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The Efficacy of Intermediary and Mitochondrial Metabolic Enzymes as Non-Estrogenic Biomarkers for the Assessment of 4-Nonylphenol Toxicity in the Freshwater Teleost *Labeo rohita*

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Abstract

4-Nonyl-Phenol (4-NP) is a breakdown product of Nonyl-Phenol Ethoxylate (NPE). It has attracted concern because of its persistence in the aquatic habitats and the toxicity to aquatic organisms. 4-NP is a known endocrine disruptor and a legendary xenoestrogen. Nevertheless, the non-estrogenic impacts of 4-NP in aquatic organisms have only been scarcely addressed. Hence, in the present study we evaluated the effects of sub-lethal concentrations of 4-NP (1.5, 2.0 and 2.5 µg/L) in the major carp *Labeo rohita* in the intermediary and mitochondrial metabolism. We found that exposure to 4-NP produced remarkable alteration in the activities of: 1. enzymes of intermediary metabolism such as glucose 6 phosphatase, lactate dehydrogenase, cytosolic malic enzyme and isocitrate dehydrogenase, and 2. mitochondrial enzymes such as malate dehydrogenase, nicotinamide adenine dinucleotide dehydrogenase, succinate dehydrogenase and cytochrome c oxidase. Thus, the study indicates that 4-NP, at concentrations as would prevail in the environment, impairs the activities of metabolic enzymes and, thereby, affects the tricarboxylic acid cycle and electron transport chain. Alteration in the levels of these enzyme activities can thus be taken to advantage for the surveillance of the impact of 4-NP in energy metabolism in the aquatic organisms.

Keywords: 4-Nonylphenol, Electron Transport Chain, Intermediary Metabolism, Mitochondrial Enzymes

1. Introduction

4-Nonyl-Phenol (4-NP) is one of the most dangerous chemicals that are recorded in aquatic environments[1,2]. It is a degradation product of Nonyl-Phenol Eth-Oxylate (NPEO) which is widely used in the production and formulation of many commercially sold products such as industrial and commercial detergents, pesticides, spermicides, paints, wetting agents, textiles, plastics, paper products, polymer resin and cosmetic products (shampoos and deodorants)[2]. In addition, 4-NP has been reported in food, water bottles and baby bottles[3-6], the range being 64-287 µg/g[7]. Due to extensive use of such products and some anthropogenic activities like wastewater treatment, land-filling and sewage sludge recycling, it reaches the sewages where it is broken down to 4-NP by microorganisms[8]. Due to its low solubility and high hydrophobicity, 4-NP accumulates in these matrices and it persist there[9]. Thereupon, it enters the food chain and ultimately reaches the humans in additive concentrations as it gets bioaccumulated[10].

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4-NP is a known endocrine-disrupting chemical, due to its ability to mimic or partially mimic naturally occurring estrogen[11]. Hence, 4-NP competes with the native estrogen to bind with the estrogen receptor and exerts an estrogen-like transcriptional activity on expression of estrogen receptor gene which leads to the over-stimulation[11]. The upshot of 4-NP on endocrine and reproductive parameters has been surveyed in numerous research investigations in fish[11-13]. Fish represent the most diverse group of vertebrates and they occupy different ecological niches in the aquatic ecosystems and play a vital role in the transfer of energy from one trophic level to another and also serve as biological markers of environmental pollution. Hence, scrutinizing the actions of 4-NP on fish can provide key insights for evaluating the overall health of the aquatic environment[14]. Studies regarding the impact of 4-NP other than its estrogenic effects are rare. Furthermore, in India, 4-NP has been detected at very high concentrations in different river systems[15-17] and the range was 0.2 - 12 µg/L[17]. The level of 4-NP should not exceed 6.6 µg/L in freshwater[18]. From this perspective, it was considered reasonable to scrutinize the effect of 4-NP on metabolic enzymes in the freshwater fish *Labeo rohita*.

Metabolic enzymes carry out a variety of cellular functions necessary for survival and homeostasis, including proteolysis and digestion, cellular respiration, energy storage, transcription and response to the environment[19]. Since metabolic enzymes are depicted as proxies for whole animal metabolic capacity and activity[20] investigation in this area is indispensable. Therefore, this study aimed to investigate the effects of 4-NP on: 1. some enzymes of intermediary metabolism such as glucose 6 phosphatase (G6PO4-ase), Lactate De-Hydrogenase (LDH), cytosolic Malic Enzyme (cME), and Iso-Citrate De-Hydrogenase (ICDH), and 2. mitochondrial enzymes such as Malate De-Hydrogenase (MDH), Nicotinamide Adenine dinucleotide De-Hydrogenase (NADH), Succinate De-hydrogenase (SDH) and Cytochrome C Oxidase (CCO). Furthermore, the efficacy of utilizing the above-mentioned intermediary and mitochondrial enzymes as endpoints of 4-NP toxicity was also analyzed.

2. Materials and Methods

2.1 Experimental Fish

The animal model, freshwater teleost fish *Labeo rohita* was used in the experiment. Fish of average weight and length 40±5g and 25±4cm, respectively, were obtained from local vendors and brought to the laboratory. The fish were acclimatized for 30 days in dechlorinated tap water. Water temperature (28 ± 2°C), oxygen saturation (70-100%) and pH (7.6) were properly maintained. Fish were fed on protein feed *ad libitum*.

