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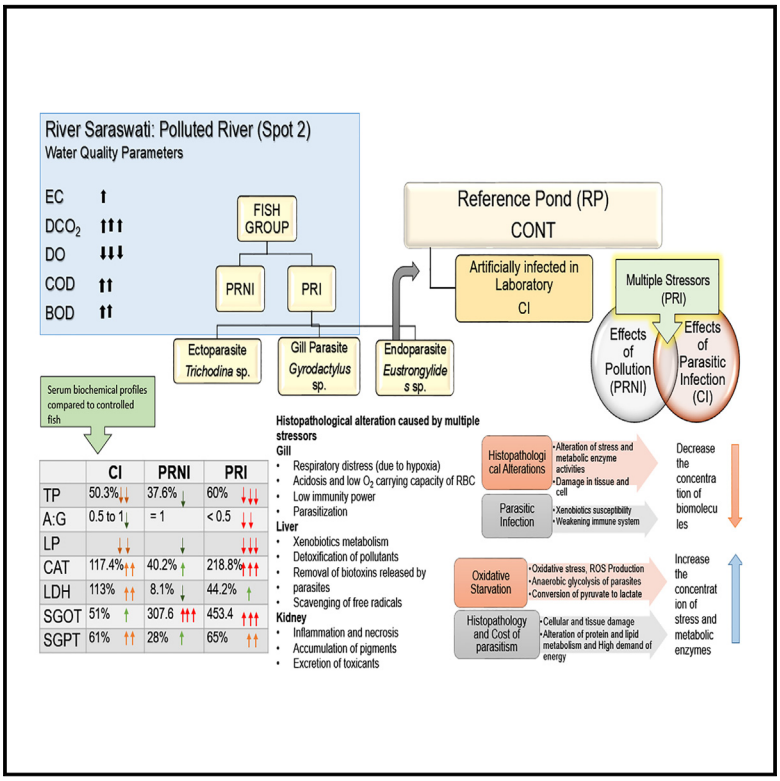
Graphical abstract



Highlights

- River water quality became degraded due to organic loading
- Tripartite interactions occurred among fish, pollution, and parasite
- Concurrent pollution and parasitization impacted fish health in a vicious cycle
- Histopathological and biochemical perturbations served as multi-stress biomarkers

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In brief

Environmental science; Pollution; Aquatic science; Aquatic biology

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Histopathological fingerprints and biochemical changes as multi-stress biomarkers in fish confronting concurrent pollution and parasitization

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SUMMARY

The current investigation examined histopathological and serum biochemical profiles to assess the interactive effects of river Saraswati's impaired physicochemical regime and parasite infection by ectoparasites (*Trichodina* sp., *Gyrodactylus* sp.) and endoparasites (*Eustrongylides* sp.) on health of fish (*Channa punctatus*). This study aimed at assessing the synergistic effects of the degraded water quality and parasitization on fish health looking through the lens of histopathology and serum biochemistry. Low dissolved O_2, high free CO_2, biochemical oxygen demand (BOD), and chemical oxygen demand (COD) indicate deteriorating water quality due to organic loading. The histopathological investigations revealed distinctive histopathological changes in gill, liver, and kidney of the fish as "signature" impairments inflicted by chronic (co) exposure to pollution and/or parasitization. Stress enzymes and altered serum biochemistry furnish excellent indicators of fish health because of their correlation with pollution and parasitization. Destined in a vicious cycle, fish health becomes adversely affected by degrading water qualities and parasitic infection in solo or combo.

