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Cytochrome P4501A responses in *Carassius carassius* in relation to nitrate and phosphate levels of Wular Lake of Jammu and Kashmir

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Abstract
Hepatic CYP1A induction in fish due to specific groups of pollutants has been proposed as an early warning system and most sensitive biological response to environmental contamination levels. Fish exposure induces the expression of the CYP1A gene through receptor-mediated mechanisms. The objective of this study was to detect Cytochrome P4501A in *Carassius* in relation to nitrate and phosphate levels of the Wular Lake. The samples (fishes and water) were collected on monthly basis from three different sites of the lake. CYP1A was detected in all the samples examined throughout the study period using Enzyme Linked Immuno Sorbant Assay (ELISA). Using one way analysis of variance, $p<0.01$ was obtained which indicated that there was a significant difference ($p<0.05$) in CYP1A levels among the 4 sampling sites *viz*., Kulhama, Laherwalpora and Ashtung and control group. There was the +ve significant correlation (r = 0.94, $p<0.01$) between total phosphate and nitrate. Further, it was observed that CYP1A showed the +ve correlation with both phosphate (r= 0.89, $p<0.01$) as well as nitrate concentrations (r=0.88, $p<0.01$). In the present study, CYP1A concentration was studied in relation to the total phosphorous and nitrate levels at the respective sites. Both CYP1A level as well as the phosphate and nitrate levels was highest in samples collected from Site B (Laherwalpora) and lowest in the samples collected from control group. It was evident from the present study that with the increase in phosphate and nitrate content, CYP1A levels also increased considerably. In other words the two parameters i,e phosphate and nitrate concentration in water showed a direct relationship with CYP1A concentration in fishes.

Keywords: Cytochrome P4501A, Wular Lake, *Carassius*, total phosphorus, nitrate nitrogen

Introduction
Cytochrome P450 enzymes (CYPs) till date evidenced as the finest susceptible biomarker for water contamination detection (Torres *et al*., 2008) [31] because of their sensitivity to extremely low rates of contaminants that could otherwise not be detected by any other lab technique (Fatima and Ahmad, 2006) [9]. In the fish, cytochrome P450 has essentially been studied as a biomarker designating pollution of the aquatic environment by industrial or agricultural sewage. Nevertheless, the responses to xenobiotics in fish may differ from those in other species. The outcome of metabolic studies of a toxin when obtained in laboratory animals are to be applied to the fish, whether Cytochrome P450 will be induced at all or, if so, which of its isoform will occur is very difficult to estimate. For example, in fish, cytochrome CYP 2B is not induced (Petrivalsky *et al.* 1997; Machala *et al.* 1997) [26, 18]. The CYP1 family members have so far been shown to be the most perceptive and sensitive markers for detecting pollutants in aquatic environments (Machala *et al*. 2000; Schlenk and Di Giulio 2002) [19, 28]. The modulation of the diverse CYP profiles in the induction of manifestations via nuclear recipients is frequently utilized as a sign of environmental contamination exposure (Miller *et al.*, 2000) [22]. The model organisms are also known as sentinel species or bioindicators (Manly, 1995) [21]. Monitoring of sentinel fish species is extensively employed to measure the extent of defilement accretion and health status impacts (Bervoets and Blust, 2003) [4]. Fishes are considered as an excellent contamination marker as they react with strong resilience to water setting variations (Kumari and Khare, 2018) [16]. Fishes at the top of food chain and top consumers in water ecology (Dallinger *et al.*, 1987) [8], are likely to accumulate micro pollutants from the surroundings, which in turn has a direct impact on individual's wellness and can negatively influence the food safety and raw materials from animal sources (fish and fish products) (Gadzala *et al.*, 2004) [11].

These biochemical markers can detect the type of poisoning while in several others, the magnitude of their reactions coordinate with the pollution rates (Kilemade *et al*., 2009) [14]. The preeminent biomarker studied, till now, is the CYP P450 initiation (Klemz *et al*., 2010) [15]. Contamination of aquatic bodies is an everlasting concern because of its implications for marine and freshwater ecosystems in addition to human welfare. A wide variety of potentially toxic organic persistent contaminants called Xenobiotics like planar polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), pesticides (organochlorines, organophosphates and carbamates), effluents from mills like fertilizers, detergents, heavy metals, and surfactants, get dispersed in aquatic environment through various routes such as direct discharge, direct use, land run-off, atmospheric deposition, in situ production, abiotic and biotic movement, and food-chain transfer (Uno *et al*., 2012) [33] and are eventually picked up into the tissues of resident organisms (Kumari and Khare, 2018) [16].