2.1.1. Experimental Design and Sampling

4-NP with 90% purity was purchased from Himedia, India. The LC_{50} dose of 4-NP was determined by probit analysis using Organization for Economic Co-operation and Development (OECD) guidelines. Lethal concentration for 50% mortality (LC_{50}) value was determined for 96 hr exposure and was observed to be 3.25 µg/L 4-NP. Based on that, three sublethal concentrations,1.5, 2.0 and 2.5µg/L of 4-NP, were selected for exposure. The sublethal doses of 4-NP were weighed into separate microfuge tubes and dissolved separately by adding about 400µlethanol and diluted to 30 L in dechlorinated tap water in separate aquarium tanks (40 L) of dimension 31 x 41 x 53 cm. After acclimatization, fish weighing 40±5g were divided into 10 groups of 10 each in separate glass tanks. The fish were exposed to different concentrations of 4-NP with six replicates for each concentration, where a maximum of 6 fish were used per concentration of the test toxicant. Freshwater sample having the specified doses of 4-NP was replaced on alternate days. The first group of fish served as control and was kept in normal dechlorinated tap water. Fish in groups II, III and IV were exposed to 1.5 µg/L 4-NP for the periods 7, 14 and 21 days, respectively. Similarly, the groups V, VI and VII were exposed to 2µg/L of 4-NP, and groups VIII, IX and X were exposed to 2.5 µg/L 4-NP for 7, 14 and 21 days, respectively. After the above-said periods of exposure, fish were sacrificed and 100 mg liver tissue was dissected out and kept frozen at -20^{0}C in a deep freezer (ROTEK) for further analysis.

2.2 Assay of Enzymes of Intermediary Metabolism

Chilled liver was separately homogenized at 4^{0}C in 0.25 mL SET buffer (sucrose, Ethylene-Diaminete-Traacetic-Acid (EDTA), tris buffer; pH 7.4) for the assay of enzymes of intermediary metabolism. Homogenates were centrifuged at 2000 rpm at 4^{0}C for 10 min in a high-speed refrigerated centrifuge (Eppendorf, Germany). The supernatant collected was used as enzyme source. All the enzymes activities were assayed spectrophotometrically (Perkin Elmer, USA) at room temperature.

2.2.1 Glucose-6-Phosphatase (G-6-PO_4ase; D-Glucose-6-Phosphatase Phosphohydrolase; E.C.3.1.3.9)

The activity was assayed by the method of Swanson[21]. Two test tubes, labeled test and control, containing assay mixture of 0.3 mL of citrate buffer (pH 6.5) and 0.2 mL of glucose-6-phosphate (pH 6.5) were pre-incubated for 5 min. After mixing the content, 0.1 mL tissue extract was added. Both test tubes were incubated for 15 min at room temperature and the reaction was arrested by adding 1 mL of 10% Trichloroacetic Acid (TCA). After adding 0.1 mL tissue extract to the control, both the test and control were centrifuged for 10 min at 3000 rpm. The supernatant was collected and analyzed for inorganic phosphate by the method of Fiske and Subbarow[22]. The absorbance was measured at 640 nm in a UV-visible spectrophotometer and the activity was expressed as nanomoles inorganic phosphate liberated/min/mg protein.

2.2.2 Lactate Dehydrogenase (LDH; E.C.1.1.27)

Lactate dehydrogenase activity was evaluated according to the protocol of King[23]. The reaction mixture containing the fixed amount of tissue homogenate and NADH solution were pre-incubated in a water bath at 37^0C for 3 min. The reaction was started by adding 1 mL sodium pyruvate solution and incubated for 30 min. Then, the reaction was stopped by adding 1 mL 2, 4-dinitrophenylhydrazine and allowed to stand at room temperature for 20 min. After the incubation, 10 mL of 0.4N NaOH solution was added to each test tube and allowed to stand for 5 to 10 min. The absorbance was read at 550 nm. The specific activity of the enzyme was expressed as IU/min/mg protein.

2.3.3 Cytosolic Malic Enzyme (cME; L-Malate: $NADP^+$ Oxidoreductase; E.C.1.1.1.40)

The activity of cME was determined by the protocol of Ochoa[24]. The reaction mixture in a quartz cell (d=1cm) contained 300 mL glycylglycine buffer, 100 mL $MnCl_2$ and 200 mL of *L*- malate. The aliquot was made up to 3 mL in double distilled water. One hundred microliter tissue homogenate containing the enzyme was added to the reaction mixture. The reaction was started by the addition of $NADP^+$ and the change in absorbance was read at 340 nm against a reagent blank, at 15 sec intervals for 2 min and the activity is presented as IU/min/mg protein.

2.2.4 Iso-Citrate De-Hydrogenase (ICDH; Isocitrate Dehydrogenase $NADP^+$; E.C.1.1.1.42)

The enzyme activity was investigated by the protocol of Ochoa[25]. The reaction mixture in a quartz cell (d=1cm) consisted of 300 mL glycylglycine buffer, 100mL $MnCl_2$ and 200 mL *DL* isocitrate. The aliquot was made up to 2.8 mL by adding double distilled water. One hundred microliter tissue homogenate containing the enzyme was added to the reaction mixture. The reaction was started by the addition of 100 µL $NADP^+$ and the change in absorbance was noted at 340 nm for 2 min and the activity is presented as IU /min/mg protein.

2.3 Isolation of Mitochondria and Assay of Mitochondrial Enzymes

Isolation of mitochondria from liver was carried out according to the method prescribed by Irving and Watson[26]. Chilled liver was weighed and washed several times in SET buffer (0.25M sucrose solution containing 1mM EDTA, 20 mM tris HCl (pH 7.2). Then the liver was homogenized in 10 volumes (w/v) of SET buffer. The homogenate was centrifuged at 600 rpm for 10 min and the precipitate was discarded in order to remove the cell debris. The supernatant was centrifuged again at 12,000 rpm for 10 min, and the pellet was dissolved in homogenizing buffer and used as the enzyme source for mitochondrial enzyme assay.

2.3.1 Malate Dehydrogenase (MDH; Malate: NAD^+ Oxidoreductase; E.C.1.1.1.37)

The activity of MDH was determined by the method of Mehler *et al.*,[27]. The reaction mixture in a quartz cell (d=1cm) contained 300 µg glycylglycine buffer and 25µg oxaloacetate. The aliquot was made up to 2.8 mL in double distilled water. One hundred microlitre tissue homogenate containing the enzyme was added to the reaction mixture. The reaction was started by the addition of NADH and the change in absorbance was noted at 340 nm in a UV visible spectrophotometer against reagent blank, at 15 sec intervals, for 1 min and the activity presented as IU/min/mg protein.

