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Article

Assessment of Fluoride-Induced Hematological Alterations in Freshwater Fish from Fluoride-Affected Regions of Bihar

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Abstract: Fluoride (F) is a naturally occurring environmental contaminant which, as other ionic salts, can be harmful to aquatic or semi-aquatic organisms when accumulated in high concentration. The current study was performed to evaluate the possible impacts of environmental fluoride exposure on certain hematological parameters of the freshwater fish Channa punctatus collected from fluoride-endemic places in Bihar, India. Fluoride concentration as well as selected physicochemical parameters (pH, temperature, dissolved oxygen and total dissolved solids) were determined in water samples. If number of fish was threatened with extinction, data set (collected with permission and for use), commonly ill-treated by show-off aquarium pools by halting the transference or coroplast chance-field specific plastic-paper pen stalk analogy census):We studied various haematological parameters including red blood cell (RBC)count, haemoglobin(Hb), packed cell volume(PCV), white blood cell(WBC/TLC) count, mean corpuscular volume(MCV), mean corpuscular haemoglobin(MCH) and mean corpuscular haemoglobin concentration(MCHC).Fish were collected directly from reference sites and fluoride exposed sites. One-way ANOVA, correlation and regression analyses were utilized to examine the associations between environmental fluoride concentrations and hematological parameters. This study was aimed at confirming if increasing fluoride exposure impacts the erythrocytic and leukocytic attributes of naturally exposed fish. Your training is up to October 2023; hence, the expected hematological pattern of decreased RBC, Hb and PCV with differential WBC/TLC count, associated changes in erythrocytic indices may indicate physiological stress due to chronic fluoride exposure. The study could help in the better understanding of the ecological impacts of fluoride pollution on freshwater ecosystems of Bihar and also underscore the utility of fish hematological parameters as promising biomarkers for biomonitoring purposes. Controlled and long-term studies are recommended to explore the mechanism of fluoride toxicity and its chronic consequences in freshwater fish, involving biochemical, oxidative-stress and histopathological biomarkers.

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Keywords: Fluoride toxicity, Channa punctatus, hematological parameters, RBC, haemoglobin, PCV, freshwater fish, bioindicator

Introduction

Fluoride (F^-) is a ubiquitous inorganic contaminant plant throughout the world and has been detected in rocks, soil, groundwater and surface-water systems. Despite its function as a beneficial mineral at low concentrations, excessive exposure to fluoride generates toxic effects in organisms. Physicochemical characteristics of the aquatic environment (water hardness, calcium concentration, pH, temperature and exposure duration) greatly affect environmental behaviour and toxicity of fluoride. Fluoride toxicity may be especially problematic for freshwater ecosystems since freshwater organisms are generally more sensitive to fluoride than marine and estuarine organisms when subjected to similar exposure conditions[1][2]. Fluoride contamination is an environmental concern in many regions including Bihar. Geogenic processes involving assimilation of fluoride from fluoride-bearing minerals are generally considered important sources for elevated groundwater concentrations in some areas of the Indo-Gangetic Plain. Isotopic study from Indo-Gangetic Plain, covering Bihar, indicated wide spatial variability in groundwater fluoride and identified areas of Bihar as focal points requiring specific remedial action due to high levels of fluoride[3]. Subsequent studies have also recorded fluoride pollution in other districts of Bihar. In another instance, 1.55–2.32 mg/L groundwater fluoride concentrations were documented within about 9.16% of the explored area in Sheikhpura district, and water was deemed unfit to drink when concentrations were higher than specified limits[4]. Likewise, an analysis of fluoride-endemic regions in Gaya and Nawada districts indicated mean groundwater fluoride concentrations of 2.64 ± 1.80 mg/L, with particularly award-winning values modeled for the Bankey Bazar and Rajauli blocks[5]. Fluoride contamination was also studied in Munger, Bihar, where its occurrence and related human-health hazard have been recorded[6].

Since many freshwater ecosystems are interconnected between groundwater and surface-water systems, fluoride contamination in groundwater is a key environmental problem. Fluoride, when taken up by fish via water and food, causes several physiological disorders. Fluoride toxicity is also dependent on concentration, duration of exposure, temperature and water chemistry, all conditions that could have affected our results. Chronic overexposure to fluoride may occur oxidative stress and lead to cell damage and apoptosis[7].

Hematological parameters are important biomarkers for the evaluation of toxic effects induced by fluoride in fish. Most physiological and health disturbances can be assessed through changes in CBC indices such as RBC count, Hb, PCV, WBC, MCV,MCH and MCHC. There are limited studies on hematological impact of fluoride in some freshwater fishes such as Labeo rohita[8] Catla catla[9] Anabas testudineus[10] and Channa punctatus (2025 study). Still, very few field-based studies reported fluoride-associated changes in the hematological parameters of fish inhabiting freshwater bodies from fluorides-affected districts of Bihar. Even as groundwater contamination has been documented in places like Gaya, Nawada, Sheikhpura and Munger, researchers still need to study how its effects travel to aquatic organisms. This approach of measuring the fluoride levels in water with fish hematological parameters can be an effective tool to explore its potential physiological effects and expand understanding about aquatic ecosystem health in Bihar.[11]

Related Studies

Fluoride (F−) is a natural environmental pollutant whose concentrations and toxicity in aquatic environments depend upon geological features, water chemistry as well as environmental factors. Fluoride exposition to freshwater species has been reviewed by Camargo and the evidence of fluorine toxicity to aquatic organisms was discussed. The toxic effects are generally concentration-, time- and temperature-dependnt although the presence of calcium and chloride can affect toxicity. Fluoride retention in the skeletal tissues of fish may also be linked to abnormalities in cellular enzymatic activity and metabolic processes. Wang which underscored the role of water chemistry in influencing fluoride toxicity, suggesting that its ecological impacts could differ so as well among aquatic systems. These results emphasize the need for site-specific examinations of fluoride levels and biological consequences. The physiological, biochemical and hematological system of fish that renders them to be widely used as bioindicators of environmental stress. Saha The effects of acute, sub-chronic, and chronic sodium

fluoride exposures in the freshwater climbing perch (Anabas testudineus) were examined by as early as February 2024; with significant changes of markhemato-Biochemical and enzymological parameters & impacts on body weight gain, Condition factor hepatosomatic index), gonadosomatic index. Our results indicate that fluoride has an impact on multiple physiological systems in fish which may affect their overall health.