INTRODUCTION

Different natural and human activities lead to aquatic pollution. The water quality of aquatic system is decreased by anthropogenic activities such as wastewater discharge, poor sanitation procedures, the use of fertilizers and pesticides in agricultural fields, animal husbandry activities, ineffective irrigation techniques, industrial effluents, and domestic sewage. Physicochemical changes can affect the number, variety, and organization of living organisms in the water body, leading to biological degradation and responsible for biochemical and histopathological stress for aquatic organisms.[1] Fish are the most common aquatic animal, which are adversely affected by water pollution. *Channa punctatus* is a common freshwater edible fish that may survive in a stressful environment owing to presence of an accessory respiratory organ and associated adaptations.[2] Fish parasites have been acknowledged as important environmental monitors that can detect changes in the aquatic environment.[3] In a polluted environment, the fish are often found to be parasitized by ecto- and endoparasites such as the ciliate *Trichodina* sp., the monogenean *Gyrodactylus* sp., and the larval stage (L4) of *Eustrongylides* sp.[4,5] The parasitic load such as prevalence, mean intensity, and relative abundance of parasites can serve as potential indicators of water quality.[6]

Histopathology allows for the detection of both acute and chronic alterations in cells, tissues, and organs in the individual parasitized organisms. The gills and gastrointestinal tract are the main "window" for entry of parasites and "route" of exposure to pollutants into fish body. Internal organs such as the liver and kidney, on the other hand, are affected by those pollutants being transported by the portal circulation.[7] Gills are prime target organs of aquatic pollutants because they constantly remain in contact with the external environment.[8] The liver serves as a critical metabolic hub for pollutants transformation, whereas the kidney aids in filtration and removal of toxicants from the body.[9] Environmental stress causes histopathological changes in various organs of fish, including the gills, liver, and kidney, which allow parasites to harbor them heavily.[10] Ciliate protozoa *Trichodina* sp. and monogenean *Gyrodactylus* sp. are ectoparasites of fish that usually infest the gills, skin, and fins of freshwater fish.[11] Endoparasites and the toxic chemicals generated by ectoparasites impact internal organs such as the liver and kidney, as evidenced by histological studies.[12] Furthermore, pollutant stress biomarkers can be found in histopathological and ultrastructural alterations in different tissues and organs of aquatic animals. Therefore, histopathological changes of different organs can be employed as potential indicators of water pollution and fish health status.[7] Histopathological changes in several

internal organs in fish are linked to serum biochemical deformities and serve as a sensitive signal for detecting early signs of pollution and other stressful situations.[13]

Serum biochemical parameters of fish have been employed as biomarkers of environmental stress, parasitic infection, and health condition.[14] Accumulations of pollutants in body tissue can affect the oxidative metabolism of different biochemical molecules such as carbohydrates, protein, and lipid. Therefore, it alters the concentration of glucose, total serum protein, albumin, globulin, and lipid profile.[15] Different sources of reactive oxygen species (ROS) are superoxide anions, H_2O_2, hydroxyl radicals, NO, etc. ROS is generated by the living system as a by-product of metabolic activities. ROS is linked to some essential physiological processes when its concentration is maintained at physiologically very low level. Being energy deficient and very reactive in nature at higher concentration, following a cascade of reactions, ROS attack biomolecules such as carbohydrates, protein, lipid, and DNA, causing lipid peroxidation, membrane disintegration, interruption of gene functions, which may culminate in cell death and tissue damage in fish.[16] The impact of environmental stress and parasitic infection was investigated by Furtado et al.[17] using oxidative stress as a potential biomarker and screening tool. Two types of antioxidant systems are found as a part of antioxidative defense to keep ROS within physiological limits. One is non-enzymatic and another is enzymatic involving catalase (CAT), superoxide dismutase (SOD), lactate dehydrogenase (LDH), glutathione peroxidase, glutathione reductase, etc.[18]

The scavenging and removal of ROS and other free radicals is essential for keeping the physiological balance of the oxidative-antioxidative system in fish and preventing parasite pathogenicity. In fish, two metabolic enzymes, serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT), simultaneously operate as serum indicators for damage to internal organs such as the liver and kidney caused by multiple stressors such as water pollution and parasite infection.[19] Histopathological observation and biochemical assays may be employed to assess alterations in organ functions in fish. Analysis of the results obtained from the parasitic load may provide valuable insight in elucidating specific responses and the pathological processes involved.