2.3.2 NADH Dehydrogenase (E.C. 1.6.5.11)

The activity of NADH dehydrogenase was measured according to the method of Minakami *et al.*,[28]. The

reaction mixture contained 1.0 mL phosphate buffer, 0.1 mL potassium ferricyanide, 0.1 mL Nicotinamide Adenine Dinucleotide (NADH) and 0.2 mL mitochondrial suspension. The total volume was made up to 3 mL with double distilled water. Nicotinamide adenine dinucleotide was added just before the addition of the enzyme. A control was also treated similarly without NADH. The change in Optical Density (OD) was measured at 420nm as a function of time for 3min at 15 sec intervals in a UV visible spectrophotometer. The activity of NADH dehydrogenase was expressed as nanomoles of NADH oxidized/min/mg protein.

2.3.3 Succinate Dehydrogenase (SDH; Succinate Ubiquinone Oxidoreductase; E.C.1.3.5.1)

The activity of SDH was assayed according to the protocol of Slater and Bonner[29]. The reaction mixture containing 1 mL phosphate buffer, 0.1 mL Bovine Serum Albumin (BSA), 0.3 mL sodium succinate and 0.2 mL potassium ferricyanide, were made up to 2.8 mL in double distilled water. The reaction was started by the addition of 0.2 mL of mitochondrial suspension. A reagent blank was parallelly maintained. The change in OD was recorded at 15 sec intervals for 5min at 420nm. The SDH activity was expressed as nano-moles of succinate oxidized/min/mg protein.

2.3.4 Cytochrome c Oxidase (CCO; E.C. 1.9.3.1)

Cytochrome c oxidase activity was assayed by the method of Pearl *et al.*,[30]. The reaction mixture contained 1.0 mL phosphate buffer, 0.2 mL N-phenyl-P-phenylenediamine, 0.1 mL 0.01% cytochrome c and 0.5mL water. The sample was incubated at 25°C for 5 min. Two hundred microliters of the enzyme preparation was added. A reagent blank was parallelly maintained. The change in OD was recorded at 550 nm for 5min at 15 sec intervals in a UV visible spectrophotometer. The activity of CCO was expressed as number of µmol phenyl-p-phenylenediamine oxidized in 5 minutes per µg of protein.

3. Estimation of Protein

The protein concentration was determined according to the protocol of Bradford[31].

3.1 Statistical Analysis

Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Duncan's[32]. Multiple Range Test using PSS software. Differences were considered to be significant at $p<0.05$ against control.

4. Results

4.1 Activities of Intermediary Metabolism Enzymes

The activities of $G6PO_4$ase, LDH, cME and ICDH in *L. rohita* exposed to 4-NP for 7, 14 and 21 days are shown in Figure 1, 2, 3 and 4, respectively. A significant decrease in $G6PO_4$ase activity was observed in all groups exposed to 1.5, 2.0 and 2.5 µg/L of 4-NP with respect to control. The activity of LDH decreased significantly in all groups exposed to all sublethal doses (1.5, 2.0 and 2.5µg/L) of 4-NP after 7, 14 and 21 days of exposure. There was a significant increase in cME activity in all groups exposed to 1.5, 2.0 and 2.5µg/L of 4-NP after 7, 14 and 21 days of exposure, with respect to the control. The ICDH activity showed a significant decrease in a time-dependent manner when exposed to 1.5 and 2.5µg/L of 4-NP, with respect to the control. Further, a significant decrease was observed in all test groups exposed to 2 µg/L4-NP.

4.2 Activities of Mitochondrial Enzymes

The activity of MDH decreased significantly in all groups exposed to 1.5, 2.0 and 2.5µg/L 4-NP except in 7 days test group exposed to 2 µg/L 4-NP, where no significant change was noticed (Figure 5). The effect on NADH dehydrogenase in fish exposed to 4-NP is shown in Figure 6. There was a sharp increase in NADH dehydrogenase activity in 7 and 14 day test groups after exposure to 1.5, 2.0 and 2.5µg/L of 4-NP. However, a significant decrease was observed in 21 day group exposed to 1.5, 2.0 and 2.5 µg/L4-NP when compared to 7 and 14 day exposure groups.

A significant increase in the activity of SDH was observed in all groups exposed to 1.5, 2.0 and 2.5µg/L 4-NP, though the increase was found to be time-dependent in the test groups exposed to 2.5µg/L 4-NP (Figure 7).