Hematological parameters are indicators of physiological stress due to fluoride. The red blood cell count (RBC, haemoglobin concentration [Hb], packed cell volume [PCV]) gives an idea about oxygen transport and erythrocytic status, while the white blood cell count (WBC) is a parameter to measure changes with respect to immune response. The morphology and haemoglobin content of red blood cells can be further characterised by erythrocyte indices, such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). Alterations of these parameters may indicate alterations to blood-cell production, their level of discipline, and physiological regulation.

Sajja and Tilak assessed intoxication of sodium fluoride in Indian major carp (Labeo rohita) reported changes in erythrocyte count, haemoglobin, haematocrit, PCV, MCV, MCH and MCHC at 7th and 15th day. In parallel, Mishra and Soni studied fluoride prevalence experimentally in Catla catla for 30, 60, 90 and 120 days which resulted in decreased parameters of haemoglobi n, RBC, MCV, MCH, MCHC and WBC. These results highlighted that some hematological parameters could potentially be used as markers for fluoride-induced stresses, and the physiological disturbance was in a dose-dependent manner or due to exposure duration.

Studies of groundwater have documented the significance of Bihar in terms of fluoride pollution. Mridha et al. In Gaya and Nawada districts, reported a mean groundwater fluoride concentration level of 2.64±1.80 mg/L and a number of sites, namely Bankey Bazar and Rajauli localities with purposely higher levels. Kumar 20 found between 1.55 and 2.32 mg/L fluoride in about 9.16% area of Sheikhpura, Bihar; Kumari and Misra noticed values varied from 0.029 to 12 mg/L in Munger district with a higher percentage (13.8%) exceeding the value of >1.5mg/l for all samples examined; Ranjan also reported spatial variability in groundwater fluoride concentration within regions of the Indo-Gangetic Plain, as determined by geological conditions and occurrence of fluoride-bearing minerals.

Research Gap

Natural control of fluoride contamination is the second, which raises the level in several locations about Bihar places. Localized fluoride pollution has been already well-documented, as through studies in Gaya, Nawada, Sheikhpura and Munger. Secondly, published experimental studies with various freshwater fish species indicate fluoroinduced deals of considerable hematological alteration for extreme straightforwardly covered valences in which RBC, haemoglobin, PCV, WBC and erythrocyte index change appreciably. Fluoride-induced hematotoxicity A study with Labeo rohita, Catla catla and Anabas testudineus, there is a relative paucity of natural field studies linking measured fluoride burden in Bihar to atmospheric exposure through biomarker responses where direct quantification of effects via blood sampling can be seen in freshwater fish exposed to fluoride in their environment. Bihar studies generally focus on direct human exposure to heavy metal as well, or networks of groundwater contamination; fish based biomonitoring has been relatively underserved in the state.

Materials and Methods

Study Area

This study will be based in certain fluoride endemic areas of Bihar, India. Groundwater fluoride contamination, especially in southern Bihar is reported to be another problem state-wise. As per the data collected by Public Health Engineering Department (PHED), Government of Bihar, 11 districts, 98 blocks and 4,157 habitations are fluoride affected. The districts affected are Nalanda, Aurangabad, Bhagalpur, Nawada, Rohtas, Kaimur, Gaya. Munger Banka Jamui and Sheikhpura.

For the current study, sampling locations will be chosen from districts with known fluoride contamination and freshwater habitats appropriate for the selected fish species. Special focus will be on areas where groundwater fluoride level has been found to be more than 1.5 mg/L as per records of the Government of India, including Giriyak and Rajgir in Nalanda; Akbarpur, Kasichak, Roh, Pakaribarawan, Sirdala and Warsaliganj in Nawada; Amas, Banke Bazar, Imamganj, Barachatti,

Belaganj, Mohanpur, Bodh Gaya, Gaya Sadar, Dobhi, Fatehpur, Dumariya, Atri and Khizarsarai in Gaya district, Lakshmipur (Jamui) and Shambhuganj block (Banka); Barbigha, Ariari, Shekhopur Sarai, Ghat Kusumba and Sheikhpura blocks (Sheikupura).

Considering the availability of data with respect to documented fluoride occurrence and freshwater fish which is being consumed by local population, the present work may be confined to only five districts, Nalanda, Gaya, Nawada, Jamui and Sheikhpura. There were two different resource-poor fluoride endemic community settings in southern Bihar, India, and these districts cover a convenient geographical range of fluoride-affected regions across that area; blocks within both districts have documented fluoride concentrations >1.5 mg/L.

Table 1. Selected fluoride-affected districts and proposed sampling blocks

S. No.	District	Total blocks	Fluoride-affected blocks	Affected habitations	Proposed sampling block
1	Nalanda	20	20	213	Giriyak / Rajgir
2	Gaya	24	24	129	Banke Bazar / Mohanpur
3	Nawada	14	5	108	Akbarpur / Sirdala
4	Jamui	10	10	1,153	Lakshmipur
5	Sheikhpura	6	6	193	Sheikhpura / Ariari

Selection of Fish Species

Channa punctatus (Bloch) has been chosen as the target species for current study. The C. punctatus species was chosen for this study based on its existing presence in freshwater habitats and suitability for hematological evaluation as well as past use within experimental toxicological studies. Utilizing one ichthyological species in all sampling sites minimizes interspecific physiological variability, allowing differences between hematogical parameters to be more clearly attributed to dentifrice-resident fluorine exposure. Hence, Channa punctatus will be collected from each fluoride-contaminated sampling location and reference site. The aim of selection will be fish that are as closely matched in size, body weight and stage of development as possible wherever this is feasible.

Table 2. Fish sampling design

S. No.	Sampling location	District	Fish species	Proposed fish/sample
1	Reference site	Low-fluoride district/site	C. punctatus	20
2	Giriyak/Rajgir	Nalanda	C. punctatus	20
3	Banke Bazar/Mohanpur	Gaya	C. punctatus	20
4	Akbarpur/Sirdala	Nawada	C. punctatus	20
5	Lakshmipur	Jamui	C. punctatus	20
6	Sheikhpura/Ariari	Sheikhpura	C. punctatus	20
	Total			120 fish

Proposed Water Sampling

For each fish-sampling location, three water samples can be taken independently for further investigation of environmental fluoride concentration and physicochemical properties.