Although many researchers conducted environmental parasitological studies on host fish collected from natural habitat, i.e., their study site, few studies have undertaken microcosm study of the host-parasite interaction inducing ''artificial'' infection in the laboratory for several weeks to months, which can be very useful in identifying and partitioning effects of pollution from that of parasitic infection as well as the combined effects when the two factors act together in a synergistic manner. It might provide a different explanation for a particular phenomenon. Further, the study of a single species of parasite is not sufficient to identify the impacts of pollution on parasite, host, and their interaction. Therefore, comparative study involving different types of parasites, namely ectoparasite (e.g., *Trichodina* sp.), endoparasite (e.g., *Eustrongylides* sp.), and gill parasite (e.g., *Gyrodactylus* sp.) of fish, may provide different ways of approaching a particular problem to earn much insight.

There is very scarce information available in India on combined approach of parasitic biomonitoring coupled with biochemical stress biomarkers in general and riverine system in particular. Limited research works have been conducted to use parasite-induced alteration in histopathological and biochemical stress biomarkers as fingerprints of pollution and/or parasitic infestation as well as proxy indicators of environmental quality of the aquatic system. Paucity of studies is found pertaining to the combined effects of multiple stressors operating in an aquatic habitat (e.g., co-occurring and co-acting parasitic infection and water quality degradation) on fish. There appear very limited scientific efforts in establishing a comprehensive portfolio of stress biomarkers to unveil the solo and combo effects of the stresses in action.

The water quality can be degraded by both inorganic and organic environmental pollutants coming from diverse natural sources and anthropogenic activities. In polluted aquatic habitat, fish's health deteriorates, which can be reflected in histopathological and serum biochemical alterations. Further, the fish become more vulnerable to parasitic infection in the polluted environment. Gradual degradation in the water quality over time may intensify parasitic infection, leading to further deterioration of fish health in a ''positive feedback'' mode. Under ''business as usual'' situation, it appears as a sequence of reciprocal cause and effect in which the elements amplify and aggravate each other, leading inevitably to a deteriorating situation (called ''vicious cycle''). It was hypothesized that alteration of fish health status not only depends on a single factor, rather it results from tripartite interactions among fish, parasites, and pollution. Second hypothesis was that a ''vicious cycle'' develops as a result of the interplay of the triad involved.

To bridge the existing knowledge gaps, the present study was undertaken with the following objectives: (1) to monitor the physicochemical profile and assess aquatic health status of river Saraswati; (2) to establish the interrelationship between degraded water quality, parasitic infection, and fish health; (3) to examine the histopathological alterations in fish caused by water quality degradation and parasitic load, alone or in combination; (4) to evaluate certain biochemical stress markers such as serum biomolecules and enzymes in fish subject to ambient water quality degradation and/or parasitic infestation; and (5) to investigate the feasibility of developing fish parasite (ectoparasite such as protozoan *Trichodina*, sp. and endoparasite such as *Eustrongylides* sp.) as parasitic biomonitoring tool coupled with biochemical stress biomarkers for comprehensive assessment of aquatic health.

RESULTS AND DISCUSSION

Analysis of physicochemical profile of water from three different water sources

The seasonal data and the ANOVA of all the physicochemical parameters of water sample from three different sources (RP, PR-1, and PR-2 of the river Saraswati) are presented in Table 1.

Among the 10 parameters, significant variations were observed in eight parameters by using one-way ANOVA, which were electrical conductivity (EC), total dissolved solids (TDS), turbidity, pH, dissolved oxygen (DO), free CO_2, biochemical

Table 1. Physicochemical parameters (mean ± SE & ANOVA) of water from the reference pond and the polluted river (Saraswati)