In many lakes and rivers the nutrient level has increased productively over the past 50 years in response to tremendously increased emission of domestic wastes and non-point pollution from agricultural practices and urban development (Mainstone and Parr, 2002) [20]. The fish as a bio indicator species plays a significant role in water pollution monitoring because they are highly sensitive to changes in the aquatic environment. Biomarkers detect the type of toxicity and in some of them, the magnitude of their response correlates with the level of pollution.The gap between the cause and effect can be filled by making use of Biomarkers (Bucheli and Fent, 1995) [6]. The biochemical marker best studied so far is the induction of Cytochrome P450 (Klemz *et al.*, 2010) [15]. The presence of increased CYP levels is a biochemical marker and is accepted as an ISO standard for detection of aquatic environment contamination under certain conditions (Siroka and Drastichova, 2004) [30].

Materials and Methods

The present investigation on *Carassius* (Crucian carp) was carried out at Fish Genetics and Biotechnology Laboratory, Aquatic Environmental Management Laboratory, Faculty of Fisheries, SKUAST Kashmir, Rangil, Ganderbal and Division of Veterinary Microbiology and Immunology, FVSc. & A.H Shuhama, Ganderbal. The study involved the following steps for meeting the set objectives:

Selection of sampling sites

For the present study, three sampling sites were selected from Wular lake *viz*., Kulhama, Laherwalpora and Ashtung.

- **Site A: Kulhama:** It is the small fishing village in the district Bandipora. The site is located towards the eastern side of the lake near to the vintage park between geographic coordinates of 34º 22’ 53.1” N and 74º 39’ 11.5” E. The site has sparse growth of trapa and macrophytes which include Azolla and Ceratophyllum.
- **Site B: Laherwalpora:** Geographically this site lies between 34º 23’ 47.4” N and 74º 35’ 9.1” E. This village is among the largest fishing villages of the district Bandipora. The site has good macrophytic growth and free floating forms like Lemna, Azolla, Hydrilla and trapa being the dominant one. Moreover this site is highly influenced by agriculture and domestic sewage.
- **Site C: Ashtung:** It is a small fishing village located on the north-western side of the lake between geographic coordinates of 34º 24’ 14.8” N and 74º 32’ 34.9” E. The site is characterized by presence of abundant growth of macrophytes such as Trapa, Azolla, Nymphoides and the bottom is covered with soft sediments. These sites were selected on the basis of intense fishing activities and landing centers.
- **Site D: Control:** It was setup in polyhouse of faculty of fisheries, SKUAST-K where fishes were reared for a period of 4 months and the water supply was provided from the nearby freshwater stream which was free from anthropogenic pressure.

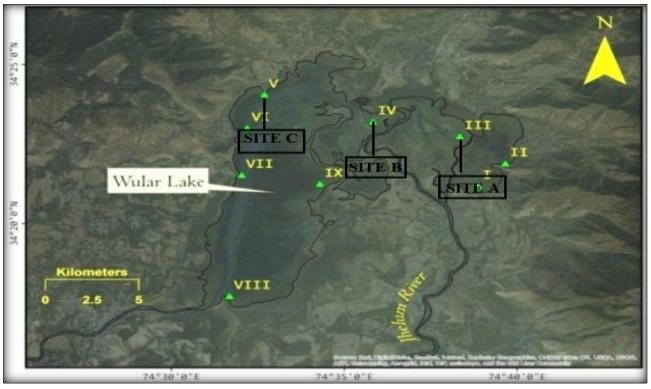

Fig 1: Location map of the study area with sampling sites.

Collection of Samples

Carassius, the experimental fish in the present study and water samples were collected from above mentioned three sites of the lake and control group from December 2019 to February 2020. Samples were collected on monthly basis.