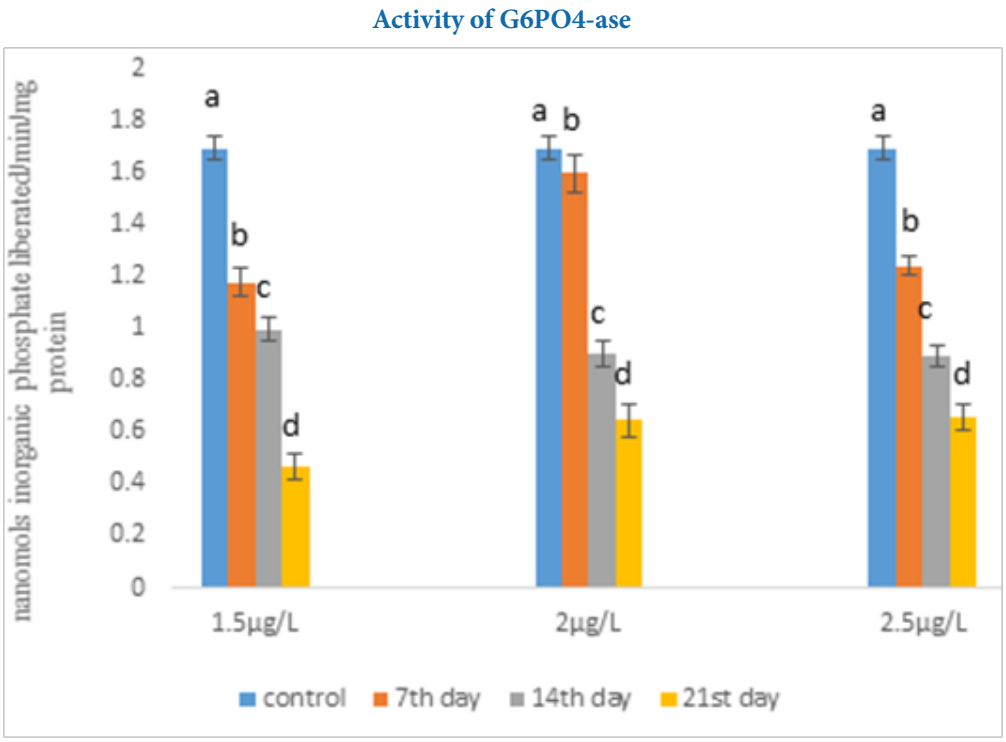

Figure 1. Effect of 4-NP (1.5, 2.0 and 2.5 µg/L) on G6PO4-ase activity in the liver of *L. rohita for* control, 7, 14 and 21 days. Each bar is mean ± SEM for six fish. Mean values of different superscript letters (a, b, c and d) were significantly different (p<0.05).

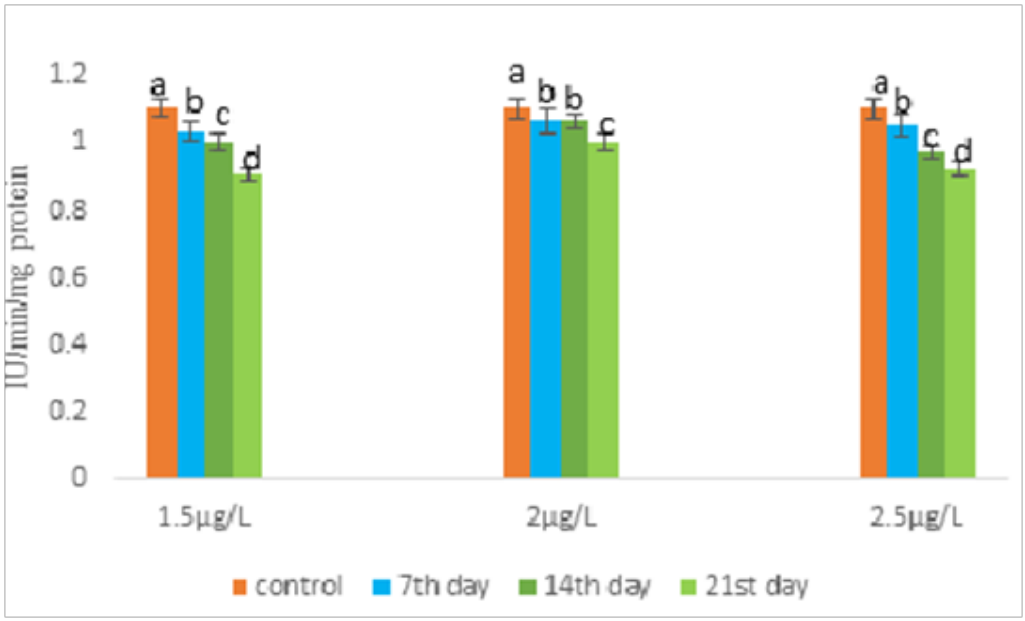

Figure 2. Effect of 4-NP (1.5, 2.0 and 2.5 µg/L) on LDH activity in the liver of *L. rohita for* control, 7, 14 and 21 days. Each bar is mean ± SEM for six fish. Mean values of different superscript letters (a, b, c and d) were significantly different (p<0.05).

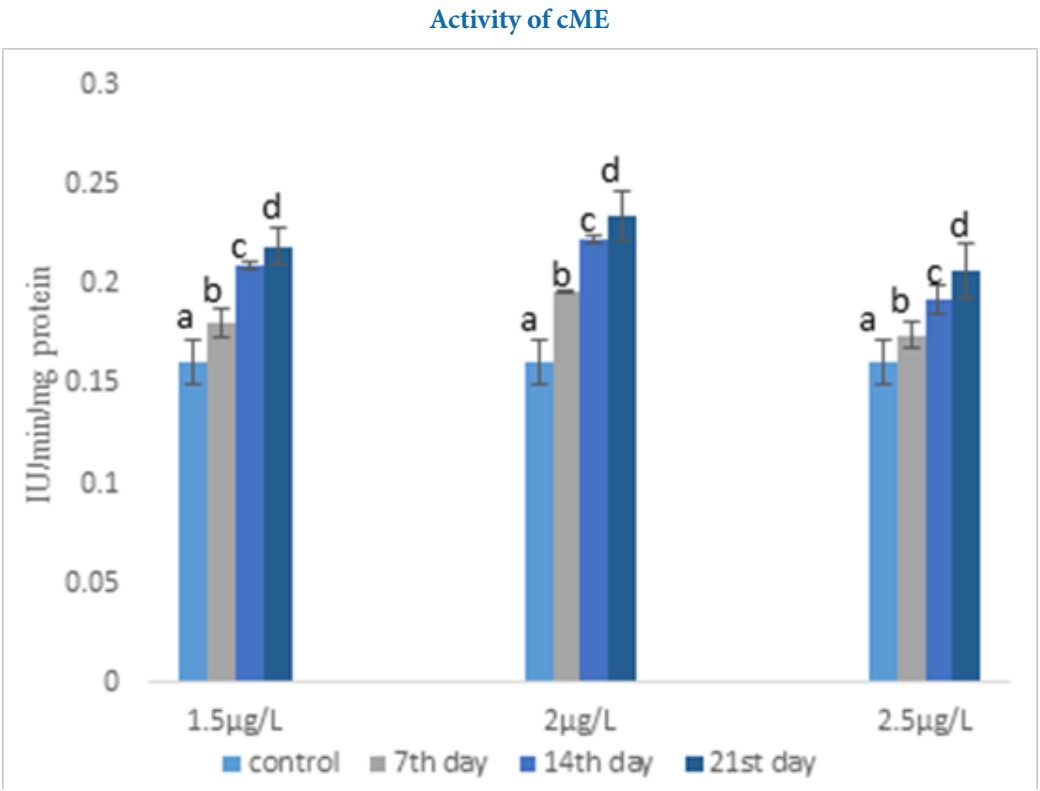

Figure 3. Effect of 4-NP (1.5, 2.0 and 2.5 µg/L) on cME activity in the liver of *L. rohita for* control, 7, 14 and 21 days. Each bar is mean ± SEM for six fish. Mean values of different superscript letters (a, b, c and d) were significantly different (p<0.05).