Water sources	Tem (°C) Mean±SE	EC (mS cm^{-1}) Mean±SE	TDS (ppm) Mean±SE	Sal (ppm) Mean±SE	Tur (NTU) Mean±SE	pH Range	DO (mgL^{-1}) Mean±SE	Free CO_2 (mgL^{-1}) Mean±SE	BOD_5 (mgL^{-1}) Mean±SE	COD (mgL^{-1}) Mean±SE
POND (RP)	30.69±0.78	520.83±13	404.72±10.53	230.56±14.26	13.02±0.67	8.38–9.05	7.13±0.45	3.59±0.54	2.22±0.1	20.59±0.49
SPOT-1 (PR-1)	29.63±0.79	404.44±25.37	291.11±16.99	172.22±18.94	29.33±2.7	7.27–7.80	3.41±0.20	34.33±0.83	5.31±0.33	18.50±0.65
SPOT-2 (PR-2)	29.35±0.83	521.94±32.25	376.38±19.57	186.11±22.93	25.41±2.23	7.15–7.59	1.92±0.19	58.59±1.51	8.06±0.38	28.02±1.08
ANOVA	$F_{2, 105} = 0.077$, $p > 0.05$	$F_{2, 105} = 7.379$, $p < 0.001$	$F_{2, 105} = 13.397$, $p < 0.001$	$F_{2, 105} = 2.559$, $p > 0.05$	$F_{2, 105} = 17.01$, $p < 0.001$	$F_{2, 105} = 194.08$, $p < 0.001$	$F_{2, 105} = 74.51$, $p < 0.002$	$F_{2, 105} = 692.52$, $p < 0.001$	$F_{2, 105} = 94.32$, $p < 0.001$	$F_{2, 105} = 40.44$, $p < 0.001$

Instead of mean, pH represents range of values monitored during different seasons. Temperature (Tem), electrical conductivity (EC), total dissolved solids (TDS), turbidity (Tur), dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD).

oxygen demand (BOD), and chemical oxygen demand (COD). On the other hand, no statistically significant difference was observed among the mean values of water temperature and salinity.

Comparison of frequency distribution of major water quality parameters (DO, free CO_2, BOD, and COD) for three different sources

In PR-2, the DO frequency distribution suggests a hypoxic situation. Most of the DO values (75%) fall between 1 and 3 mgL^{-1}, with no observation exceeding the acceptable limit (5 mgL^{-1}; Table S1). The level of DO in PR-1 is somewhat higher than in PR-2. Majority (72.2%) of the DO values in PR-1 lie between 2 and 5 mgL^{-1}, with 22.2% exceeding the acceptable limit. RP represents much better water quality in terms of DO level (Figures 1A–1C).

A huge difference is observed in the frequency distribution of free CO_2 values between RP and both the spots of the polluted river. In RP, the highest frequency (30.6%) of free CO_2 lies between 3 and 5 mgL^{-1} and altogether 70% of the values stay below the standard limit (10 mgL^{-1}; Table S1). Throughout the study period, all recorded values of free CO_2 concentration in the polluted river remained above the standard limit (Table S1). About 64% values of free CO_2 of PR-1 remains between 34 and 42 mgL^{-1}, whereas 60% values in PR-2 remain within the range of 57 and 70 mgL^{-1} (Figures 1D–1F).

The frequency distribution of BOD value represents a better outcome in PR-1 than PR-2 because 41.7% values have crossed the standard limit (Table S1) in PR-1, whereas in PR-2, the value (86.1%) is more than two times higher than PR-1. On the other hand, the highest frequency (PR-1 = 50% and PR-2 = 38.9%) of BOD ranges from 4 to 5 mgL^{-1} in PR-1 and 8 to 10 mgL^{-1} in PR-2. The BOD value frequency distribution demonstrates a varied relationship between both the sampling sites of the polluted river (Figures 1G–1I).

The COD level of PR-1 stands in between that of RP and PR-2. The highest frequency (50%) of COD for RP lies between 20 and 24 mgL^{-1}, and 25% values stay below the benchmark value (20 mgL^{-1}; Table S1). A huge difference is observed in the frequency distribution between the two spots of the polluted river. The highest frequency (38.9%) of COD value in PR-1 stays above the standard value of 20 mgL^{-1} as encountered in RP (50%) but 44.5% value in PR-1 stays under standard limit (Table S1), indicating even an occasional better COD level compared to RP. On the other hand, in PR-2, the highest COD frequency (41.6%) lies in between the range of 28 and 36 mgL^{-1}; only 5.6% COD value remains below the standard limit (Table S1) throughout the study period (Figures 1J–1L).