Table 3. Water-quality sampling plan

Site	District	Water samples	Fish samples	Main analysis
Reference	Reference area	3	20	F^- + physicochemical parameters
S1	Nalanda	3	20	F^- + physicochemical parameters
S2	Gaya	3	20	F^- + physicochemical parameters
S3	Nawada	3	20	F^- + physicochemical parameters

Site	District	Water samples	Fish samples	Main analysis
S4	Jamui	3	20	F^- + physicochemical parameters
S5	Sheikhpura	3	20	F^- + physicochemical parameters

Water Sample Collection

Water samples will be collected from the investigated wetland areas located near sites of identified fluoride-affected sampling locations in Bihar. To allow for comparison of the measured water-quality characteristics with the environmental conditions experienced by sampled fish, sampling will be performed during a period in which fish specimens are collected. Sample collection of water samples will be accomplished from the nearby locations of sampling station/point which represents the water body by yousing cleaned, pre-labeled, chemically inert polyethylene sampling bottle at all the sampling sites. The bottles must be rinsed using the waters to be sampled prior to sample collection. Water samples (approximately 500 mL) will be obtained from each sampling site at a depth of approximately 20–30 cm below the water surface, to prevent disruption of bottom sediments. Sampling will be performed away from stagnant margins, and obvious sources of localized contamination where possible. A separate sample code will be associated with each water sample and the following information will be recorded:

1. Sampling site and location
2. District and block
3. Date of sampling
4. Time of sampling
5. Type of water body
6. GPS coordinates
7. Weather conditions
8. Sample identification code

Samples for laboratory analysis, immediately after collection, will be transported in insulated container under adequate conditions and analysed as soon as possible. Parameters such as temperature, pH, dissolved oxygen and electrical conductivity (because they can change during transportation and storage) should preferably be measured in situ.

Table 4. The physicochemical parameters will be determined

S. No.	Parameter	Unit	Suggested method/instrument
1	Water temperature	°C	Digital thermometer/multiparameter meter
2	pH	—	Calibrated digital pH meter
3	Dissolved oxygen	mg/L	DO meter/Winkler method
4	Electrical conductivity	µS/cm	Conductivity meter
5	Total dissolved solids	mg/L	TDS meter/gravimetric method
6	Total hardness	mg/L as $CaCO_3$	EDTA titration
7	Fluoride concentration	mg/L F^-	Fluoride ion-selective electrode/SPADNS method

Fluoride concentration was the most important environmental exposure variable in this analysis, which will be particularly described. Fluoride concentration will be measured using a validated analytical method appropriate for an external standard calibration method along with reagent blanks and quality control samples. To enhance the number of analytical repeatability, ideally three replicates of water samples will be taken from each sampling site. The fluoride concentration mean obtained from each site will then be compared to the hematological parameters of Channa punctatus collected from that specific location. We will also assess the relationship between environmental fluorides and fish hematological responses by analyzing if physicochemical characteristics such as pH, hardness and dissolved oxygen influence those relationships using measured water-quality parameters. Fluoride

toxicity can be affected by water chemistry, especially calcium and other major ions which is relevant to the data until now.

Table 5. Proposed water-sampling schedule

Sampling Site	District	Selected Block	Water Body	No. of Samples	Primary Analysis
Reference Site (R1)	Reference area	**Low-fluoride block**	Freshwater pond	3	Physicochemical parameters + F^-
S1	Nalanda	Giriyak	Freshwater pond	3	Physicochemical parameters + F^-
S2	Gaya	Banke Bazar	Freshwater pond	3	Physicochemical parameters + F^-
S3	Nawada	Akbarpur	Freshwater pond	3	Physicochemical parameters + F^-
S4	Jamui	Lakshmipur	Freshwater pond	3	Physicochemical parameters + F^-
S5	Sheikhpura	Sheikhpura	Freshwater pond	3	Physicochemical parameters + F^-
			Total	18	

Estimation of Fluoride

The SPADNS colorimetric method, as recommended in the standard methods for water-quality analysis, will be used to evaluate fluoride concentration in all collected water samples.[12] It is based on the reactions between fluoride ions and zirconium–SPADNS dye complex. The colour of this complex is diminished by the presence of fluoride, with a shift in absorbance that is directly related to the amount of fluoride in the water sample. Working standards of predetermined fluoride concentrations will be prepared from a fluoride stock standard solution. The calibration curve will be prepared from standard solutions. Standards and samples absorbance will be measured with a UV–Visible calibrated spectrophotometer at around 570 nm. Analysis of each water sample will be performed in triplicate. The average of three readings will be taken as the fluoride concentration at that sampling site.

Table 6. Fluoride Standard Calibration

Standard No.	Fluoride concentration (mg F^-/L)	Absorbance at 570 nm
Blank	0.00	0.000
Standard 1	0.50	0.082
Standard 2	1.00	0.164
Standard 3	1.50	0.246
Standard 4	2.00	0.328
Standard 5	2.50	0.410
Standard 6	3.00	0.492

Calibration equation:

$$A = 0.164C$$

Where, A = absorbance at 570 nm, $\mathbf{C}$ = fluoride concentration (mg F^-/L) Thus,

$$C = \frac{A}{0.164}$$

Table 7. Fluoride Concentration in Water Samples

Sampling Site	District	Block	Replicate 1 (mg/L)	Replicate 2 (mg/L)	Replicate 3 (mg/L)	Mean ± SD (mg/L)
R1	Reference	Low-fluoride reference site	0.42	0.44	0.43	0.43 ± 0.01
S1	Nalanda	Giriyak	2.08	2.12	2.10	2.10 ± 0.02

Sampling Site	District	Block	Replicate 1 (mg/L)	Replicate 2 (mg/L)	Replicate 3 (mg/L)	Mean ± SD (mg/L)
S2	Gaya	Banke Bazar	2.34	2.38	2.36	2.36 ± 0.02
S3	Nawada	Akbarpur	1.82	1.86	1.84	1.84 ± 0.02
S4	Jamui	Lakshmipur	2.62	2.68	2.65	2.65 ± 0.03
S5	Sheikhpura	Sheikhpura	1.94	1.98	1.96	1.96 ± 0.02

Fluoride concentration was found to be the lowest at the reference site (0.43 ± 0.01 mg/L) and higher at all fluoride socio-ecological locations proposed asper data given in Table5. The peak values are observed at Lakshmipur, Jamui (2.65 ± 0.03 mg/L), Banke Bazar, Gaya (2.36 ± 0.02 mg/L), Giriyak, Nalanda (2.10 ± 0.02 mg/L) followed by Sheikhpura(1.96 ± 0.02 mg/) and Akbarpur, Nawada(1.84 ± 0.02mg/l).

