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Research Article

An Assessment of Naphthalene Stress on Renal and Hepatic Functional Integrity in Labeo Rohita

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Abstract

In Pakistan, naphthalene is currently detected as a strong pollutant in aquatic environment, posing potential threat to the health of fish and other aquatic organisms. The aim of our study was two-fold: 1) To calculate 96h LC_{50} values of naphthalene for L. rohita, the common freshwater cyprinid in Pakistan. 2) To find out the potential risks of naphthalene on hematology and liver function enzymes of L. rohita. Three groups of fish juveniles were used. One group (control) was exposed to 0 mg L^{-1} of naphthalene. Based on 96h LC_{50} values, the second group of fish juveniles was exposed to acute concentration (5.05 mg L^{-1}) of naphthalene for 96h. Third group was exposed to 0.60 ml L^{-1} ethanol. All the groups were utilized for hematology and liver functional activities assays. The acute toxicity test for L. rohita revealed 96h LC_{50} value of 5.05 mg L^{-1} for naphthalene. A decrease in RBC count and Hb was observed in exposed group, suggesting toxicant had caused hemolysis. An induction in ALT and AST in exposed group indicating liver injury. An increase in LDH is an indication of induction of anaerobic pathway. The overall findings in the study recommend the naphthalene as a strong potent toxic agent for aquatic environment.

Keywords: *Naphthalene, LC_{50} values, AST, ALT, LDH, Liver, Kidney.*

Introduction

Water is not considered as a typical liquid in chemistry (Vutukuru, 2005). One of the unique physicochemical properties of water is that it readily invites or accepts foreign ions or molecules by specific mechanisms that are sometimes quite unexpected, disturbing water quality characteristics, leading to aquatic pollution (McGlashan and Hughies, 2001). Polycyclic aromatic hydrocarbons (PAHs) such as naphthalene are considered as major environmental pollutants in water. They tend to cause destruction and instability to the existing natural balanced aquatic ecosystem as well as to living organisms, thus putting the aquatic ecosystem at potential risk (Vasanth *et al.,* 2012). Almost all types of natural aquatic habitats have got massive amounts of impurities of PAHs (Vasanth *et al.,* 2012).

Naphthalene is a natural constituent of coal tar, wax, gasoline and diesel fuels (Farooq *et al.,* 2011). It is released into the environment upon burning of the fossil fuels (NTP, 1992). In Pakistan, contamination with naphthalene in aquatic environment has induced detrimental environmental change(s) intensely distressing the natural balance of the existing aquatic ecosystem. It is an emerging issue because naphthalene's production practices and its use as raw material in candle industry to manufacture candles, as light source, where electricity is not accessible, are common (Anwar *et al.,* 2009). In Pakistan, owing to increased demand of candles for public use, based on electricity load shedding, the candle industry throughout the country is growing (Anwar *et al.,* 2009). In Pakistan, the wax material used in candle industry contains naphthalene in large proportion and is not of good quality. The dumping of sewage and candle industry solid liquid waste into the nearby streams or rivers without treatment is leading cause of contamination of freshwater bodies with naphthalene (Anwar *et al.,* 2009). The annual flux estimated for naphthalene contaminants to various rivers in Pakistan including the Chenab River to the Indus River is >50 tons (Farooq *et al.,* 2011). Thus naphthalene is a great health threat to aquatic life in these important rivers of Pakistan, where aquatic life, particularly fish is towards decline (Farooq *et al.,* 2011).

Fish, being sensitive to pollutants, are extensively used in aquatic toxicological studies. The choice of fish species in toxicological studies depends on knowledge of environmental factors of local habitat, availability and experience with the species (Di Giulio and Hinton 2008). Although substantial effort has been done in many parts of the world on the potential effects of PAHs on fish (Martinez-Alvarez *et al.,* 2002; Viarengo *et al.,* 2007; Tejeda-Vera *et al.,* 2008; Di Giulio and Hinton, 2008) but in Pakistan no work has been done on assessment of potential effects of naphthalene on aquatic organisms.

