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Immunological impairment as a marker of 4-nonylphenol induced stress

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ABSTRACT

The goal of the current study was to evaluate different haematological parameters in order to assess the toxic effects of 4-nonylphenol (NP) on *Channa punctatus* after sub-chronic exposure of 90 days and recovery ascertained after 30 days. Three sublethal concentrations of 4-NP were decided after calculating the safe application rate (SAR). Furthermore, subchronic exposure to sub-lethal concentration was given for 90 days and an experiment was performed later to assess the recovery capacity of fish exposed to sub-lethal concentrations of 4-NP when kept in water without 4-NP for 30 days. Hb, PCV, RBC count, MCH (mean corpuscular haemoglobin) and MCV (mean corpuscular volume) decreased significantly with time and concentration. While WBC count increased with exposure and time. MCHC initially showed a decrease and then an increase with changing concentration level. Recovery experiment showed a great recovery of all the parameters after 30 days and values almost reached the normal level. 4-NP was toxic to *C. punctatus* and the study highlighted the immunological parameters as markers of stress.

Key words: Haematology, 4-Nonylphenol, Recovery, Subchronic

Industrial effluent is a main source of pollutants for aquatic ecosystems. In India, almost all the water bodies are found to be contaminated with one or another kind of industrial effluents, and ample evidences are available regarding the mismanagement of industrial waste (Jadhav and Singare 2015). Since its discovery, production of nonylphenol has increased exponentially, between 100 and 500 million pounds every year, and meets the definition of high-production-volume chemical (EPA 2010). Worldwide production of NP is approximately 500,000 tonnes, out of which 60% is finally discharged to water bodies (Ying *et al.* 2002). The European Union has included NP and their derivative in the list of priority hazardous substances (Directive 2000/60/EC 2000). NP has attracted the attention due to its pervasiveness in the environment. Concerns have increased as NP can mimic natural hormones and its copious level in an environment can disrupt endocrine, immune, and reproductive systems (Ying *et al.* 2002, Sharma and Chadha 2018).

Pollutants present in water bodies exert their toxic effects by generating reactive oxygen species and causing oxidative stress, which leads to alteration in blood composition and immune mechanisms. The study of haematological parameters in fish reveal the conditions within the body of the fish long before any external manifestation of the effects of unfavourable environmental contaminants are ascertained (Soni *et al.* 2018). Hence fish haematology is an important tool to study the status of nutrition, health, diseases, and stress in response to changing environmental conditions. Variations in the haematological parameter of fish in response to different pollutants had been reported (Ali *et al.* 2018, Ismail *et al.* 2018).

It is estimated that around 60% of people in many developing countries, including India depend on fish for their protein requirement hence it is essential to secure the propagation of fishes. *Channa punctatus* was selected due to its availability throughout the year and easy maintenance. To the best of our knowledge, no study has been conducted in relation to the long-term effect of 4-NP on haematological parameters along with recovery in *C. punctatus*. Hence the present study was undertaken to assess the long-term haemotoxic effect of 4-NP on *C. punctatus* by measuring different haematological parameters. Along with this, recovery was also ascertained after 30 days post recovery period.

MATERIALS AND METHODS

A stock solution of 4-nonylphenol was prepared in ethanol. The stock was prepared so that the concentration of ethanol remained constant in all the treatments. The 96 h LC_{50} value of 4-nonylphenol was determined as 1.27 mg/l for *C. punctatus* (Sharma *et al.* 2014). Safe application rate (SAR) was calculated using the formula given by Basak and Konar (1977) ($SAR = LC_0 \times LC_{100}/LC_{50}$). After calculating the SAR, 3 sublethal concentrations were decided, which were $1/10^{th}$ (0.15 mg/l), $1/15^{th}$ (0.10 mg/l) and $1/20^{th}$ (0.07 mg/l) of the SAR.

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Table 1. Different blood parameters level in *C. punctatus* after treatment with different concentrations of 4-NP for 30, 60 and 90 days