Preparation of liver homogenates

Liver homogenates were prepared as per the protocol mentioned by the manufacturer (Cusabio Taiwan) on the “Cytochrome ELISA (Enzyme Linked Immunosorbent Assay) detection kit”

Principle of the Assay

The assay used the competitive inhibition enzyme immunoassay technique. The microtiter plate issued in the kit had been pre-coated with antibodies which are specific to CYP1A1.To each microtiter plate well equipped with Biotin Conjugated CYP1A, standards or samples were added in an appropriate amount. There occurs a competitive inhibition reaction between CYP1A1 (standards or samples) and Biotin conjugated CYP1A1 with the pre-coated antibody specific for CYP1A1. More the amount of CYP1A in samples, fewer antibodies will bind to Biotin-conjugate CYP1A. After proper washing, addition of avidin conjugated Horseradish peroxidase (HRP) to each well was done. Eventually, at the end, substrate solution was added to the wells and the color developed in opposite to the amount of CYP1A1 in the sample. The development of color was stopped and the color intensity was measured.

Detection and quantification of Cytochrome P4501A using ELISA reader (Assay procedure)

As per the instructions given on the “Cytochrome detection kit” all reagents and samples were prepared. 50µl of standard or sample was added per well (in duplicates) followed by 50µl of conjugate to each well, except to blank well. Complex was properly mixed and then incubated for 60 minutes at 37 ˚C.

Using a multi-channel pipette, each well was filled with 1X wash buffer (200 µl) and aspirated and washed three times. After the final wash, remaining wash buffer was aspirated or decanted out. Then, to each well (except for the blank well), 50µl of HRP-avid in was correctly mixed before being incubated once more for 30 minutes at 37 °C. Wash process was again repeated for three times. After washing, 50µl of substrate A and substrate B were added to each well and then again incubated for 15 minutes at 37 ˚C. 5Following the addition of 50µl of Stop solution, the plate was lightly pounded to ensure proper mixing. Using an ELISA reader (Labsystems, Multiskan) set at 450 nm, the optical density of each well was estimated in less than 10 minutes.

Fig 2: ELISA detection Kit with microtiter plate

Estimation of total phosphorous and nitrate nitrogen concentrations

Samples of surface water were collected from the each sampling site and from experimental control in one litre polyethylene bottles marked distinctly. The sampling was done usually between 11:00am to 2:00 pm. Parameters like water temperature and fixing of Dissolved oxygen were determined at the respective sampling sites and detailed analysis of samples were carried out in AEM Laboratory at Faculty of fisheries, SKUAST- Kashmir using the methods outlined in American Public Health Association (2012).

Statistical Analysis

The data collected were statistically analyzed by "PAST-3" software and "Curve expert software". Data was subjected to one-way analysis of variance (ANOVA) and correlation, $p<0.005$ was considered as statistically significant.

Results & Discussion

Detection and quantification of CYP1A

Cytochrome P4501A was detected in all the samples examined throughout the study. From the samples collected from Site A, the level of CYP ranged from a minimum of 58.61pg/l to maximum of 118.88 pg/l with the mean value of 88.74±2.53 pg/l. The CYP concentration of samples collected from Site B ranged from a minimum of 97.01pg/l to a maximum of 190.11pg/l with mean value of 143.56±3.01 pg/l. While as, for Site C it ranged from minimum of 71.12pg/l to a maximum of 125.74pg/l with the mean value of 98.43±1.95. However, CYP1A concentration was much lesser in samples collected from control group which ranged between 19.57 pg/l (minimum) to37.18 pg/l (maximum) with mean value of 28.37±0.90 pg/l. Various authors across the world have reported the persistence of cytochrome P450 levels as an organism's response to the presence of pollutants in aquatic environment (Aas *et al*. 2001; Bard *et al*. 2002; Moore *et al*. 2003) [1, 3, 23].The findings of the present study are in agreement with Celander *et al*., (1993) [7] while studying on the induction of cytochrome p450 1Al and conjugating enzymes in rainbow trout (*Oncorhynchus mykiss*) liver when exposed to isosafrole (ISF) or β-naphthoflavone (β -NF). reported Propiconazole (fungicide) induced the hepatic cytochrome P4501A (CYP1A) in brown trout (*Salmo trutta*).Statistically significant correlation was reported by Perez *et al*., (2002) [25] and Fisher *et al*., (2006) [10] between the CYP1A and Pyrene in Nile Tilapia; CYP1A and PCBs in common carp respectively.