Water quality of a water body is determined by a set of major physicochemical and biological quality parameters and serves as the *prime determinants of* its aquatic health and ecosystem services.[20] The mean EC value of PR-1 (404.44 ± 25.37 mS cm^{-1}) was lower compared to PR-2 (521.94 ± 32.25 mS cm^{-1}) and RP (520.83 ± 13 mS cm^{-1}), which exceeded the standard limit (300 mS cm^{-1}; Table S1) in all the sampling sites. The mean turbidity value was higher in both the spots (PR-1 = 125.26% and PR-2 = 95.16%) of the polluted river compared to RP and crossed the standard limit (10 NTU; Table S1).

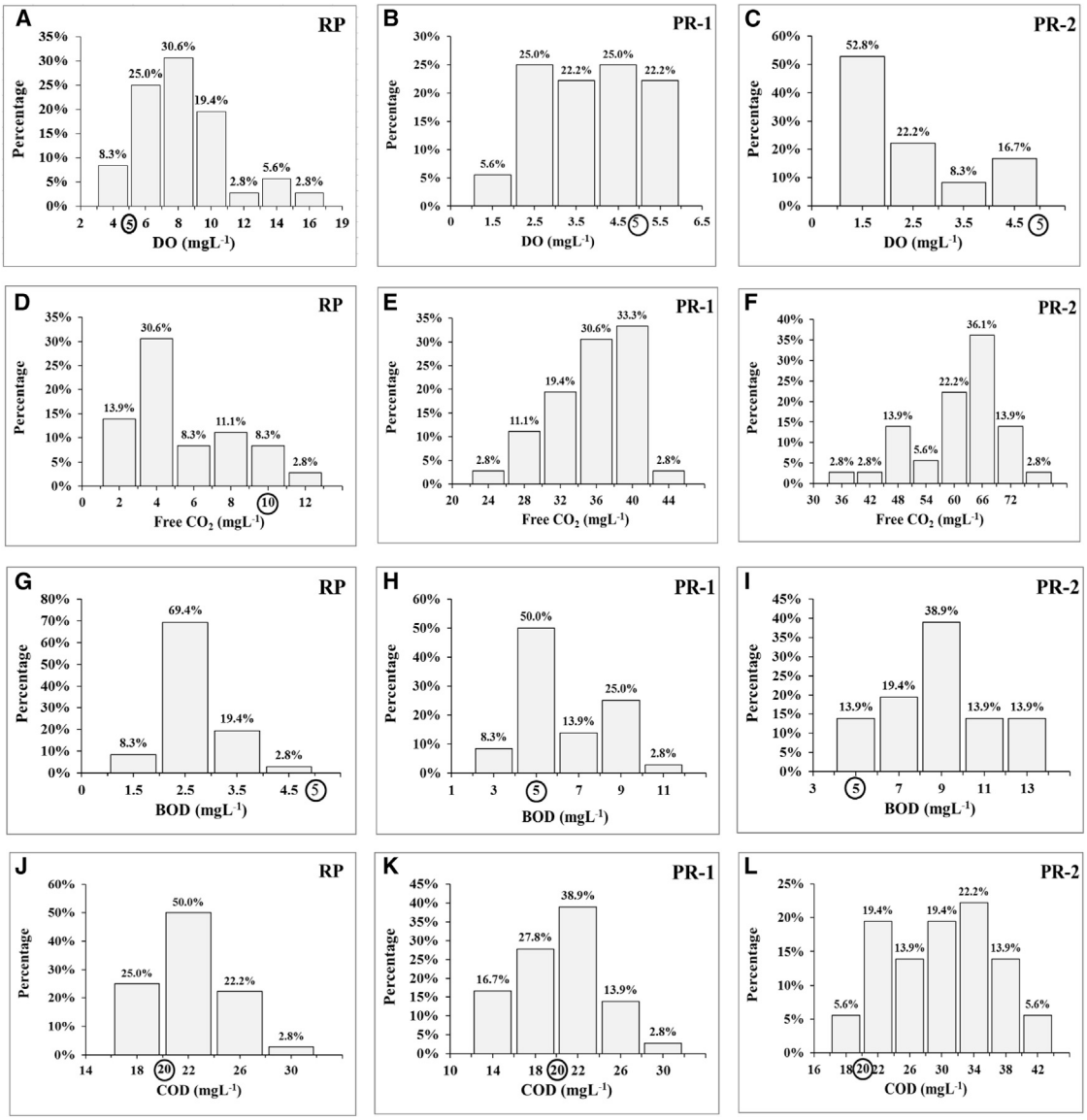

Figure 1. Frequency distribution of water quality parameters for three sampling sites

Frequency distribution analysis of physicochemical parameters of water collected from reference pond (RP), spot 1 (PR-1), and spot 2 (PR-2) of polluted river from March 2017 to February 2020: DO (A–C); free CO_2 (D–F); BOD (G–I); COD (J–L). The numbers within the circle (O) indicate respective standard value of the physicochemical parameter shown.

Turbidity of a water body is determined by the suspended solid particles. Higher turbidity *prima facie* indicates lower water quality, which cannot be taken alone as a reliable indicator of water pollution. Although, it is considered as a subordinate or associate indicator of water quality in addition to the principal water quality parameters.[21] In riverine system, high turbidity is caused by the suspended solids that become mixed with water being generated from riverbed material by heavy precipitation.[22] Additionally, loading of industrial wastes, sewage-borne planktons, and nutrient enrichment (eutrophication) also contribute to elevated turbidity of the water body.