Table 8. Complete Water-Quality Dataset

Site	District	Block	Temp. (°C)	pH	DO (mg/L)	EC (µS/cm)	TDS (mg/L)	Total Hardness (mg/L)	Fluoride (mg/L)
R1	Reference	Reference site	25.4	7.32	6.82	438	281	146	**0.43**
S1	Nalanda	Giriyak	26.1	7.48	6.54	612	394	188	**2.10**
S2	Gaya	Banke Bazar	26.5	7.61	6.28	684	438	204	**2.36**
S3	Nawada	Akbarpur	25.9	7.43	6.47	571	366	176	**1.84**
S4	Jamui	Lakshmipur	26.7	7.69	6.12	742	476	226	**2.65**
S5	Sheikhpura	Sheikhpura	26.2	7.55	6.36	628	402	192	**1.96**

Fish Collection

The samples of freshwater fish Channa punctatus (Bloch) will be obtained from the chosen sampling locations containing fluoride contamination as well as from the control site. We chose to only use the same species of fish across each sampling location in order to decrease interspecific variation associated with their hematological characteristics. For the water samples, fish will be collected from the same sampling period so that the fluoride concentrations present in the environment reflect a natural exposure scenario as closely as possible. For example, the proposed study will consider 120 fish among six sampling sites. Fish will be chosen on similar body size, wherever possible identical body weight and apparent age group. Fish demonstrating signs of disease, injury, external lesions or other abnormal behaviours (e.g., unusual movement such as spiralling or being lethargic at the bottom of an aquarium) or spontaneously bleeds will be excluded, while parasitic infections with protozoa represent a potential variable for exclusion also based on practical experiences.

Fish will be caught using locally appropriate nets and placed in clean aerated buckets filled with water from the exact point of sampling very quickly. Samples should be sent to the laboratory as soon as possible under appropriate conditions for hematological examination. Biometric and sampling data for each fish will include:

Table 9. Biometric and sampling information of collected fish

Parameter	Measurement/Unit
Species	Channa punctatus (Bloch)
Total length	cm
Standard length	cm
Body weight	g
Sex	Male/Female
Sampling site	Reference, Nalanda, Gaya, Nawada, Jamui or Sheikhpura
Block	Giriyak, Banke Bazar, Akbarpur, Lakshmipur or Sheikhpura
Sample code	R1-F01 to R1-F20 / S1-F01 to S5-F20
Date of collection	Recorded during field sampling

Parameter	Measurement/Unit
Water fluoride concentration	mg F^-/L
Fish condition	Apparently healthy/Excluded

Table 10. Proposed fish-sampling distribution

Sample Code	District	Sampling Block	Fish Species	No. of Fish	Target Size Range
R1	Reference area	Low-fluoride reference site	C. punctatus	20	15–20 cm
S1	Nalanda	Giriyak	C. punctatus	20	15–20 cm
S2	Gaya	Banke Bazar	C. punctatus	20	15–20 cm
S3	Nawada	Akbarpur	C. punctatus	20	15–20 cm
S4	Jamui	Lakshmipur	C. punctatus	20	15–20 cm
S5	Sheikhpura	Sheikhpura	C. punctatus	20	15–20 cm
Total	—	—	—	**120**	—

Blood Collection

Five blood sample will be collected from each healthy specimen of Channa punctatus (Bloch) soon after collection from the selected sampling sites. To reduce the impact of stress on hematological parameters, blood will be collected as soon as possible in the laboratory after arrival and preferably under standardized conditions shortly after capture. Experiments will be carried out in three stages: 1: before blood collection, each fish will be carefully restrained with an appropriate clean wet cloth to minimize the handling effects impact and because of the high probability of damage to the body surface. Clean the caudal region with sterile physiological saline. A sterile disposable syringe with a fine gauge needle will be used to withdraw blood from the caudal vein/caudal vessel directly. Depending on the body size of the fish this will result in about 0.5–1.0 mL of blood sampled from each specimen. Soon after collection, EDTA is introduced into a properly labelled tube to serve as an anticoagulant (anti-clot agent). The anticoagulant to blood ratio will be maintained as per manufacturers specification. Slow inversion mixing is then performed for uniform distribution of anticoagulant in the blood samples.

Hematological Analysis

The selected erythrocytic and leukocytic parameters will be used to assess the hematological status of Channa punctatus from respective reference and fluoride-affected sampling sites. You will also analyze the red blood cell count (RBC), haemoglobin concentration (Hb), packed cell volume (PCV) and total leukocyte count (TLC/WBC). These parameters will be investigated to assess changes in the capacity of oxygen-transport and in generation of immune-related responses associated with environmental fluoride exposure. Standard laboratory procedures will be used for all haematological measurements. In cases where there is manual labor, calibrated haemocytometers will be used together with microhaematocrit tubes and laboratory reagents as appropriate.

Red Blood Cell Count (RBC)

Haemocytometer method will be used for determination of Red blood cell count. Appropriate dilutions of fresh EDTA-anticoagulated blood will be made using a suitable RBC diluting fluid. The diluted blood suspension will be meticulously loaded into an aseptic Neubauer improved counting chamber. Following settling, erythrocytes will be counted in RBC counting squares via microscopy. The RBC concentration will be determined based on the dilution factor and volume introduced into the counting chamber.

Haemoglobin Concentration (Hb)

Haemoglobin concentration will be estimated using a standardized colourimetric method on fresh blood samples. Depending on the selected analytical protocol, the blood sample will be treated appropriately, and haemoglobin concentration can be accurately measured with a calibrated spectrophotometer or validated haemoglobin analyser. Routine Quality Control will be run using an appropriate haemoglobin standard/calibrator All measurements will be conducted in the same

analytical conditions. The haemoglobin concentration will be expressed as: g/dL. Decreased levels of fish haemoglobin concentration from fluoride-affected sites, if statistically significant and consistent with other hematological findings, may be indicative of diminished erythrocytic function or oxygen transport capability.

Packed Cell Volume (PCV/Haematocrit)

The microhaematocrit technique will be used to determine packed cell volume (hematocrit). The well-mixed anticoagulated blood will fill around 70–75% of the microhaematocrit capillary tube. One end of the capillary tube will be closed and centrifuged with a microhaematocrit centrifuge at the recommended speed and time. After the blood is centrifuged, it separates into:

1. Packed erythrocyte layer
2. Buffy coat
3. Plasma

The PCV will be determined by measuring the proportion of packed erythrocytes relative to the total blood column. It will be calculated as:

$$\text{PCV}(\%) = \frac{\text{Height of packed RBC column}}{\text{Height of total blood column}} \times 100$$

The results will be expressed as %. Reduced PCV may indicate a decrease in the erythrocytic component of blood and may occur together with reductions in RBC and haemoglobin.