Table 2: The data provides the univariate (descriptive) statistical of 4 different sites.

	Kulhama	Laherwalpora	Ashtung	Control
N	45	45	45	45
Min	58.61	97.01	71.12	19.57
Max	118.88	190.11	125.74	37.18
Stand. Dev.	16.97	20.20	13.14	6.04
Median	82.33	119.66	83.71	24.43
Skewness	0.54	1.85	1.52	0.52
Geom. Mean	85.47	120.47	86.07	26.96
Coeff. Var	19.51	16.57	15.89	21.90

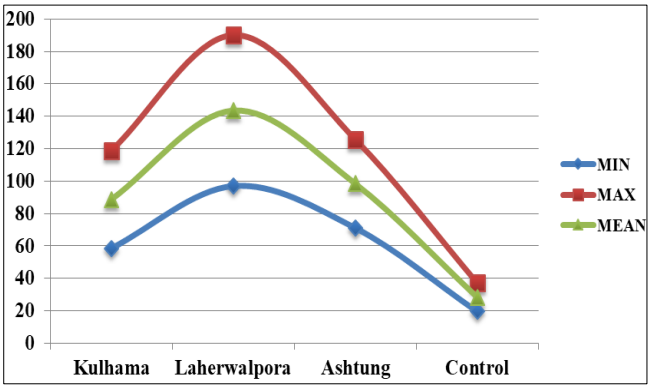

Fig 3: Minimum, maximum and mean values of CYP1A (pg/ml) level at 4 different sites.

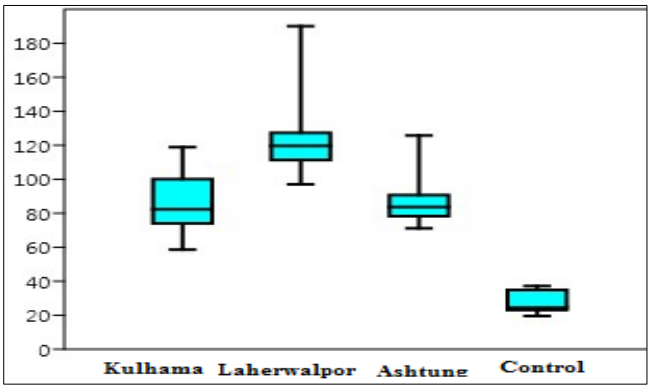

Fig 4: Box plot showing minimum, maximum and mean values of CYP1A levels

Table 3: Distribution of average CYP1A levels at four sites under study

Site	Mean	Std. error	F value	P value
Kulhama	88.74	2.53	304.5	<0.01
Laherwalpora	143.56	3.01		
Ashtung	98.43	1.95		
Control	28.37	0.90		

Using one way analysis of variance, $p<0.01$ was obtained which indicated that there was a significant difference ($p<0.05$) in CYP1A levels among the 4 sampling sites *viz*., Kulhama, Laherwalpora and Ashtung and control.

Total Phosphorus

In the present study, Site A recorded the total phosphorus concentration of minimum 225.6µg/l in the month of January to a maximum of 262.3 µg/l in the month of February with a mean value of 238.7±11.8 µg/l, For Site B it ranged from a minimum of 311.8 µg/l (January) to a maximum of 375.7 µg/l (February) with a mean value of 347.2±18.7 µg/l, while as, for Site C it ranged from a minimum of 201.6 µg/l (December) to a maximum of 298.3 µg/l (February) with a mean value of 259.8±29.6 µg/l. However, in control group the total phosphorous content was recorded in the range of 87µg/l (minimum) in month of January to 91µg/l (maximum) in the month of February with a mean value of 89±1.15µg/l. There was a +ve significant correlation (r = 0.89*, $p<0.01$) between total phosphate and CYP1A. Minimum values of total phosphorus during winter could be ascribed to the absence of planktonic material in the water column and reduced pesticide and fertilizer use in agricultural fields in lake catchment may be due to the quick uptake and subsequent storage of phosphate by the plankton, locking up of phosphate in the dense macrophytic vegetation that abounds in the lake. (Khan *et al*., 2014) [13]. Similar results were found by Kaul *et al*, 1980 [12] who reported appreciable decline in total phosphorus values during winter. While as maximum values of total phosphorus during spring month could be due to anthropogenic inputs of fertilizers and pesticides rich in phosphate applied to agriculture fields surrounding the lake. Agriculture run-off, untreated domestic sewage, washing of clothes and vehicles are the primary sources of $PO4^{-3}$ in spring (Bhat *et al*., 2017) [5].