[23] In both the spots of the polluted river, the mean DO concentration always remained below the standard limit (5 mgL^{-1}; Table S1) considered conducive for fish. In PR-2, the mean concentration of DO was precipitously low (1.92 ± 0.19 mgL^{-1}), indicating hypoxic condition stressful for aquatic biota. The free CO_2 concentration was 15-fold higher (58.59 ± 1.51 mgL^{-1}) in PR-2 in respect to RP (3.59 ± 0.54 mgL^{-1}) and about 2x compared to PR-1 (34.33 ± 0.83 mgL^{-1}) of the polluted river. The mean concentration of COD (28.02 ± 1.08 mgL^{-1}) was moderately higher in PR-2 but the mean concentration of BOD was 263.06% higher in PR-2 (8.06 ± 0.38 mgL^{-1}) and 139.19% in PR-1 (5.31 ± 0.33 mgL^{-1}) compared to RP (2.22 ± 0.1 mgL^{-1}). In PR-2, mean COD value crossed the standard limit (Table S1) in all the seasons. BOD

Table 2. Number of both the infected and non-infected host fish (*Channa punctatus*), mean (±SD), prevalence, mean intensity, and abundance of three parasites (*Trichodina* sp., *Gyrodactylus* sp. and *Eustrongylides* sp.) collected from spot-2 (PR-2) of river Saraswati in monsoon, post-monsoon, and winter seasons

Parasite	Year	No. of fish examined	Total number of fish infected	Number of parasites collected from host fish	Prevalence %	Mean intensity	Abundance
Trichodina sp.	2017–18	118	42	900	35.59	21.42	7.62
	2018–19	150	52	1061	34.66	20.40	7.07
	2019–20	126	49	1011	38.88	20.63	8.02
	Mean ± SD				**36.38±2.21**	**20.82±0.53**	**7.57±0.47**
Gyrodactylus sp.	2017–18	118	17	114	14.40	6.70	0.96
	2018–19	150	28	221	18.66	7.89	1.47
	2019–20	126	20	135	15.87	6.75	1.07
	Mean ± SD				**16.31±2.16**	**7.11±0.67**	**1.17±0.26**
Eustrongylides sp.	2017–18	118	21	96	17.79	4.57	0.81
	2018–19	150	39	132	26.00	3.38	0.88
	2019–20	126	23	109	18.25	4.73	0.86
	Mean ± SD				**20.68±4.60**	**4.23±0.73**	**0.85±0.03**