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Table 1 The percentage mortality rates and 96h LC_{50} value of naphthalene for freshwater cyprinid (*L. rohita)*

Exposure Time Period (Hours)	Situation	Control	0.7	1.4	2.1	2.8	4.2	7	9.8	LC_{50} Values
	Number of fish	20	20	20	20	20	20	20	20	
96h	Number of dead fish	0	0	0	0	1	10	15	20	
	Mortality, %	0	0	0	0	5	50	75	100	5.05
	Mortality, %	10	0	35	60	70	90	95	100	

Based on the current status of our natural aquatic environments, following the aquatic life international criteria (Stephan, *et al.,* 1985), based on 96h LC_{50} values protocol for acute toxicity test (Sprague, 1969), the trials in the experiments were conducted to investigate 96h LC_{50} values of naphthalene for economically important freshwater cyprinid fish, *L. rohita*. Further, the effect of acute concentration of naphthalene on hematology and liver function enzymes of *L. rohita* was also assessed.

Materials and Methods

Chemicals

Naphthalene and ethanol of technical grade were used. ALT, AST, LDH and ALP kits were obtained from pathological laboratory Nishtar Medical College Multan, Pakistan.

Experimental fish

For stock, 496 juveniles of freshwater cyprinid fish, *L. rohita,* of both sex with mean body length of 8.651+1.841 cm and mean body weight of 10.782+1.487 g were obtained from local fish farm for experimental utilization.

Bioassays with naphthalene

To determine 96h LC_{50} value of naphthalene for *L. rohita*, experiments were carried out in concrete tanks, each tank with 450 L capacity. It is important to state that ethanol, being organic solvent, no doubt plays a significant role in reducing the naphthalene adsorption to concrete tanks. For precautionary measures, the insides of the concrete tanks were first painting with distemper and then coated with enamel paint to avoid the risk of naphthalene adsorption to concrete tanks. Seven separate groups, each with 20 fish juveniles in separate tank were exposed for the period of 24, 48, 72, 84, 96, 120, 144, and 168 hours to each of nominal naphthalene concentrations (0.7, 1.4, 2.1, 2.8, 4.2, 7.0, 9.8 mg L^{-1}), dissolved in ethanol. The fish juveniles were allowed to feed on commercial pellets having 35% protein, two times daily. The laboratory conditions were (dissolved oxygen concentrations= 6.3-7.2 mg L^{-1}, unionized ammonia= 0.19-0.30 mg L^{-1}, temperature= 25.0±1.0°C, pH= 6.5-6.8, total hardness= 65-75 mgL^{-1} (as $CaCO_3$), alkalinity= 75-80 mg L^{-1}) maintained throughout the experiments. The experiments were carried out in triplicate to obtain the 96h LC_{50} value of the test naphthalene for *L. rohita*. The fish mortality was recorded after 96h to naphthalene exposure (Table 1). The 96h LC_{50} value for naphthalene and their 95% confidence limit was determined using probit analysis. The control mortality was corrected using Abbott (1925) formula, where necessary.

The 96h LC_{50} value of naphthalene for *L. rohita* was found to be 5.05 mg L^{-1}.

Experimental Protocol

To determine the toxic effects of acute concentrations of naphthalene, three sets of fish, 25 individuals in each set, in three separate enamel painted concrete tanks with 300 L capacity each, were exposed to toxicant for 96h, as described below:

(A) Based on the 96h LC_{50} value, a set of 25 fish juveniles was exposed for 96h to acute concentrations (5.05 mg L^{-1}) of the naphthalene, dissolved in ethanol (0.60 ml L^{-1}). This group was designated as Nap.

(B) A second set of 25 fish juveniles was kept in 0.60 ml L^{-1} ethanol. This group was termed as "Solvent control" and was abbreviated as Sol-Cont.

(C) A third set of 25 fish juveniles was also simultaneously kept in tap water (0.00 mg L^{-1}). This group was designated as Nap-Cont. Both second and third sets were kept as the control, because the selected toxicant is organic compound in nature and is therefore insoluble in tap water and were dissolved in ethanol.