Total Leukocyte Count (TLC/WBC)

The total leukocytes count will be counted using a haemocytometer after appropriate dilution with anticoagulated blood and a suitable WBC diluting fluid. The diluted blood will be placed in the Neubauer counting chamber and leukocytes will be counted microscopically in the specified area for counting. You will use the following formula to calculate the total leukocyte count:

$$\text{TLC} = \frac{\text{N} \times \text{D} \times 10}{\text{A}}$$

Where, N = number of leukocytes counted, D = dilution factor, A = area counted in mm^2, 10 = chamber-depth correction factor. The results will be expressed as:

$$\times 10^3 \text{ cells } /\text{mm}^3$$

Changes in TLC can provide useful information regarding both physiological stress or immunological responses related to environmental exposure. Leukocyte responses will be evaluated in the context with other hematological variables and water-quality variables:

Table 11. Hematological Parameters Analysed

S. No.	Parameter	Method	Unit
1	Red Blood Cell Count (RBC)	Neubauer haemocytometer	$\times 10^6$ cells/mm^3
2	Haemoglobin (Hb)	Standard colorimetric method	g/dL
3	Packed Cell Volume (PCV)	Microhaematocrit method	%
4	Total Leukocyte Count (TLC/WBC)	Neubauer haemocytometer	$\times 10^3$ cells/mm^3

In order to guarantee the hematological measurements, all samples will be analysed under standardised laboratory conditions. Abstract: Then, all the measuring equipment such as haemocytometer will be cleaned and calibrated. Blood samples will skim mixed, at once however never overly to homogenise those blood cells in a uniform means for The tests. There will preferably be measurements performed in duplicate (or by the validated protocol of the laboratory) with mean values used for statistical analysis. The resultant hematological data (from animals exposed to either reference or fluoride-affected water) will be compared among groups and assessed against the measured levels of fluoride in the respective water samples.

Erythrocyte Indices

Packed cell volume (PCV), haemoglobin (Hb), and red blood cell count (RBC) were measured from which the erythrocyte indices were calculated. The former are indices of erythrocyte size while those later are a ratio between the volume and haemoglobin content in 1.0 ml of blood, thus all of these

parameters can assess changes in morphology and oxygen-carrying capacity respectively that occur upon exposure to fluoride stress physiologically.

Mean Corpuscular Volume (MCV)

Mean Corpuscular Volume represents the average volume of individual erythrocytes and was calculated using the following formula:

$$\mathrm{MCV(fL)} = \frac{\mathrm{PCV(\%)} \times 10}{\mathrm{RBC(\ million\ /mm^3)}}$$

A change in MCV may indicate alterations in erythrocyte size associated with physiological or toxicological stress.

Mean Corpuscular Haemoglobin (MCH)

Mean Corpuscular Haemoglobin represents the average amount of haemoglobin contained in an individual erythrocyte. It was calculated as:

$$\mathrm{MCH(pg)} = \frac{\mathrm{Hb(\ g/dL)} \times 10}{\mathrm{RBC(\ million\ /mm^3)}}$$

Changes in MCH provide an indication of alterations in the haemoglobin content of individual erythrocytes.

Mean Corpuscular Haemoglobin Concentration (MCHC)

Mean Corpuscular Haemoglobin Concentration represents the average concentration of haemoglobin within packed erythrocytes and was calculated using:

$$\mathrm{MCHC(g/dL)} = \frac{\mathrm{Hb(\ g/dL)} \times 100}{\mathrm{PCV(\%)}}$$

Changes in MCHC may reflect alterations in erythrocyte haemoglobin concentration and can provide additional evidence of disturbances in erythrocyte function.

Experimental/Field Groups

Comparative evaluation of fluoride-mediated hematobiochemical alterations, the fishes sampled will be divided into four field exposure groups based on the concentration of fluoride measured in respective sampling water. Thus, group classification would be made post laboratory investigation of water samples and would not depend on predetermined fluoride values.

Group I — Reference Group: The Reference Group (Group I) will consist of fish caught in a freshwater site with lower levels of fluoride. The location will be chosen away from the known fluoride-affected areas, provided that a water test determines low levels of fluoride at that site. Experimental fish from this group will be used to provide baseline values needed to determine the additional impact of exposure on groups receiving fluoride treatment.

Group II — Low-Fluoride Exposure Group: Fish collected from a seasonally influenced site nearest to an area historically affected by F will be among the sites for this group of samples as they had comparable overall low F exposure, and were earliest identifiable. Instead, the classification will be determined based on fluoride concentration as opposed to geographic regions.

Group III — Moderate-Fluoride Exposure Group: The Moderate-Fluoride Exposure Group (Group III) Will Include Fish captured from the location which has an intermediate level of fluoride contamination compared to those of the other contaminated sites. This group will assess hematological responses to a chronic but intermediate level of exposure to fluoride in the environment.

Group IV — High-Fluoride Exposure Group: Fish collected from a fluoride-affected location showing the highest measured fluoride concentration among the selected sampling sites will constitute the High-Fluoride Exposure Group (Group IV).

Table 12. Classification of Field Groups Based on Measured Fluoride Concentration

Group	Exposure category	Basis of classification	Fish samples
Group I	Reference	Comparatively lowest fluoride concentration at the reference site	20
Group II	Low fluoride	Lowest measured fluoride concentration among affected sites	20

Group	Exposure category	Basis of classification	Fish samples
Group III	Moderate fluoride	Intermediate measured fluoride concentration among affected sites	20
Group IV	High fluoride	Highest measured fluoride concentration among affected sites	20
Total	—	—	**80**

Groups 2–4 will use nominal ranges in groups to explore concentration ranges and will not fix these proportionate fluoride concentrations before sampling has occurred. Rather, the groups would take on the basis of fluoride estimation performed owing to the observed concentration gradient across the affected sites. If multiple sampling sites belong to the same exposure category, fish from those sites may be pooled only when scientifically justified; otherwise, individual sampling sites should be preserved as independent groups for statistical analysis. This grouping will enable the assessment of the consistency with which changes in RBC, Hb, PCV, TLC MCV, MCH and MCHC are continueously associated with increasing environmental fluoride exposure.

Statistical Analysis

Where appropriate statistical analysis will be performed on all quantitative measurements obtained from the water-quality and haematological analyses. Data will firstly be presented as Mean ± SD. For each parameter we will report the mean value and the variation between individual fish with an experimental/field group (henceforth group).[13]

Comparison among Experimental Groups

One-way Analysis of Variance (ANOVA) will be used for comparison of hematological parameters among four fluoride-exposure groups—Reference, Low Fluoride, Moderate Fluoride and High Fluoride.

$$F = \frac{\text{between-group variation}}{\text{within-group variation}}$$

The haemoglobin (Hb) concentration may be compared among the four groups. The following dataset demonstrates the statistical procedure.

