogen nonylphenol and its biodegradation by the alga *Cyclotella caspia. J. Environ. Sci.,* **25**, 1662-1671 (2013).
Lotfi, M., A.H. Hasanpour, A.A. Moghadamnia and S. Kazemi: The investigation into neurotoxicity mechanisms of nonylphenol: a narrative review. *Curr. Neuropharmacol.,* **19**, 1345 (2021).
Meseguer, J.: Melano-macrophages of the seawater teleost, sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*): morphology, formation and possible function. *Cell Tissue Res.,* **277**, 1-10 (1994).
Midhila, E. M. and K.C. Chitra: Nonylphenol-induced hepatotoxicity in the freshwater fish, *Oreochromis mossambicus. Int. J. Sci. Res. Pub.,* **5**, 1–5 (2015).
Mukherjee, U., A. Samanta, S. Biswas, S. Das, S. Ghosh, D.K. Mandal and S. Maitra: Bisphenol A-induced oxidative stress, hepatotoxicity and altered estrogenreceptor expression in *Labeo bata*: impact on metabolic homeostasis and inflammatory response. *Ecotoxicol. Environ. Saf.,* **202**, 110944 (2020).
Mukherjee, U., A. Samanta, S. Biswas, S. Ghosh, S. Das, S. Banerjee and S. Maitra: Chronic exposure to nonylphenol induces oxidative stress and liver damage in male zebrafish (*Danio rerio*): Mechanistic insight into cellular energy sensors, lipid accumulation and immune modulation. *Chem. Biol. Interact.,* **35**, 109762 (2022).
Noorimotlagh, Z., S.A. Mirzaee, S.S. Martinez, D. Rachoń, M. Hoseinzadeh and N. Jaafarzadeh: Environmental exposure to nonylphenol and cancer progression Risk–A systematic review. *Environ. Res.,* **184**,109263 (2020).
OECD: Test guideline No. 203: Fish, Acute Toxicity Test. OECD guidelinesfor the testing of chemicals, Section 2, OECD Publishing, Paris (2019).
Pandit, D.N. and M.L. Gupta: Hepato-somatic index, gonado-somatic index and condition factor of *Anabas testudineus* as bio-monitoring tools of nickel and chromium toxicity. *Int. J. Innov. Eng. Technol.,* **12**, 25-28 (2019).
Press, C.M. and Ø. Evensen: The morphology of the immune system in teleost fishes. *Fish Shellf. Immunol.,* **9**, 309-318 (1999).
Raju, S., M. Sivamurugan, K. Gunasagaran, T. Subramani and M. Natesan: Preliminary studies on the occurrence of nonylphenol in the marine environments, Chennai-a case study. *J. Basic Appl. Zool.,* **79**, 52 (2018).
Rocha, R.M., H.S. Leme - Dos Santos, C.A. Vicentini and C. Da Cruz: Structural and ultrastructural characteristics of interrenal gland and chromaffin cell of Matrinxã, *Brycon cephalus* Gunther 1869 (Teleostei - Characidae). *Anat. Histol. Embryol.,* **30**, 351-355 (2001).
Ruggeri, B., M. Ubaldi, A. Lourdusamy, L. Soverchia, R. Ciccocioppo, G. Hardiman, M.E. Baker, F. Palermo and A.M. Polzonetti-Magni: Variation of the genetic expression pattern after exposure to estradiol-17β and 4-nonylphenol in male zebrafish (*Danio rerio*). *Gen. Comp. Endocrinol.,* **158**, 138–144 (2008).
Santos, A.A., R.C. Gutierre, M.M. Antoniazzi, M.J. Ranzani - Paiva, M.R. Silva, C.T. Oshima and M.I. Egami: Morpho-cytochemical, immunohistochemical and ultrastructural characterization of the head kidney of fat snook *Centropomus parallelus. J. Fish Biol.,* **79**, 1685-1707 (2011).
Sayed, A.E.D.H., C. Kataoka, S. Oda, S. Kashiwada and H. Mitani: Sensitivity of medaka (*Oryzias latipes*) to 4-nonylphenol subacute exposure; erythrocyte alterations and apoptosis. *Environ. Toxicol. Pharmacol.,* **58**, 98-104 (2018).
Schwaiger, J., O.H. Spieser, C. Bauer, H. Ferling, U. Mallow, W. Kalbfus and R.D. Negele: Chronic toxicity of nonylphenol and ethinylestradiol: haematological and histopathological effects in juvenile Common carp (*Cyprinus carpio*). *Aquat. Toxicol.,* **51**, 69-78 (2000).
Selvaraj, K.K., G. Shanmugam, S. Sampath, D.G.J. Larsson and B.R. Ramaswamy: GC–MS determination of bisphenol A and alkylphenol ethoxylates in river water from India and their ecotoxicological risk assessment. *Ecotoxicol. Environ. Saf.,* **99**, 13–20 (2014).
Sharma, M., P. Chadha and M.K. Borah: Fish behaviour and immune response as a potential indicator of stress caused by 4-nonylphenol. *Am. J. Biosci.,* **3**, 278-283 (2015).
Sharma, M., P. Chadha and M.K. Borah: Histological alterations induced by 4-Nonylphenol in different organs of fish, *Channa punctatus* after acute and sub chronic exposure. *J. Entomol. Zool. Stud.,* **6**, 492-499 (2018).
Sinha, R. and D.K. Mandal: Immune system of fish with special reference to estrogenic immune regulation: A review. *Acta Zool.,* **106**, 121-131 (2024).
Steinel, N.C. and D.I. Bolnick: Melanomacrophage centres as a