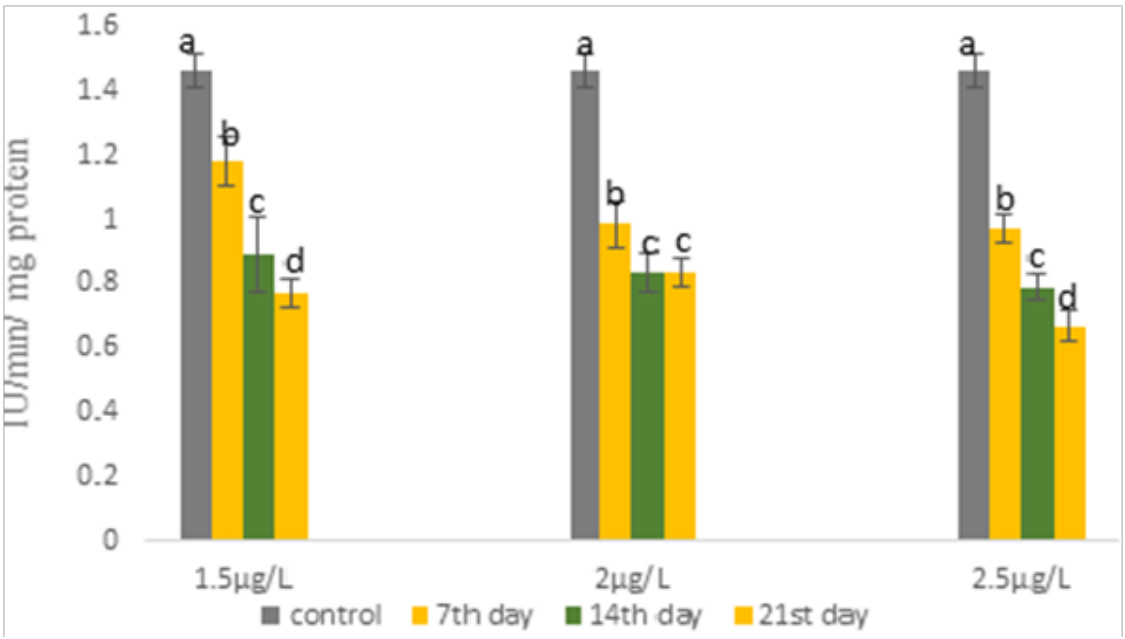

Figure 4. Effect of 4-NP (1.5, 2 and 2.5µg/L) on ICDH activity in the liver of *L. rohita for* control, 7, 14 and 21 days. Each bar is mean ± SEM for six fish. Mean values of different superscript letters (a, b, c and d) were significantly different (p<0.05).

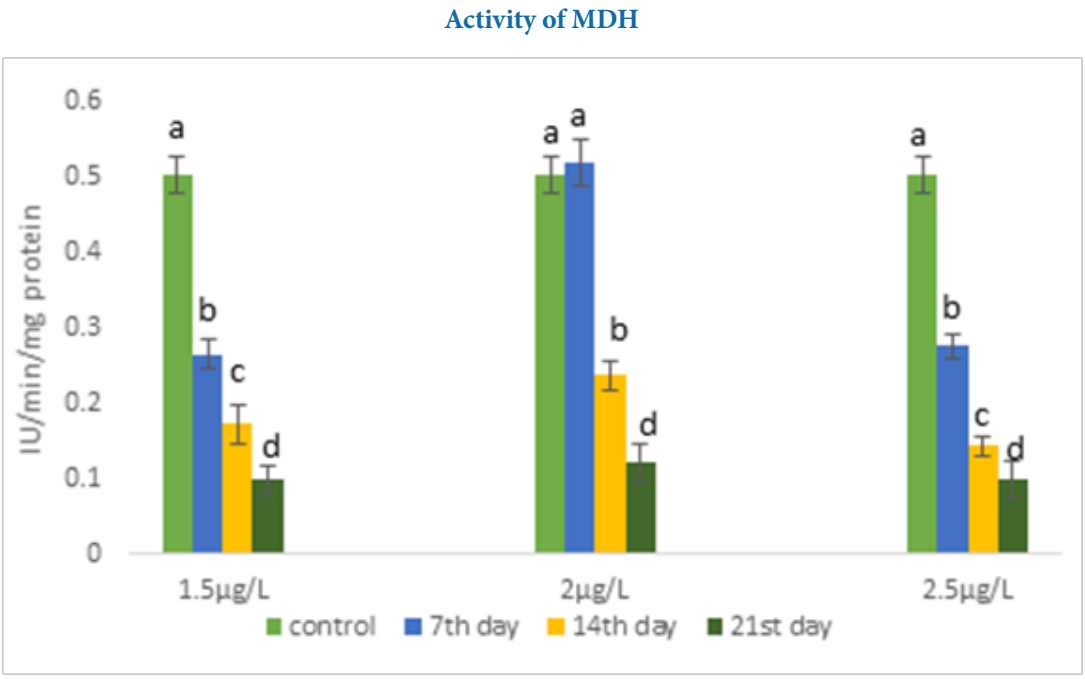

Figure 5. Effect of 4-NP (1.5, 2.0 and 2.5 µg/L) on MDH activity in the liver of *L. rohita* for control, 7, 14 and 21 days. Each bar is mean ± SEM for six fish. Mean values of different superscript letters (a, b, c and d) were significantly different (p<0.05).