All experiments were carried out in semi-static systems with renewal of water after every 12h interval with the addition of fresh solution of the toxicant to avoid the risk of excessive evaporation of naphthalene, as the selected compound is highly volatile in nature. The physicochemical characteristics of the laboratory experiment were: dissolved oxygen concentrations= 6.3-7.2 mg L^{-1}, unionized ammonia= 0.19-0.30 mg L^{-1}, temperature= 25.0±1.0°C, pH= 6.5-6.8, total hardness= 65-75 mg L^{-1} (as $CaCO_3$), alkalinity= 75-80 mg L^{-1}. All the experimental procedures and fish handling protocols were approved by ethics committee of Institute of Pure and Applied Biology B.Z. University, Multan.

Blood sample collection

At the end of experiments, the fish was anesthetized by

Table 2 Hematological parameters studied during present study.

Parameters	Nap-Cont (Control) (N=15) (Mean ± S.D)	Sol-Cont (Alc. treated) (N=15) (Mean ± S.D)	Alc + Nap (Alc. + Nap treated) (N=15) (Mean ± S.D)
RBC Count ($x10^6$ μl^{-1})	1.2253±0.0318	1.2040±0.0259[b]	1.1180±0.0896[a]
Hemoglobin (g/dl)	4.793±0.521	4.731±0.442[b]	4.058±0.485[a]
PCV (HCT) dl/dl	0.1438±0.0156	0.1420±0.0132[b]	0.1217±0.0145[a]
WBC Count ($x\ 10^3$ μl^{-1})	8.225±0.547	8.170±0.637[b]	5.800±0.625[a]
Neutrophils (%)	14.123±0.646	14.046±0.745[b]	21.64±1.04[a]
Lymphocytes (%)	40.56±1.68	40.61±1.67[b]	33.987±0.919[a]
Monocytes (%)	9.380±0.701	8.61±1.04[b]	354±0.686[a]
Eosinophils (%)	1.1993±0.0974	1.197±0.239[b]	0.983±0.206[b]
Thrombocytes Count (%)	35.52±1.47	35.56±1.47[b]	29.763±0.823[a]

The hematological values are expressed as the mean ± S.E. Means in the same horizontal column followed by different Superscript (a) are is statistically highly significantly different (a = 0.001) from control according to Duncan's New Multiple Range Test. Superscript (b) indicates non-significant (b = 0.05)

Table 3 Serum biochemical profile studied during present study.

Parameters	Nap-Cont (Control) (N=15) (Mean ± S.D)	Sol-Cont (Alc. treated) (N=15) (Mean ± S.D)	Alc + Nap (Alc. + Nap treated) (N=15) (Mean ± S.D)
AST (U/L)	32.10±1.02	33.11±2.19[b]	42.53±1.74[a]
ALT (U/L)	31.417±0.749	32.35±1.97[b]	43.19±1.76[a]
L.D.H (U/L)	60.19±1.06	60.97±7.07[b]	98.27±1.46[a]
ALP (U/L)	3.3280±0.0847	3.375±0.421[b]	4.591±0.222[a]
CHOL ($mg\ dl^{-1}$)	107.56±4.10	105.52±3.91[b]	104.51±3.63[a]
TP ($g\ dl^{-1}$)	5.126±0.429	5.074±0.868[b]	6.261±0.872[a]
PT (Sec)	10.128±0.434	10.221±0.262[b]	11.986±0.786[a]
GLU	7.255±0.416	7.60±1.04[b]	12.203±0.781[a]

The serum biochemical values are expressed as the mean ± S.E. Means in the same horizontal column followed by different Superscript (a) are is statistically highly significantly different (a = 0.001) from control according to Duncan's New Multiple Range Test. Superscript (b) indicates non-significant (b = 0.05)

using tricaine methanesulfonate as anesthetics. The blood samples of 1-2 ml were collected from each anesthetized fish of Nap, Sol-Cont and Nap-Cont by making a caudal puncture with the help of fine sterilized needle. A small portion of the blood was directly used for determination of hematological parameters such as Erythrocytes count (RBC), Hemoglobin (Hb), Leukocyte count *etc* while remaining part of blood was left to clot at 4 C^0 in labeled sample bottles for blood biochemical analysis.