histological indicator of immune function in fish and other poikilotherms. *Front. Immunol.,* **8**, 827 (2017).

Suman, P. Gaurav, M. Joshi, R. Chaube and G. G. Jiwatram: Toxicogenomic profiling of endocrine disruptor 4-Nonylphenol in male catfish *Heteropneustes fossilis* with respect to gonads. *Sci. Rep.,* **15**, 14307 (2025).

Tsujii, T. and S. Seno: Melano - macrophage centres in the aglomerular kidney of the sea horse (Teleosts): Morphologic studies on its formation and possible function. *Anat. Rec.,* **226**, 460-470 (1990).

Vega-López, A., I. Lara-Vega, G. Atonal-Brioso and M. Nájera-Martínez: Neurotoxicant effects of Bisphenol A, nonylphenol and tert butyl phenol in the Nile tilapia (*Oreochromis niloticus*). *Aquat. Toxicol.,* **268**, 106868 (2024).

Witeska, M., E. Kondera and B. Bojarski: Hematological and hematopoietic analysis in fish toxicology-A review. *Animals,* **13**, 2625 (2023).

Won, H., S. Woo and S. Yum: Acute 4-nonylphenol toxicity changes the genomic expression profile of marine medaka fish, *Oryzias javanicus. Mol. Cell. Toxicol.,* **10**, 181-195 (2014).

Yu, J., W. Li, L. Tang, Y. Luo and J. Xu: *In vivo* and *in vitro* effects of chronical exposure to nonylphenol on lipid metabolism. *Environ. Sci. Eur.,* **32**, 87 (2020).

Yu, J., X. Yang, X. Yang, M. Yang, P. Wang, Y. Yang, J. Yang, W. Li and J. Xu: Nonylphenol aggravates non-alcoholic fatty liver disease in high sucrose-high fat diet-treated rats. *Sci. Rep.,* **8**, 3232 (2018).

Yu, J., X. Yang, Y. Luo, X. Yang, M. Yang, J. Yang, J. Zhou, F. Gao, L. He and J. Xu: Adverse effects of chronic exposure to nonylphenol on non-alcoholic fatty liver disease in male rats. *PLOS ONE,* **12**, e0180218 (2017).

Zhu, Z. and Y. Zuo: Bisphenol A and other alkylphenols in the environment-occurrence, fate, health effects and analytical techniques. *Adv. Environ. Res.,* **2**, 179-202 (2013).