Table 4: Monthly variation in total Phosphorous (µg/L) at 4 different sites

Site	December	January	February	Mean±S.E
Kulhama	228.2	225.6	262.3	238.7±11.8
Laherwalpora	354.3	311.8	375.7	347.2±18.7
Ashtung	201.6	279.5	298.3	259.8±29.6
Control	89	87	91	89±1.15

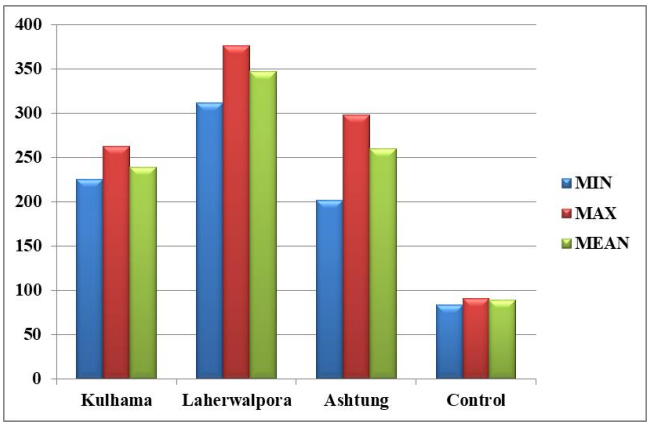

Fig 5: Minimum, maximum and mean values of Total Phosphorus (µg/l) at 4 different sites

4.2.2 Nitrate nitrogen

The site A in the present study recorded the minimum of 331.8 µg/l nitrate nitrogen in the month of February to a maximum of 375.6 µg/l in January with a mean value of 355.8±12.8 µg/l. For Site B it ranged from a minimum of 506.4 µg/l in the month of February to a maximum of 544.4 µg/l in December with a mean value of 522.2±11.4 µg/l, while as, for Site C it ranged from a minimum of 419 µg/l (February) to a maximum of 458.8 µg/l (January) with a mean value of 441.2±11.7 µg/l. However, in control group the nitrate nitrogen content was recorded in the range of 82µg/l (minimum) in month of February to 88µg/l (maximum) in the month of December with a mean value of 84.3±1.85 µg/highly elevated levels of nitrate nitrogen during winter is due to a decreased biological activity (bacterial denitrification and algal assimilation) in winter (Ratnayaka, 2009) [27]. The elevated nitrate-nitrogen concentration during winter is the combining effect of nitrification in the water column and mud water interface (Trisal, 1977) [32]. The nitrate- nitrogen levels>150µg/L is an indicator of Eutrophication and as such the Wular Lake falls under Eutrophic category as reported by Kundangar, 1996 [17] and Shah and Pandit, 2012 [29]. Ratnayaka, 2009 [27] reported higher levels of nitrate in winter are due to decreased biological activities (bacterial denitrification and algal assimilation). Mushtaq, 2017 [5] reported the same from the Wular lake and attributed it to the cumulative effect of nitrification in the water column and mud water interface and vigorous stirring of lake waters.