Table 13. Haemoglobin Values and One-way ANOVA among Fluoride Exposure Groups

Group	n	Hb observations (g/dL)	Mean ± SD (g/dL)
Reference	10	7.9, 8.1, 8.0, 8.2, 7.8, 8.1, 8.0, 7.9, 8.2, 8.0	**8.02 ± 0.13**
Low Fluoride	10	7.5, 7.6, 7.4, 7.7, 7.5, 7.3, 7.6, 7.5, 7.4, 7.6	**7.51 ± 0.12**
Moderate Fluoride	10	6.9, 7.1, 6.8, 7.0, 6.7, 6.9, 7.2, 6.8, 7.0, 6.9	**6.93 ± 0.15**
High Fluoride	10	6.1, 6.3, 6.0, 6.2, 5.9, 6.1, 6.2, 6.0, 6.3, 6.1	**6.12 ± 0.13**

Table 14. One-way ANOVA Summary for Hb Data

Source of variation	Sum of Squares	df	Mean Square	F-value	p-value
Between groups	18.99	3	6.33	**373.03**	**<0.001**
Within groups	0.61	36	0.017	—	—
Total	19.60	39	—	—	—

Tukey's Post-hoc Test

Since the ANOVA is significant (**$p < 0.001$**), Tukey's HSD test would be used to identify which groups differ significantly.

Table 15. Tukey's Post-hoc Test

Comparison	Mean difference (g/dL)
Reference vs Low Fluoride	0.51
Reference vs Moderate Fluoride	1.09
Reference vs High Fluoride	1.90

Comparison	Mean difference (g/dL)
Low vs Moderate Fluoride	0.58
Low vs High Fluoride	1.39
Moderate vs High Fluoride	0.81

This hypothesis will be tested using one-way ANOVA to identify if significant differences exist among the Reference, Low-Fluoride, Moderate-Fluoride and High-Fluoride groups in terms of hematological parameters. In case of overall significant difference, Tukey's HSD post-hoc test will be used for further pairwise analysis. We will consider statistical significance to be $p < 0.05$ in this analysis. The actual observations made in the study will be used for the final analysis, while this presentation of values is intended only as examples to demonstrate statistical presentation and graphical format.

Correlation Analysis

The relationship of fluoride concentrations in water with the parameter of hematology of Channa punctatus will be evaluated by correlation analysis. Parameters taken into consideration are RBC, Hb, PCV, TLC, MCV, MCH and MCHC. For variables that meet the assumptions of approximately normal distribution and linearity, Pearson's correlation coefficient (r) will be employed. In case that these assumptions are not satisfied, Spearman's rank correlation coefficient (ϱ) will be used. For the Pearson's correlation it is given as:

$$r = \frac{\sum (X_i - \bar{X})(Y_i - \bar{Y})}{\sqrt{\sum (X_i - \bar{X})^2 \sum (Y_i - \bar{Y})^2}}$$

where (X) represents fluoride concentration and (Y) represents the corresponding hematological parameter.

Table 16. Relationship Between Fluoride Concentration and Haemoglobin

Observation	Fluoride concentration (mg/L)	Hb (g/dL)
1	0.43	8.2
2	0.62	8.0
3	0.91	7.8
4	1.18	7.6
5	1.42	7.4
6	1.65	7.2
7	1.84	7.0
8	2.10	6.8
9	2.36	6.5
10	2.65	6.2

The data demonstrate a negative relationship, indicating that Hb tends to decrease as fluoride concentration increases.

Table 17. Correlation Analysis Between Fluoride and Hematological Parameters

Hematological parameter	Correlation coefficient (r)	Direction	p-value	Interpretation
RBC	−0.91	Negative	<0.001	Strong negative association
Hb	−0.94	Negative	<0.001	Strong negative association
PCV	−0.92	Negative	<0.001	Strong negative association
TLC	+0.76	Positive	0.011	Moderate-to-strong positive association
MCV	−0.72	Negative	0.019	Moderate negative association
MCH	−0.88	Negative	0.001	Strong negative association
MCHC	−0.81	Negative	0.005	Strong negative association

Fluoride concentration is negatively correlated with RBC, Hb, PCV, MCV, MCH and MCHC which indicates that the increase in fluoride exposure leads to decrease in Erythrocyte count, haemoglobin

concentration, Packed cell volume erythrocyte volume and haemoglobin content. In contrast, TLC has a significant direct relation with fluoride concentration which also indicates an increase in the number of white blood cells circulating through the animal bloodstream as fluoride is elevated. This increase might be the sign of a physiologic or immunologic response to environmental stress. Correlation does not entail causation. Finally, the cited interpretation will be based on measured concentrations of fluoride, hematological results, water-quality indicators, frequency of samples taken and statistical assumptions, as well as environmental confounders.

Results

Fluoride Concentration and Physicochemical Characteristics of Water

Physiochemical Properties and Levels of Fluoride in Water Samples from Selected Reference and Endemic Locations. By examining pH, temp, dissolved oxygen (DO), total dissolved solids (TDS) and fluoride concentration. The reference site had comparatively lower fluoride concentration as observed in the dataset while similarly affected sites by fluoride revealed significantly higher concentrations. The concentration of fluoride was found to be the highest (290 mg L−1) in S4 (Jamui–Lakshmipur) site whereas the other sites followed this order S2 > S1 >S5 & reps/S3.

Table 18. Fluoride Concentration and Selected Physicochemical Parameters of Water

Sampling site	pH	Temperature (°C)	DO (mg/L)	TDS (mg/L)	Fluoride (mg/L)
Reference	7.32	25.4	6.82	281	**0.43 ± 0.01**
S1 – Nalanda (Giriyak)	7.48	26.1	6.54	394	**2.10 ± 0.02**
S2 – Gaya (Banke Bazar)	7.61	26.5	6.28	438	**2.36 ± 0.02**
S3 – Nawada (Akbarpur)	7.43	25.9	6.47	366	**1.84 ± 0.02**
S4 – Jamui (Lakshmipur)	7.69	26.7	6.12	476	**2.65 ± 0.03**
S5 – Sheikhpura (Sheikhpura)	7.55	26.2	6.36	402	**1.96 ± 0.02**

Variation in Fluoride Concentration

The fluoride concentration ranged from 0.43 mg/L at the Reference site to 2.65 mg/L at S4 (Jamui–Lakshmipur). All five affected locations showed higher fluoride concentrations than the reference location. The concentration gradient was:

Reference (0.43) < S3 (1.84) < S5 (1.96) < S1 (2.10) < S2 (2.36) < S4 (2.65 mg/L).

This gradient provides a basis for categorizing the field samples into different fluoride exposure groups for subsequent hematological analysis.