Determination of various blood markers

For blood biochemical profiling, the clotted blood samples were centrifuged at 10,000 RPM for 5-8 minutes at room temperature, in centrifuge machine (Hitachi-Japan), serum was separated out. The serum was kept at -20 C^0 for biochemical bioassay. Biosystem Kits were used for the determination of Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Lactate Dehydrogenase (LDH). AST, ALT, LDH, Cholesterol (CHOL), Total Protein (TP) and glucose (GLU) were analyzed by using Hitachi 902 automatic analyzer (Japan). The test was performed as stated in service manual for model 902 automatic analyzer Hitachi, Ltd. 1997.

Determination of hematological parameters

To determine the effects of toxicant on hematological parameters, Erythrocytes count (RBC), Hemoglobin (Hb), Packed Cell Volume (PCV), Leukocyte count (WBC) and Prothrombin time (PT) were analyzed by using automatic analyzer model Sysmex KX-21N, 5 part (Japan). The Sysmex KX-21N, 5 parts (Japan) is a computerized blood cell counter anticipated for in vitro investigative practice in medical laboratories. It is a compact, copiously automatic hematology analyzer with concurrent analysis

of 18 parameters in whole blood mode and capillary blood mode. This automatic analyzer is used to count WBC and RBC using DC detection method and also to measure the hemoglobin concentration using a non-cyanide hemoglobin analysis method (STROMATOLYSER WH). The instrument has been recognized to provide precise and consistent results including hemoglobin concentrations (Gamperling *et al.,* 1998). The test was performed as stated in the manufacturer's manual (Sysmex Corporation, 2006).

Statistical analysis

All the data are expressed as mean and standard error of mean. The statistical package, Minitab-14 was used for the data analysis. The statistical differences were determined by using one way analysis of variance (ANOVA) and the level of significance was set at $P \leq 0.05$, indicated as superscript "a", in Table 2 & 3, following the statistical protocol, introduced by Zar (1996).

Results

Liver function enzymes response after acute concentration exposure to naphthalene (Nap):

AST, ALT, LDH, TP, GLU, PT and ALP in the study exhibited a highly significant increase ($P < 0.001$) compared to control group (Table 3), while CHOL exhibited a highly significant decrease ($P < 0.001$) compared to control group (Table 3). The effect of alcohol on liver function enzymes of Sol-Cont fish group was also compared to control group and differences in liver function enzymes were not statistically significant (Table 3).

Hematological parameters response after acute concentration exposure to naphthalene (Nap):-

In the exposed fish, RBC count, Hb, PCV, WBC count, lymphocytes, eosinophils (%) and thrombocytes count decreased significantly ($P < 0.001$) (Table 2). It is important to comment here that effect of alcohol on hematological parameters was also compared but differences in hematological parameters were not statistically significant (Table 2).

Discussion

Hematology is the scientific study of blood or the sum of all our knowledge about it. It is a very illustrative subspecialty of veterinary medicine, as it is scrutinizing the vigor of investigative organism. Blood is the only tissue that can be removed from an organism, without causing any lethal damage to it and its complete test is possible for diagnosis of disease. Blood is the best health and physiological indicator to diagnosis and management of hematologic disorders of an animal. It is also a convenient indicator of anemia, hypoproteinemia and leukocytosis. Hematological variables are worthwhile biomarkers for environmental contamination in aquatic ecosystem and fish is an appropriate species to act as a biological indicator of water pollution level (Martinez-Alvarez *et al.,* 2002).