Table 5: Monthly variations in Nitrate nitrogen (µg/L) at 4 different sites

Site	December	January	February	Mean±S.E
Kulhama	360	375.6	331.8	355.8±12.8
Laherwalpora	544.4	516	506.4	522.2±11.4
Ashtung	458.8	445.9	419	441.2±11.7
Control	88	83	82	84.3±1.85

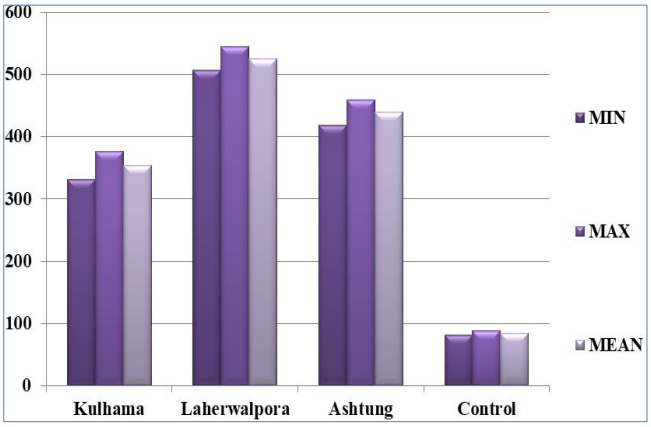

Fig 6: Minimum, maximum and mean values of Nitrate nitrogen (µg/l) at 4 different sites.

In the present study, CYP1A concentration was studied in relation to the total phosphorous and nitrate levels at the respective sites. Both CYP1A level as well as the phosphate and nitrate levels was highest in samples collected from Site B (Laherwalpora) and lowest in the samples collected from control. From the present study, it was evident that with the increase in phosphate and nitrate content, CYP1A levels also increased considerably. In other words the two parameters i, e phosphate and nitrate content in water show a direct relationship with CYP1A level in fishes.

Table 6: Correlation between different parameters

	Total phosphorus	Nitrate
Nitrate	0.94*	
CYP1A	0.89*	0.88*

***=** significant at 0.01 (1%) level of significance

The data presented in the table reveals that there was a +ve significant correlation (r = 0.94, $p<0.01$) between total phosphate and nitrate. Further, it was observed that CYP1A showed the + ve correlation with both phosphate (r= 0.89, $p<0.01$) as well as nitrate (r= 0.88, $p<0.01$).

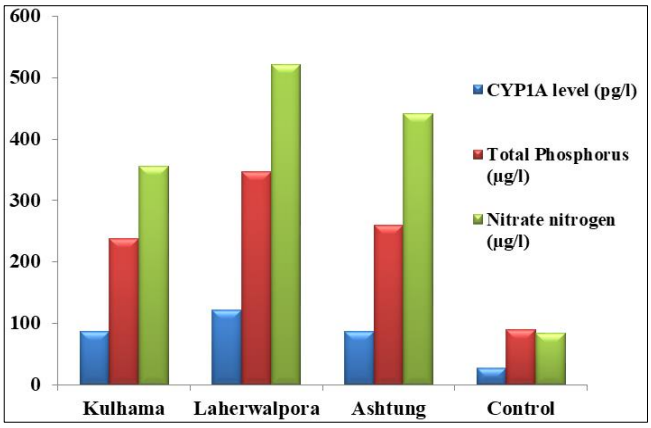

Fig 7: Comparative CYP1A level (in fishes) and total phosphorus, nitrate nitrogen (in water) collected from respective sites**.**

Conclusion

From the present investigation, it was concluded that with the increase in phosphate and nitrate concentration (in water), CYP concentration (in fishes) also increased considerably. It was evident from present study that the two parameters i,e phosphate and nitrate levels in water signifies a direct relationship with CYP1A level in fishes. It was inferred from the present research that there was a +ve significant correlation (r= 0.94, $p<0.01$) between total phosphate and nitrate levels. Further, it was observed that CYP1A showed +ve correlation with both phosphate (r= 0.89, $p<0.01$) as well as nitrate (r=0.88, $p<0.01$).Assessment of histopathological changes can be done in relation to induction of CYP1A. Fishes at the top of food chain and top consumers in water ecology, are likely to accumulate pollutants from the surroundings under eutrophic conditions, and the resultant elevated CYP levels, could be used as a potential indicator of stress in fish. This in turn has a direct impact on individual's wellness and can negatively influence the food safety and raw materials from fish and fish products. Moreover, the increased level of CYP1A could be used to assess the overall health status of the aquatic ecosystem.

Conflict of interest

The authors do not have any conflict of interest in the present work. The funding agency had no involvement in this work.

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