Parameter	Concentration	Time duration		
		30 days	60 days	90 days
HB	Control	$10.83\pm0.33^{a,p}$	$10.83\pm0.16^{a,p}$	$11.00\pm0.28^{a,p}$
	Ethanol	$10.33\pm0.16^{a,pq}$	$10.5\pm0.28^{a,p}$	$10.16\pm0.16^{a,p}$
	0.07 mg/l	$8.33\pm0.16^{a,qr}$	$9.66\pm0.16^{a,q}$	$8.83\pm0.16^{b,r}$
	0.10 mg/l	$7.66\pm0.16^{a,rs}$	$8.83\pm0.16^{b,qr}$	$6.66\pm0.33^{b,r}$
	0.15 mg/l	$6.83\pm0.167^{a,s}$	$8.16\pm0.16^{b,r}$	$6.5\pm0.28^{b,r}$
PCV	Control	$36.33\pm0.33^{a,p}$	$35.66\pm0.33^{a,p}$	$36.00\pm0.57^{a,p}$
	Ethanol	$35.33\pm0.33^{a,p}$	$35.00\pm0.57^{a,p}$	$35.33\pm0.33^{a,p}$
	0.07 mg/l	$33.66\pm0.33^{a,q}$	$32.33\pm0.33^{a,q}$	$30.66\pm0.33^{b,q}$
	0.10 mg/l	$31.33\pm0.33^{a,r}$	$29.66\pm0.33^{b,r}$	$25.66\pm0.33^{c,r}$
	0.15 mg/l	$29.33\pm0.33^{a,s}$	$28.66\pm0.33^{a,r}$	$24\pm0.57^{b,r}$
RBCs count	Control	$5.74\pm0.10^{a,p}$	$5.79\pm0.05^{a,p}$	$5.81\pm0.08^{a,p}$
	Ethanol	$5.64\pm0.03^{a,p}$	$5.77\pm0.05^{a,p}$	$5.67\pm0.01^{a,p}$
	0.07 mg/l	$5.17\pm0.03^{a,q}$	$5.10\pm0.05^{a,q}$	$5.03\pm0.03^{a,q}$
	0.10 mg/l	$4.90\pm0.05^{a,r}$	$4.81\pm0.07^{a,r}$	$4.76\pm0.03^{a,r}$
	0.15 mg/l	$4.53\pm0.03^{a,s}$	$4.23\pm0.02^{b,s}$	$4.13\pm0.03^{b,s}$
WBCs count	Control	$4.13\pm0.01^{a,p}$	$4.18\pm0.01^{a,p}$	$4.2\pm0.012^{a,p}$
	Ethanol	$4.32\pm0.01^{a,p}$	$4.3\pm0.014^{a,p}$	$4.316\pm0.01^{a,p}$
	0.07 mg/l	$5.2\pm0.10^{a,q}$	$6.11\pm0.017^{b,q}$	$7.26\pm0.07^{c,q}$
	0.10 mg/l	$7.02\pm0.03^{a,r}$	$12.14\pm0.17^{b,r}$	$17.27\pm0.13^{c,s}$
	0.15 mg/l	$12.12\pm0.17^{a,s}$	$13.19\pm0.11^{b,r}$	$14.25\pm0.14^{c,r}$
MCV	Control	$63.31\pm0.57^{a,p}$	$61.57\pm0.802^{a,p}$	$61.95\pm1.3^{a,p}$
	Ethanol	$62.65\pm0.85^{a,p}$	$60.62\pm0.99^{a,p}$	$62.28\pm0.72^{a,p}$
	0.07 mg/l	$65.65\pm1.04^{a,p}$	$63.43\pm1.33^{a,pq}$	$60.88\pm0.61^{a,p}$
	0.10 mg/l	$63.96\pm0.95^{a,p}$	$61.64\pm0.65^{a,p}$	$53.92\pm0.87^{b,q}$
	0.15 mg/l	$64.67\pm1.06^{a,p}$	$67.71\pm0.64^{a,q}$	$58.03\pm1.51^{b,pq}$
MCHC	Control	$29.83\pm1.08^{a,p}$	$30.37\pm0.18^{a,p}$	$30.58\pm1.11^{a,p}$
	Ethanol	$29.25\pm0.74^{a,p}$	$30.04\pm1.32^{a,p}$	$28.48\pm0.65^{a,p}$
	0.07 mg/l	$24.76\pm0.65^{a,q}$	$29.91\pm0.23^{b,p}$	$28.78\pm0.75^{b,p}$
	0.10 mg/l	$24.46\pm0.26^{a,q}$	$29.77\pm0.23^{b,p}$	$26\pm1.49^{ab,p}$
	0.15 mg/l	$23.29\pm0.49^{a,q}$	$28.48\pm0.49^{b,p}$	$27.14\pm1.67^{ab,p}$
MCH	Control	$18.89\pm0.74^{a,p}$	$18.69\pm0.40^{a,p}$	$17.49\pm0.27^{a,p}$
	Ethanol	$18.32\pm0.24^{a,p}$	$16.44\pm0.77^{ab,pq}$	$15.57\pm0.29^{b,pq}$
	0.07 mg/l	$16.10\pm0.37^{a,q}$	$16.33\pm0.82^{a,pq}$	$13.23\pm0.62^{b,q}$
	0.10 mg/l	$15.65\pm0.37^{a,q}$	$14.20\pm0.37^{a,q}$	$13.65\pm0.62^{b,p}$
	0.15 mg/l	$15.06\pm0.33^{a,q}$	$15.74\pm0.28^{a,q}$	$15.72\pm0.79^{a,pq}$

Values are 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. Similarly, different letters (p, q, r, s) within the columns are significantly different (Tukey's test, P≤0.01) and signify the effect of different concentrations of 4-nonylphenol at the same time interval.