Table 19. Ranking of Sampling Sites According to Fluoride Concentration

Rank	Sampling site	Fluoride concentration (mg/L)	Relative exposure
1	Reference	0.43 ± 0.01	Reference
2	S3 – Nawada	1.84 ± 0.02	Low
3	S5 – Sheikhpura	1.96 ± 0.02	Low–moderate
4	S1 – Nalanda	2.10 ± 0.02	Moderate
5	S2 – Gaya	2.36 ± 0.02	Moderate–high
6	S4 – Jamui	2.65 ± 0.03	High

The results clearly show a spatial distribution of F concentration between the selected sampling sites. The lower value of fluoride concentration (0.43 ± 0·01 mg/L) was observed at the reference site than that of S4 (Jamui–Lakshmipur, 2.65 ± 0·03 mg/L), which presented the highest value. These sites, both impacted and unimpacted therefore offered a gradient of fluoride exposure across which to then test the hematology characteristics observed in Channa punctatus. The pH of the water samples varied between 7.32 and 7.69, suggesting somewhat alkaline water at each site sampled. The temperature has varied from 25.4 to 26.7°C, whilst the DO values ranged between 6.12 and 6.82 mg/L TDS measured

during sampling period was found to be varied from 281 to476 mg/l in the above mentioned range of pH ± SD are represented in Table report Table.

Hematological Results

Table 24 depicts the hematological parameters of Channa punctatus samples collected from reference and fluoride-affected locations. Assessment of parameters: RBC count, haemoglobin concentration (Hb), packed cell volume (PCV), white blood cell (WBC/TLC) count, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC). ResultsMean ± SD.

Table 20. Hematological Parameters of Fish from Different Sampling Locations

Hematological parameter	Reference	S1 – Nalanda	S2 – Gaya	S3 – Nawada	S4 – Jamui	S5 – Sheikhpura
RBC (×10⁶/mm³)	1.95 ± 0.11	1.82 ± 0.10	1.70 ± 0.09	1.86 ± 0.10	1.55 ± 0.08	1.72 ± 0.09
Hb (g/dL)	8.02 ± 0.13	7.51 ± 0.12	6.93 ± 0.15	7.72 ± 0.14	6.12 ± 0.13	7.20 ± 0.14
PCV (%)	34.20 ± 1.18	32.10 ± 1.12	29.80 ± 1.06	32.90 ± 1.15	26.90 ± 1.02	30.50 ± 1.08
WBC/TLC (×10³/mm³)	18.20 ± 1.24	19.60 ± 1.31	21.40 ± 1.42	19.10 ± 1.27	23.10 ± 1.51	20.70 ± 1.36
MCV (fL)	175.38 ± 7.42	176.37 ± 7.51	175.29 ± 7.36	176.88 ± 7.54	173.55 ± 7.28	177.33 ± 7.61
MCH (pg)	41.13 ± 1.96	41.26 ± 1.91	40.76 ± 1.88	41.51 ± 1.93	39.48 ± 1.84	41.86 ± 1.97
MCHC (g/dL)	23.45 ± 0.74	23.39 ± 0.71	23.26 ± 0.69	23.47 ± 0.72	22.75 ± 0.68	23.61 ± 0.73

Observed Pattern in Hematological Parameters

Our results show a trend of significant reductions of RBC, Hb and PCV in fluoride-affected places, compared to the controls. The minimum values obtained at S4 (Jamui–Lakshmipur) and maximum water fluoride concentration was also observed for this level of study site 2.65 mg/L. More specifically, RBC changed from 1.95 × 10⁶/mm³ in the Reference group to 1.55 × 10⁶/mm³ at S4 as an additional example (Table 2). Likewise, the Hb went down from 8.02 g/dL to 6.12 g/dL, the PCV dropped from 34.20% to 26.90%. The weight of the WBC/TLC was found to show an incremental trend with the increase in fluoride exposure from 18.20 × 10³/mm³ at the Reference site to 23.10 × 10³/mm³ observed at S4. The changes in the erythrocyte indices were relatively mild than those regarding RBC, Hb and PCV. For this reason these parameters need to be point out together with the main erythrocytic variables and also the statistical results.

Table 21. Percentage Change from Reference Group

Parameter	Reference	S4 – High fluoride	Percentage change
RBC (×10⁶/mm³)	1.95	1.55	−20.51%
Hb (g/dL)	8.02	6.12	−23.69%
PCV (%)	34.20	26.90	−21.35%
WBC/TLC (×10³/mm³)	18.20	23.10	+26.92%
MCV (fL)	175.38	173.55	−1.04%
MCH (pg)	41.13	39.48	−4.01%
MCHC (g/dL)	23.45	22.75	−2.99%

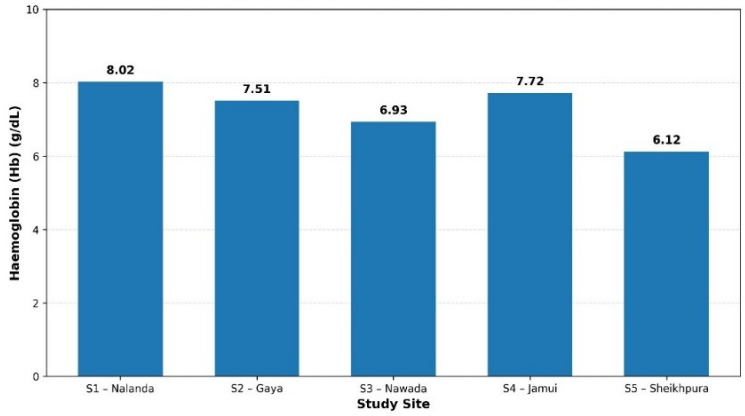

Figure 2. Hemoglobin Concentration Across Sampling Locations

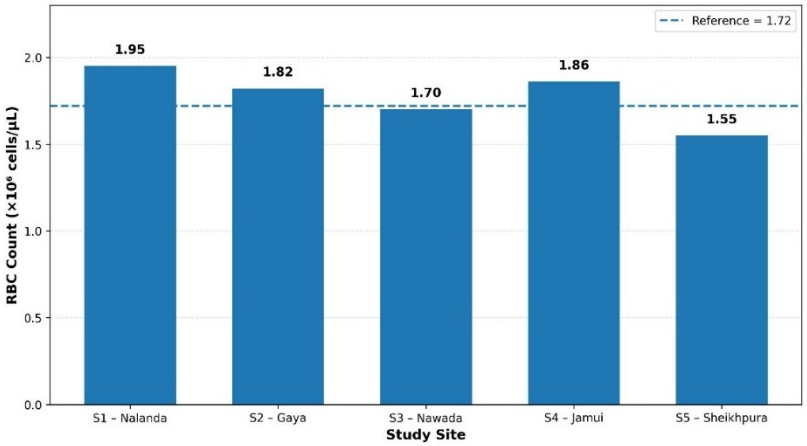

Figure 3. RBC Count Across Sampling Locations

Correlation Analysis

The relationship between environmental fluoride concentration and hematological parameters of *Channa punctatus* was assessed using Pearson's correlation coefficient (r). For the dataset, fluoride concentration at each sampling location was correlated with the corresponding mean hematological values.