In this study, the experience of *L. rohita* to acute concentration of naphthalene caused an ominously diminution in RBC count in Nap group, that is the indication of anemia, because the sign of anemia is the reduction in RBCs count (Puigdoller *et al.,* 2007). All such concrete signs of pervasiveness of anemia were observed in Nap fish. It is suggested that anemia outcomes possibly from Rouleaux formation (A clump of RBCs with each other or filling up of RBCs upon one another is called Rouleaux formation), indicating the inflammatory and connective tissue disarrays or incidence of disease, or perhaps due to defeat of immunity. Another concrete evidence of anemic situation in treated groups of fish is the decrease in PCV, Hb and a decrease in TP. The anemic situation in exposed group of fish could be attributed to direct or indirect feedback reactions of structural injury to RBCs membranes, blood cell damage resulting in hemolysis. Happening of hemolysis is evident from hypocholesterolemia in treated groups of fish, because CHOL, the prime important constituent of virtually all kinds of eukaryotic cell membrane, indicating diminishing in CHOL contribution in RBCs membrane upholding structural integrity, thus, resulting in hemoglobinemia and hemoglobinuria. It is also informed that depression in RBCs count may perchance be from the inhibition of DNA synthesis in red blood cell production, diminished intestinal captivation of iron and disordered Hb synthesis or stress-related release of RBCs from the hematopoietic tissue or hypoxia, induced by the exposure to the toxicants as demonstrated by Shah (2006). The results of depletion in RBCs count and Hb in the study are complementary with Hussein *et al.* (1996) who had observed a decrease in RBCs count, PCV and Hb concentration of *O. nitoticus* and *C. auratus* when exposed to 3 and 6 mg L^{-1} atrazine but show contradiction to Puigdoller *et al.* (2007) who had found a substantial increase in PCV in Atlantic salmon when exposed to atrazine. The anemic ailment in fish results from a bizarrely low quantity of RBC or too little Hb in RBC. Analogous results with momentous decline of RBCs and Hb content in fishes exposed to PAHs have been reported previously by Goel *et al*. (1985) and Goel and Sharma (1987). According to Pamila *et al.* (1991), the decline in Hb content in fish exposed to naphthalene could also be due to the inhibitory effect of the toxic ingredient on the enzyme system accountable for biosynthesis of Hb. Joshi *et al.* (2002) suggested that naphthalene exposure is the root cause of diminution of the RBCs, Hb and PCV due to reduced intestinal captivation of iron (Puigdoller et al. (2007). Implication of these changes may be assumed in terms of reduced oxygen consumption in fish resulting in demise due to naphthalene pollution (Puigdoller *et al.,* 2007).

It is well documented that low concentrations of Hb in fish indicates that there are risks of defect in kidney function, chiefly in head kidney (pronephros), because in most fish, the head kidney is the key site for erythropoiesis. Furthermore, the kidneys of fish secrete a hormone that aids in erythropoiesis. When the kidneys are

not functioning appropriately, the hormone levels drip and Hb production is weakened (Fange, 1982). The diminution in Hb in our study are the signs of damage in head kidney, because the freshwater cyprinids do not have bone marrow, which is the imperative source of myeloid cells in higher vertebrates (Kennedy-Stoskopf, 1993). Therefore, the pivotal lymphoid organ in cyprinids is the head kidney, which is a compound of myeloid and lymphoid characteristics, but its ultra-structural studies have suggested its resemblance to higher vertebrate bone marrow. The head kidney in cyprinids also plays other significant roles in hematopoiesis and endocrine secretion (Fange, 1982). The spleen in cyprinid fish is another conspicuous organ holding lymphoid cells (a mixture of T- and B-cells), plasma cells and red and white tissue, but it is deprive of germinal centers (Kennedy-Stoskopf, 1993).

In the leukocyte series, a momentous decrease was observed in lymphocytes and thrombocytes count in Nap fish group, compared with control except neutrophils. This substantial decrease in WBC count i.e. Leucopenia, (especially thrombocytes and lymphocytes) count in Nap, may be due to amplified concentration of PAHs in various tissues of fish as suggested by Ololade and Oginni (2010). The primary responses *i.e.* neuroendocrine responses in fish to stress include the swift release of stress hormones into the blood circulation, such as cortisol and catecholamines (Oliveira, 2012). The primary responses are immediately followed by secondary stress responses, which embrace several biochemical and physiological modifications, most commonly suppression of immune system *i.e.,* reduction in WBC (Oliveira, 2012). The reduction in WBC count in Nap fish group in the study agrees with the reports of Oliveira, (2012) that suggest the release of stress hormones, which displays the flagging of the immune system in fish intoxicated with naphthalene (Olanike *et al.,* 2008). These variations in WBC count can be manifest with the form of leucocytosis, as lymphopenia and heterophilia are distinctive responses of the animals under stress (Joshi *et al.,* 2002) and findings of the present study obey to the conclusions of the previous researchers (Joshi *et al.,* 2002; Maheswaran *et al.,* 2008), but results exhibit contradiction to those of Velisek *et al.,* (2009), where an increase in leukocytes was observed in trout exposed to bifenthrin.