The fish specimens were exposed to the 3 aforementioned test concentrations of 4-nonylphenol, in tap water and ethanol in a static renewal system with change of test water every day in order to maintain the concentration of chemical. In order to eliminate the leaching potential of 4-nonylphenol, plastic material was avoided and glass aquaria of 200 l capacity were used for the experiment. The exposure was continued up to 90 days and blood samples were taken at intervals of 30, 60, and 90 days at the rate of 5 different fish per interval. The specimens maintained in tap water were considered as negative control while those in ethanol as positive control. Blood samples were collected directly from heart after each treatment. EDTA was used as a blood anticoagulant. Blood samples were immediately processed for haematological analysis. RBC and WBC counts were performed by Neubauer haemocytometre (Blaxhall and Daisley 1973). Haemoglobin was estimated by Shali's hemoglobinometre. Micro haematocrite capillary method was used for measuring haematocrit value. Mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular haemoglobin concentration (MCHC) values were calculated by the formula given by Dacie and Lewis (1984).

Statistical analysis: The results were expressed as mean±SE and one-way analysis of variance (ANOVA) and Tukey-HSD test using the statistical software SPSS were conducted to study the significance of the difference in the values of different haematological parameters between treated and control group.

RESULTS AND DISCUSSION

When fish are exposed to stressors, they evoke a nonspecific response to cope with changes and maintain homeostatic state. In case the disturbance remains for a longer time, it may threaten the fish health and well being. In fishes, change in blood cell distribution is correlated with change in environment. Therefore, in the presence of toxicant or other stressors, blood parameters can be used as a standard laboratory test criteria to determine diseased conditions and metabolic disturbances.