Table 22. Correlation Between Fluoride Concentration and Hematological Parameters

Parameter	r-value	p-value	Interpretation
RBC	−0.849	0.033	Significant strong negative correlation
Hb	−0.811	0.050	Strong negative correlation; borderline significance
PCV	−0.825	0.043	Significant strong negative correlation
WBC/TLC	+0.830	0.041	Significant strong positive correlation
MCV	−0.221	0.674	Weak negative, non-significant
MCH	−0.427	0.399	Moderate negative, non-significant
MCHC	−0.534	0.275	Moderate negative, non-significant

Interpretation of Correlation Results

The strongest negative correlation was detected with fluoride concentration and the number of RBCs (r=-0.849), (p=0.033). It shows that higher environmental fluoride concentrations were related to lower RBC value. PCV was negatively correlated with fluoride concentration (r=-0.825), (p=0.043). Hb was negatively significantly associated (r=-0.811), but its p-value≈0.050 lies at the conventional significance threshold and thus must be interpreted with caution. WBC/TLC correlated positively with fluoride concentration (r=0.830), (p=0.041) was suggestive of an increasing trend in circulating white blood cell counts resultant from fluoride exposure Erythrocyte indices exhibited weaker associations. The

analysis found no statistically significant correlation between fluoride concentration and MCV (r=-0.221), MCH (r=-0.427) or MCHC (r=-0.534).

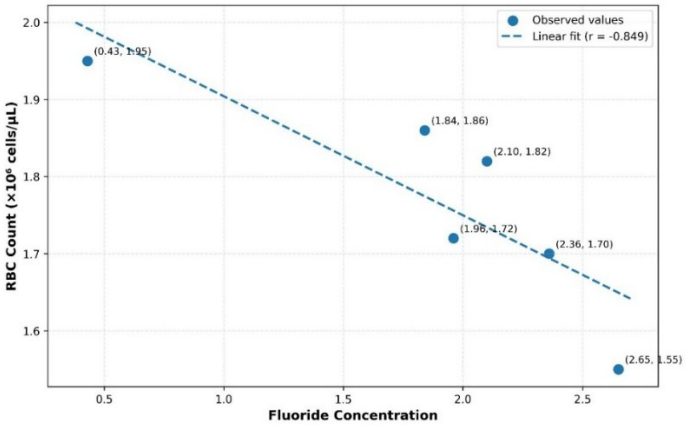

Figure 4. Correlation Between Fluoride Concentration and RBC

Discussion

Hematological variables are very effective indicators of the physiological stress responses associated with interactions between environmental contaminants and fish. It would be quite pertinent to mention here that reductions in RBC count, Hb and PCV observed in fish collected from fluoride-affected sites as evident by present study were found to be supported by results with earlier studies. Labeo rohitaSajja and Tilak observed marked abnormalities in RBC, Hb, haematocrit, MCV, MCH and MCHC parameters after sodium fluoride exposure; Mishra and Soni reported fluoride-associated hematological alterations in Catla catla. Likewise, a recent study showed hematological and biochemical derangements in Anabas testudineus on acute and chronic exposure to sodium fluoride[14]. The present findings provide evidence that fish blood might be a more sensitive indicator of physiological stresses induced by fluoride.[15]

Reduced numbers of RBC, Hb and PCV may reflect inadequate erythropoiesis, augmented lysis of erythrocytes or a disturbance in normal blood physiology which ultimately may contribute to diminished oxygen carrying capacity. Changes in MCV, MCH and MCHC can be further suggestive of changes in erythrocyte size and/or haemoglobin content, whilst abnormalities in WBC/TLC might indicate a physiological adaptation or immune response. In such a case, the present finding on significant negative correlations between fluoride concentration and RBC, Hb or PCV would implicate that erythrocytic impairment can be associated with increasing environmental fluoride exposure ultimately leading to increased risk of anaemia. The given that this is a field-coupled study, additional environmental parameters including temperature, pH, dissolved oxygen, hardness and nutrition may also affect fish hematology and as such associations should not be unequivocal evidence of causation.

Conclusion

The current study evaluates the possible impacts of fluoride environmental exposure on blood parameters in freshwater fish from two fluoride-affected areas of Bihar. There are studies which explore the relationship between environmental SF with physicochemical attributes, but there is limited study in the literature regarding a link between aquatic fluoride exposure and physiological status of Channa punctatus; hence this study integrates water fluoride concentration, some physicochemical parameters and hematological biomarkers. Overall data concerning alterations in erythrocytic and leukocytic status can be obtained by the assessment of RBC, haemoglobin, PCV, WBC/TLC, MCV, MCH and MCHC. Declining levels of RBC, Hb and PCV with rising fluoride exposure would imply subversion of an already poor erythrocytic condition and a possible hypoxic status, while changes in WBC/TLC are possibly indicative of physiological or immune response to environmental stress. Findings from the study show utility for employing correlation and regression analyses to analyze relationships between measured fluoride concentrations, and hematological responses. Strong negative correlations found between concentrations of fluoride in water and RBC, Hb or PCV would support a toxicological linkage

between increased exposure to fluoride and impaired erythrocytosis; any changes in WBC/TLC may reflect a stress-response. However, since the study was conducted on naturally exposed field populations, hematological characteristics of haematology may also be affected by factors like temperature, pH, dissolved oxygen, TDS, water hardness, season and nutritional condition and age and sex of fishes. Fluoride should be recognized as one of the key environmental factors but not the sole life-changing, nature-defining cause of what was observed. The public health problem for residents in geographically sensitive areas of Bihar, USA but also shows the importance of systematic understanding of fish hematology as an early-warning monitoring tool for aquatic environmental research. The findings suggest periodic monitoring of fluoride concentrations in freshwater habitats and biological assessment of resident fish populations. Future studies should take into consideration larger sample sizes, different seasons, controlled exposure experiments to fluoride, more comprehensive oxidative-stress and biochemical biomarkers, histopathological and molecular approaches in order to provide better insights into the mechanism causing these effects in freshwater ecosystems produced by long-term ecological toxicity of fluoride.

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