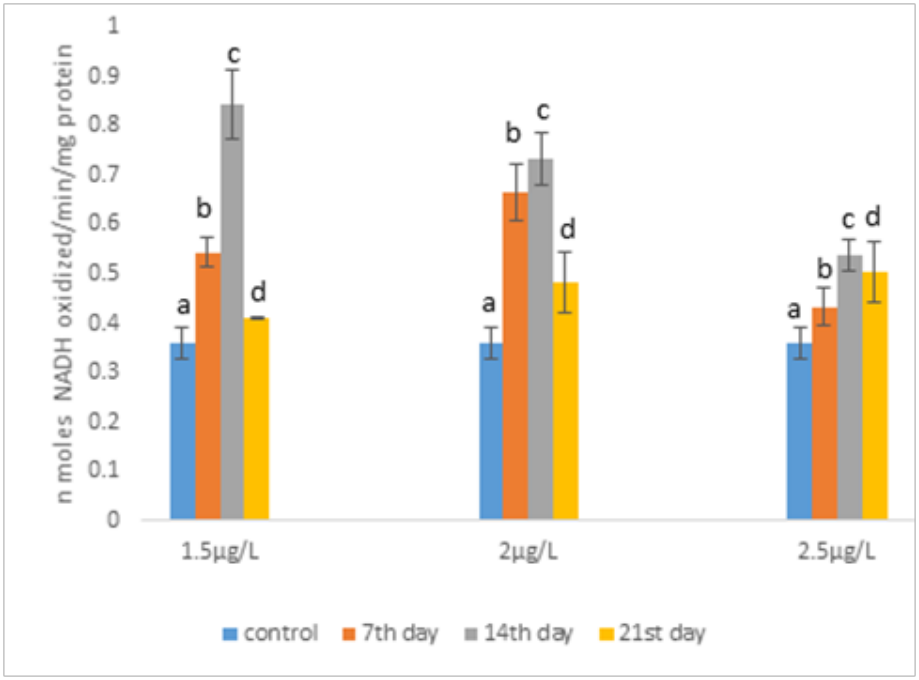

Figure 6. Effect of 4-NP (1.5, 2.0 and 2.5 µg/L) on NADH dehydrogenase activity in the liver of *L. rohita* for control, 7, 14 and 21 days. Each bar is mean ± SEM of six fish. Mean values of different superscript letters (a, b, c and d) were significantly different (p<0.05).

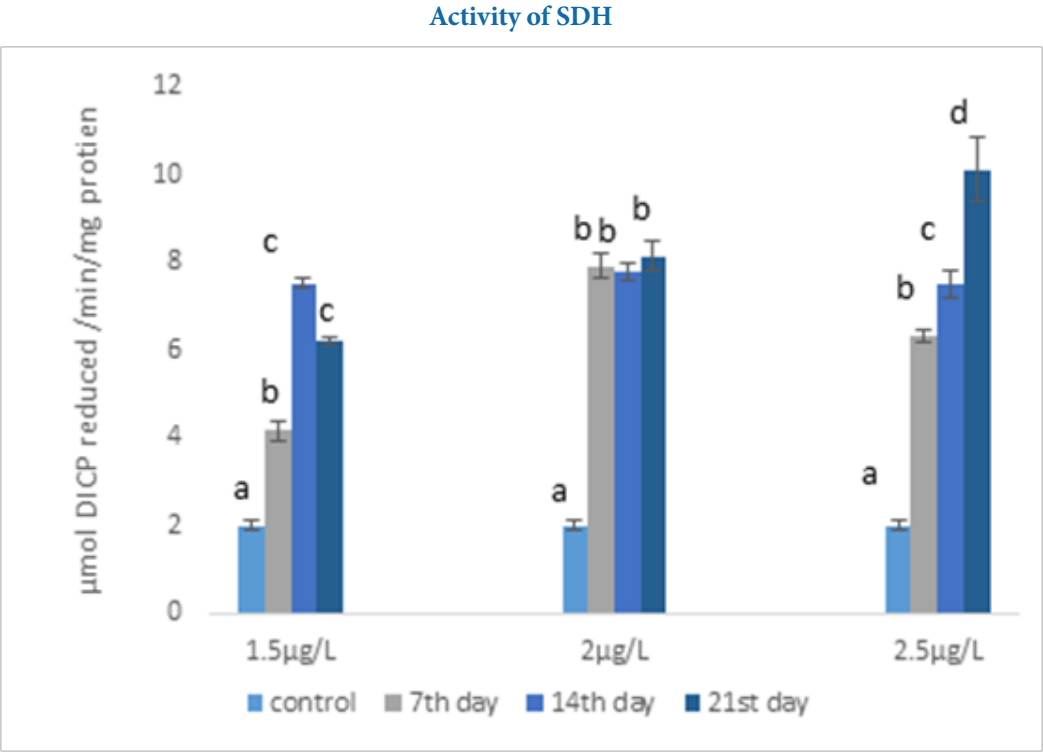

Figure 7. Effect of 4-NP (1.5, 2.0 and 2.5 µg/L) on SDH activity in the liver of *L. rohita* for control, 7, 14 and 21 days. Each bar is mean ± SEM of six fish. Mean values of different superscript letters (a, b, c and d) were significantly different (p<0.05).