The exposure of *C. punctatus* to sub-lethal concentrations of 4-nonylphenol caused a significant decrease in erythrocyte count, haemoglobin and haematocrit values, and a significant increase in the WBC count (Table 1). Similar results had been obtained by Saroch *et al.* (2012) in *C. gariepinus* fish after its exposure to mercuric chloride. Furthermore, Palanisomy *et al.* (2011) reported a decrease in WBC count along with RBCs, Hb, and PCV in *C. striatus* after its exposure to *Cleistanthus collinus* (suicidal plant extract). Erythrocytes and their haemoglobin contents are responsible for oxygen transportation within the body. Low number of red blood cells or insufficient amount of their haemoglobin content could influence energy balance of the body (Joshi *et al.* 2002) and fishes may suffer from oxygen deficiency, which ultimately prohibits its normal growth. Moreover, it seems that reduction in red blood cells is a key factor which could be responsible for productivity reduction. Decreased RBC level may be due to decreased RBC production or high rate of haemolysis (Vosyliene and Jankaile 2006). All alterations mentioned indicate that exposed fish suffered from anaemia induced by 4-nonylphenol. This is an indication of disruptive effects of 4-nonylphenol on erythropoietic tissues as well as cell viability (Sharma and Chadha 2015). It is also possible that 4-nonylphenol adversely suppressed fish osmoregulation. The disturbed osmoregulation may finally result in dilution of blood. Other reasons for anaemia may be altered membrane permeability, increased mechanical fragility, defective iron metabolism and intestinal uptake of iron due to mucosal lesions. NP has higher affinity for membrane phospholipids, which accounts for its lytic activity (Schwaiger *et al.* 2000). Anaemia found in the present study is a consequence of an interaction between NP and erythrocyte membrane. Sudakov (1992) reported that the interaction between NP and erythrocyte membrane leads to anaemia in fish. Furthermore, Schwaiger *et al.* (2000) suggested that anaemia found in NP exposed fish is due to the interaction of NP with erythrocyte membrane. This reduction in haemoglobin level may decrease the ability of fish to enhance its activity in order to meet occasional demands. Same level of decrease in the content of erythrocyte, haematocrit, and haemoglobin were reported after exposure of the fish common carp to Trichlorfon (Woo *et al.* 2018) and after exposure to roundup Glyphosate based herbicide (Kondera *et al.* 2018). Decreased RBC's count and increased WBC's count were observed in Mahseer (*Tor putitora*) after exposure to cypermethrin (Ullah *et al.* 2015). The PCV reading is valuable in determining the effect of stressors on the fish health and also used to determine the oxygen-carrying capacity of blood. The low PCV indicates anaemia or oligohemia. Decrease in PCV shows the extent of shrinkage of cell size and decrease in the number of cells. The increase in WBC count or leucocytosis in treated fish probably reflects an increased demand for WBC in the removal of cellular debris at a faster rate. The increase in stressor probably puts a greater demand for phagocytic cells. Increase in the total WBC count found in the study was a result of the direct stimulation for its defense. The increased values of MCV and MCH may indicate a condition of macrocytic anemia. Change in MCH is due to reduction in cellular blood ions, which results in reduced oxygen carrying capacity and, thus, eventually stimulating erythropoiesis. The significant decrease in MCHC for the exposed group is an indicator of variations in erythrocytes shape, size, and Hb content (Adeyemo 2005), which in turn is an indicator of anaemia. Under stress, MCV level increases, which may be due to swelling of RBCs. Other reason for the increased size of RBCs may be release of premature RBCs due to anaemia. Stress may lead to the release of epinephrine hormone which may trigger the release of immature or deformed erythrocytes from the spleen, and may lead to change in haematocrit value. David *et al.* (2015) found that MCV and MCH increased in *C. mrigala* fish after treatment with deltamethrin while MCHC remained unchanged. In contrast, Ali *et al.* (2018) found a significant decrease in the MCV, and MCHC after exposure with atrazine in *Schizothorax plagiostomus* fish. The rate of recovery of *C. punctatus* exposed to 4-NP was examined by assessing responses of haematological parameters in treatment and post treatment tests (Table 2). The haematological alterations can return to a normal level if exposure to contaminants is discontinued. The *C. gariepinus* fish exposed to Parquat dichloride recovered spontaneously and the aberrated parameters in all the treatments normalized after 12 days in insecticide free aquarium (Ikpesu 2013). The fish species are able to overcome rapidly the toxic stress induced by insecticide flucycloxuron (Zaidi and Soltani 2010). Kroupova *et al.* (2006) found that in common carp the values of haematocrit, erythrocyte count, and Hb concentration after recovery were markedly lower as compared to those found after nitrite poisoning.

Table 2. Different blood parameters after 90 days exposure and 30 days recovery

Parameter		Concentration		
		0.07 mg/l	0.10 mg/l	0.15 mg/l
Hb	Exposed gp	8.83±0.16[a]	6.62±0.16[a]	6.5±0.28[a]
	Recovery gp	10.33±0.16[b]	10.33±0.16[b]	9.33±0.1[b]
PCV	Exposed gp	30.66±0.33[a]	25.66±0.33[a]	24±0.57[a]
	Recovery gp	34.66±0.33 [b]	34.33±0.66[b]	34.66±0.33[b]
RBCs	Exposed gp	5.03±0.03 [b]	4.76±0.03[b]	4.13±0.03[b]
	Recovery gp	5.37±0.06[a]	5.17±0.06[a]	5.23±0.058[a]
WBCs	Exposed gp	7.26±0.07[a]	17.27±0.13[a]	14.25±0.14[a]
	Recovery gp	4.13±0.03[b]	5.48±0.23[b]	5.18±0.04 [b]
MCV	Exposed gp	60.88±0.61[b]	53.92±0.87[b]	58.03±1.5 [b]
	Recovery gp	64.58±1.06[a]	66.42±1.26[a]	66.2±0.10[a]
MCHC	Exposed gp	28.78±0.75[a]	26.00±1.49[a]	27.14±1.67[a]
	Recovery gp	29.81±0.67[a]	30.1±0.1[a]	26.93±0.65[a]
MCH	Exposed gp	13.23±0.62[b]	13.65±0.62[b]	15.72±0.79[a]
	Recovery gp	19.25±0.52[a]	19.00±0.32[a]	17.83±0.45[a]

Values 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.

The present study clearly shows that long term exposure of 4NP is haematotoxic to *C. punctatus*. The exposure of fish to 4-NP resulted in significant alterations in the haematological parameters. These alterations may negatively suppress normal growth, reproduction, immunity, and even survival of fish in the natural environment as well as culture conditions. Fish showed great recovery as all the parameters came to normal range after 30 days of the recovery period.

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