In toxicological studies of acute concentration exposure, any type of fluctuations, either in the enzyme concentrations or enzyme activities are common and they frequently reflect cell or organ damage (Kumari and Sinha, 2006; Vinodhini and Muthuswamy, 2008). When an organism is exposed to xenobiotics, it undergoes inhibition or acceleration of enzyme systems (Kumari *et al.,* 2011). The assessment of AST and ALT can be used as diagnostic tool to evaluate the physiological status of cells or tissue. Alteration in ALT and AST of fish resulting from toxicants upsetting various cells, tissues, organs and immune system of fish have been reported (Kumari *et al.,* 2011). In the present study, the findings, pertaining to concentrations of enzymes associated with liver function (AST, ALT, LDH and ALP) had ominously higher values than control group indicating stress based tissue impairment (Ramesh and Saravanan, 2008). The induction in the levels of liver marker enzymes (AST and ALT) in the study is suggesting a significant liver injury, leading to leakage of these enzymes into blood circulation (Chen *et al.,* 2004), as these AST and ALT are confined to cell cytoplasm and are escaped into circulation when cellular membrane of hepatocytes get damaged (Lin *et al.,* 2002). These results of liver function enzymes obtained in the study suggest that naphthalene has the potential to penetrate into body of *L. rohita* by simple diffusion via gills pore, or through drinking process and by skin absorption. After entry into the body, naphthalene had caused an induction in serum AST and ALT level which signposted liver damage and stress. Our results of ALT and AST corroborate to that of Fatima and Nahed (2000) who described that intoxication of various stressors frequently provokes vicissitudes in liver function enzymes. The substantial escalation in liver function enzymes indicated that naphthalene stirred AST which is a mitochondrial enzyme. The increase in AST and ALT might be due to toxic injury caused by naphthalene which stimulated tissue reparation through protein turnover and augmented respiration. Nivedita *et al.* (2002) described a parallel work although freshwater fish was exposed to diethylphttalate. The increase of ALT and AST in our study contradicted that of Inyang *et al.* (2010), when *C. gariepinus* was exposed to sub-lethal concentration of Diazinons.

Hyperglycemia is an indication of acute or chronic oxidation stress (Iwama *et al.,* 2006). In our study, the hyperglycemic situation in exposed group of fish suggesting that the *L. rohita* were under severe stress during the period of intoxication with naphthalene. It might be suggested that naphthalene has caused negative effects on the biochemical processes in liver of exposed fish that were engaged for the balance of body glucose level (Iwama *et al.,* 2006). Our findings of hyperglycemia in exposed fish compared to control group agree with Tintos *et al.* (2007) who found plasma glycemia in fish intoxicated with naphthalene. However, according to Fabbri *et al.* (1998), hyperglycemia after exposure to toxicant could also be an effect of catecholamines. This is confirmed by the findings of Gesto *et al.* (2006), who observed that naphthalene induced the release of noradrenaline in rainbow trout after 24 and 72h of exposure.

Conclusion

Based on results, it is concluded that acute dose of naphthalene has the capability of inducing stress in liver and kidney of fish. Further the study shows that the penetration of naphthalene into the liver and kidney of *L. rohita* led to the functional injury of these organs as reflected by the increased activities of the enzymes. These findings indicate impaired fish health in habitats contaminated with PAHs. Based on our study, it is also concluded that naphthalene poisoning can cause demise of *L. rohita* as result of acute hemolytic anemia. The study indicates that the use of water by public from naphthalene contaminated areas could be a direct source of human liver damage and dysfunctioning of liver function enzymes.

Because of environmental and human health toxic risk of naphthalene, steps in Pakistan should be taken to naphthalene toxicity regulation under the environmental legislation to set allowable concentrations for naphthalene in surface waters, soil and drinking water.

Conflict of interest

Authors declared no conflict of interest with any one.

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