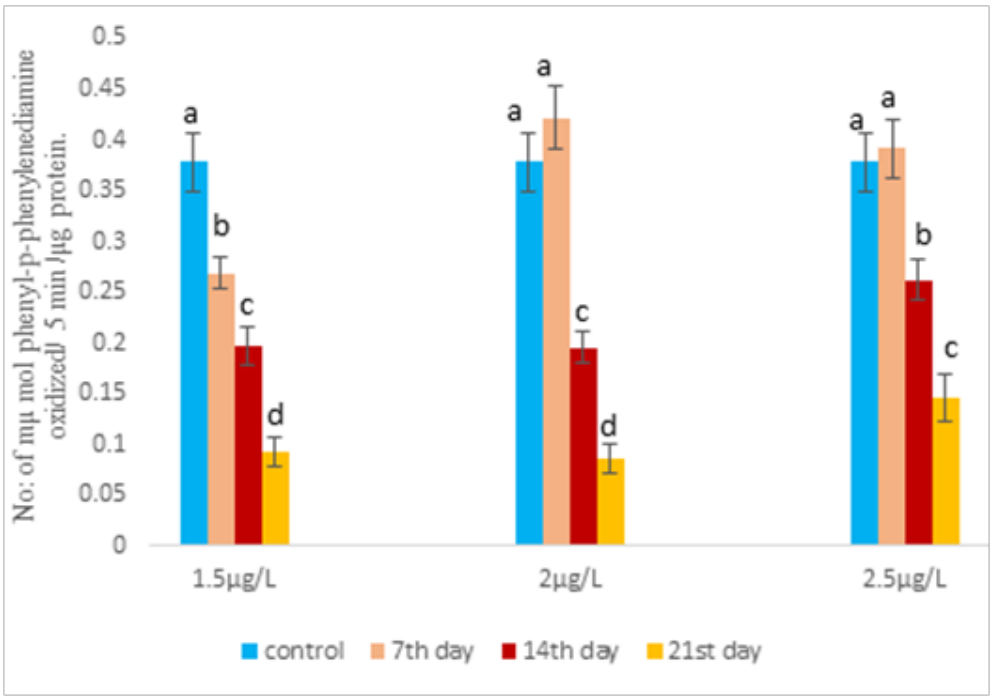

Figure 8. Effect of 4-NP (1.5, 2 and 2.5µg/L) on CCO activity in the liver of *L. rohita* for control, 7, 14 and 21 days. Each bar is mean ± SEM of six fish. Mean values of different superscript letters (a, b, c and d) were significantly different (p<0.05).

The effect of sub-lethal doses of 4-NP in CCO activity was determined in the hepatic tissue of fish and the result is given in Figure 8. The CCO activity was found to be significantly decreased in all groups exposed to 1.5µg/L 4-NP in a time-dependent manner. A significant increase in CCO activity was observed after 7 day exposure to sub-lethal doses of 2 and 2.5 µg/L4-NP, followed by a significant decrease in 14 and 21 day exposure groups with respect to the control.

4.3 Discussion

4-nonylphenol is one of the major xenobiotics, mainly in the aquatic systems[2]. According to Kumari and Sinha[33], xenobiotics probably induce inhibition or acceleration of whole enzyme system by either changing the enzyme activity, the biochemical processes or by directly affecting the enzyme molecule. Variations in the respiratory enzyme activities are considered as early biomarkers to assess the extent of pollution in the exposed organisms[34]. The present study focused on the influence of 4-NP on some enzymes in the intermediary metabolism and mitochondrial pathways in the liver of *L. rohita*.

The activity of the G_6PO_4-ase was found to be significantly reduced after exposure to 4-NP. The main role of G_6PO_4-ase is to liberate free glucose from glycogen pool and some other precursors of glucose-6-phosphate. It also catalyses a critical step in gluconeogenesis and also regulates glucose homeostasis[35]. The G_6PO_4-ase activity was found to be decreased significantly in *Aplocheilus lineatus* exposed to glyphosate[36] and in *Oreochromis mossambicus* exposed to bisphenol S[37]. The significant decrease in G_6PO_4-ase activity was also reported in *Mystus vittatus* on exposure to metasystox and sevin[38]. According to Alexander[39] decrease in the activity of G_6PO_4-ase may be caused by microsomal membrane damage as this enzyme is distributed exclusively in the membranes of the endoplasmic reticulum. In the present investigation, the decreased activity of G_6PO_4-ase in *L. rohita* may interfere with the balance between glucose storage and glucose production and may also affect glucose homeostasis[40].

In the present study, the activity of LDH was found to be significantly decreased in fish exposed to 4-NP. Lactate dehydrogenase catalyses the reversible conversion of lactate to pyruvate. The data regarding the adverse effects of 4-NP or its allies on the LDH activity of fishes are rare but there are many studies about the effects of other chemical pollutants on this enzyme[41]. Amali[43] has reported a significant decrease in LDH activity in the tissues of *L. rohita* in response to quinolphos, padan and their mixture. A significant decrease in LDH activity was also observed in Nile tilapia, *Oreochromis niloticus* exposed to cadmium[44], in *Channa punctatus* after exposure to mercuric chloride[45] and monocrotophos[46] and in *O. mossambicus* exposed to bisphenol S[37]. Lactate dehydrogenase is generally associated with cellular metabolic activities and, hence, its inhibition may be due to ion imbalance or plasma membrane damage[47] and may also be due to the formation of enzyme inhibitor complex[48]. Thus, the significant changes in the activity of LDH indicate damage to any or all organs producing this enzyme[49]. Changes in LDH activity may provide direct and indirect evidence of the cellular damage and can indicate the toxic mechanism[49]. The present investigation indicates that the decrease in LDH activity shifts the tissue metabolism towards glycolysis[45]. Thus, the decreased activity of LDH in *L. rohita* may be due to interference of 4-NP with energy metabolism.

The activity of the cME in the liver showed significant increase due to sub-lethal exposure to 4-NP. Cytosolic malic enzyme is involved in catalyzing the NADPH production for reductive fatty acid biosynthesis and also catalyses the reversible decarboxylation of malate to form pyruvate in the presence of NADP coenzyme. This enzyme is particularly interesting since it uses pyruvate as a substrate and provides an alternative route for pyruvate metabolism in fish during the active mobilization of protein as an energy source or supports gluconeogenesis in the liver[50]. Malic enzyme activity has an important role in complete oxidation of intermediate compounds in Tri-Carboxilic Acid (TCA) cycle for energy production[51]. A significant increase in the activity of cME was reported in *A. lineatus* after exposure to glyphosate[36] and in *O. mossambicus* exposed to bisphenol S[37]. The present study indicated that increase in cME activity oxidizes malate rapidly which might lead to accelerated reduction of malate in cells and thereby disturbs redox status in fish.

In the present study, 4-NP exposure significantly reduced ICDH activity. Isocitrate dehydrogenase is a key enzyme which catalyses the reversible oxidation of isocitrate to oxalosuccinic acid, followed by decarboxylation, leading to the formation of alpha ketoglutarate. Isocitrate dehydrogenase is found only in the mitochondrion and this enzyme appears to participate in the TCA cycle. On the view point of Benderdour *et al.*[52], because of its role in intermediary metabolism and energy production,

ICDH has been extensively studied with respect to its isoenzymes, regulation, kinetics, and its presence in other species including prokaryotes. The significant reduction in the activity of ICDH was observed in common carp, *Cyprinus carpio*, on exposure to cadmium[53], in *Larimichthys croceus* exposed to copper[54], in *A. lineatus* exposed to glyphosate[36] and in *O. mossambicus* exposed to bisphenol S[37]. The reduced activity of ICDH indicates that 4-NP exposure in fish may influence energy and redox status. It may lead to an interruption of steroid-regulated cellular and physiological process in fish considering that steroid hormones are known to produce variation in fish intermediary metabolism[55]. The reduced ICDH level might inhibit the activation energy of mitochondrial metabolism and thereby it may lead to diminished ATP synthesis by uncoupling of oxidative phosphorylation.

Malate dehydrogenase activity was significantly decreased when the fish was exposed to sub-lethal doses of 4-NP. Malate dehydrogenase is an enzyme that reversibly catalyses the oxidation of malate to oxaloacetate. This reaction is a part of many metabolic pathways, including the citric acid cycle. Several studies have connected the significant decrease in activities of TCA cycle enzymes to the changes in the integrity of mitochondria[36,37,56]. The significant decrease in activity of MDH was observed in matrinxa, *Brycon cephalus,* exposed to Folidol 600[57], in *L. rohita* on exposure to endosulfan[58] and cypermethrin[59] and in *O. mossambicus* exposed to bisphenol S[37]. The decreased level of MDH activity may lower the level of functioning of Krebs cycle due to inadequate supply of substrate or decreased oxygen uptake at the tissue level during toxicity stress[56].

The activity of NADH dehydrogenase increased significantly in *L. rohita* exposed to sublethal doses of 4-NP. NADH dehydrogenase is the first enzyme complex in the respiratory chain for generation of ATP by mobilizing cytosolic NADH to mitochondria[60]. Significantly elevated activity of NADH dehydrogenase was reported in grass shrimp, *Palaemonetes pugio*[61], in *Danio rerio*[62] and in *Mytilus galloprovincialis*[63] exposed to hypoxia. In an anaerobic condition, vast amount of NADH was produced by anaerobic respiration[64]. The excess amount of NADH is oxidized by the mitochondrial respiratory chain through NADH dehydrogenase distributed on the inner mitochondrial membrane[65]. Enhanced activity of NADH dehydrogenase may be due to the switching of aerobic metabolism to anaerobic pathway[64]. The increase in the activity of NADH dehydrogenase in the present study might also lead to the imbalance of mitochondrial electron transfer activities and ATP synthesis.

A significant increase in SDH activity was noticed in fish after exposure to 4-NP. Succinate dehydrogenase is an important enzyme of citric acid cycle and catalyzes the reversible oxidation of succinate to fumarate. Succinate dehydrogenase is a primary enzyme in the oxidative catabolism of sugars and is used effectively as a marker of mitochondrial activity[66]. Significant elevation in the level of SDH was observed in *Cirrhina mrigala* exposed to diethyl phthalate[67] and in *L. rohita* on exposure to hexavalent chromium[68]. It clearly indicates that stress produced by a prejudicial effect on energy metabolism causes the increased SDH activity[68].

In the present study, the activity of CCO decreased significantly after exposure to 4-NP. Cytochrome c oxidase activity has been shown to reflect the aerobic metabolic rates of many tissues and whole organisms, and its use as a metabolic indicator in animals has been suggested by Gagnon and Holdway[69]. Cytochrome c oxidase is one of the important enzyme complexes of mitochondrial oxidative phosphorylation system. It is the terminal enzyme of the electron transport system and is distributed in the inner membrane of the mitochondria. This enzyme is also considered as a reliable indicator of metabolic capacity in fish[70]. The significant decrease in CCO activity was also observed in wild yellow perch exposed to cadmium contaminated water[71], *L. rohita* exposed to cypermethrin[59], *O. mossambicus* in response to octylphenol[72], *A. lineatus exposed* to glyphosate[36] and *O. mossambicus* exposed to bisphenol S[37]. The significant inhibition of CCO by 4-NP in the present study suggests that the oxidative metabolism of exposed fish might have been altered due to the reduced capacity of the Electron Transport System (ETS) to produce ATP molecules[73]. Hence, 4-NP can be considered as an uncoupler of oxidative phosphorylation which results in reduced synthesis of ATP.

Based on the present study, it is logical to conclude that 4-NP hinders intermediary and mitochondrial metabolism in the experimental fish. In the current investigation, decreased activities of $G6PO_4$ase, LDH and ICDH and increased cME activity disrupted intermediary metabolism. Similarly, it was observed that 4-NP interfered with the electron transfer chain by decreasing the activities of MDH and CCO, while increasing the activities of NADH dehydrogenase and SDH which might have an impact on an organism's ability to obtain energy

by the way of ATP production and oxygen consumption. Henceforth, the enzymes in the intermediary and mitochondrial metabolic pathways can be used as non-estrogenic biomarkers in the assessment of 4-NP toxicity in fish. Several authors have reported that the disturbance in oxidative metabolism leads to alteration in the whole animal oxygen consumption in different species of fish[74-76].

5. Conclusion

4-NP exhibits a non-estrogenic effect on both intermediary and mitochondrial metabolism in fish. 4-NP can interfere with both TCA cycle and oxidative phosphorylation which lead to impaired ATP production. 4-NP can be regarded as an uncoupler of oxidative phosphorylation which inhibits energy production by suppressing ATP synthesis and leads to pushing aerobic pathway of fish respiration towards anaerobic pathway. In conclusion, the persistence of 4-NP in the environment is a serious threat to the aquatic organisms, especially fish, and thereby can potentially affect the ecological balance.

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7. References

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