and COD represent the amount of oxygen utilized by the microorganisms for oxidation of organic matter and for oxidization of oxidizable organic and inorganic material available in water, respectively. Both the values are negatively correlated with DO concentration.[24] High concentration of free CO_2 and BOD level indicated an increase in pollution, owing to organic loading from neighbor catchment areas.[25] High organic loading consumed dissolved oxygen to reduce it considerably, with concomitant increase in free CO_2 concentration culminating to a semi-anoxic to almost anoxic condition.

Parasitic load: Prevalence, mean intensity, and abundance

Prevalence, mean intensity, and abundance of parasites were calculated for quantification of parasite number in a host sample or population. The descriptive statistics associated with the parasitic load of host fish are represented in Table 2.

The mean values of prevalence are higher in *Trichodina* sp. (36.38 ± 2.21%) compared to *Gyrodactylus* sp. (16.31 ± 2.16%) and *Eustrongylides* sp. (20.68 ± 4.60%). The mean intensity and abundance differed significantly ($p < 0.001$) in the order of *Trichodina* sp.> *Gyrodactylus* sp.> *Eustrongylides* sp. by one-way ANOVA (Figure S1).

The ectoparasite Trichodinids require a single host for completion of their life cycle, hence considered as monoxenic parasite. Their reproduction is accomplished mainly by binary fission.[26] *Trichodina* sp. and *Gyrodactylus* sp. grow and reproduce ideally in a temperature range of 21°C–26°C; hence winter season is considered as most suitable for their reproduction. The process of binary fission is inhibited by high temperature.[27] Higher EC value indicates the nutrient enrichment condition of the aquatic habitat, which can cause suppression of immunity in the host fish. Thus, parasitic load was found to be intensified in the immunocompromised fish host living in a polluted environment.[28] Growth, metabolism, and swimming behavior of host fish are directly impacted by the higher BOD level and lower DO concentration, whereas availability of nutrients is indirectly associated with those parameters.[3] On the other hand, hypoxic condition of water is responsible for respiratory distress; therefore, the host fish lose their mobility and become susceptible to parasitic infection.[29] In PR-2, the free CO_2 concentration was much higher throughout the season, which restricted diffusion rate of CO_2 from fish blood during exhalation. Consequently, the CO_2 level of fish blood rises, which can lead to acidosis and reduce oxygen carrying capacity of hemoglobin.[30] Gaseous exchange from the respiratory surface (gill) is restricted by low DO and high free CO_2 level. Most of the time the DO level remains below the threshold value (4 mgL^{-1})[31] in PR-2. Under such adverse ambience, the host fish (*Channa punctatus*) survives owing to activities of accessory respiratory organ and increase in the ventilation rate but becomes restless, exhausted, and weak as a fallout. *Eustrongylides* sp. is a heteroxenic endoparasite of fish and wading bird (*Ardea alba*, *Anas platyrhynchos*, and *Ardeidae* sp.).[32] Prevalence, mean intensity, and abundance of *Eustrongylides* sp. were not affected by the physicochemical parameters of water. Both prevalence and efficiency of intermediate hosts play an important role in the transmission of endoparasite.[33] The prevalence, mean intensity, and abundance of endoparasite depend on its life cycle, physicochemical parameters, and quality of water.[34] It also depends on intermediate host where life cycle of parasite needs more than one host. Life cycle of *Eustrongylides* sp. requires three hosts. Fish acts as the second intermediate host, where encapsulated 3rd stage larva (L3) is transformed into 4th stage larva (L4).[35]

Histopathological and serum biochemical alterations

The diagnosis of fish health and environmental stress is considered to be aided by histopathological study and serum biochemical analyses of host fish.

Histopathology of gill

The polluted river non-infected (PRNI) fish showed that the primary lamellae were disorganized, and the central core of cartilage was not uniform. In contrary to the normal structure, their

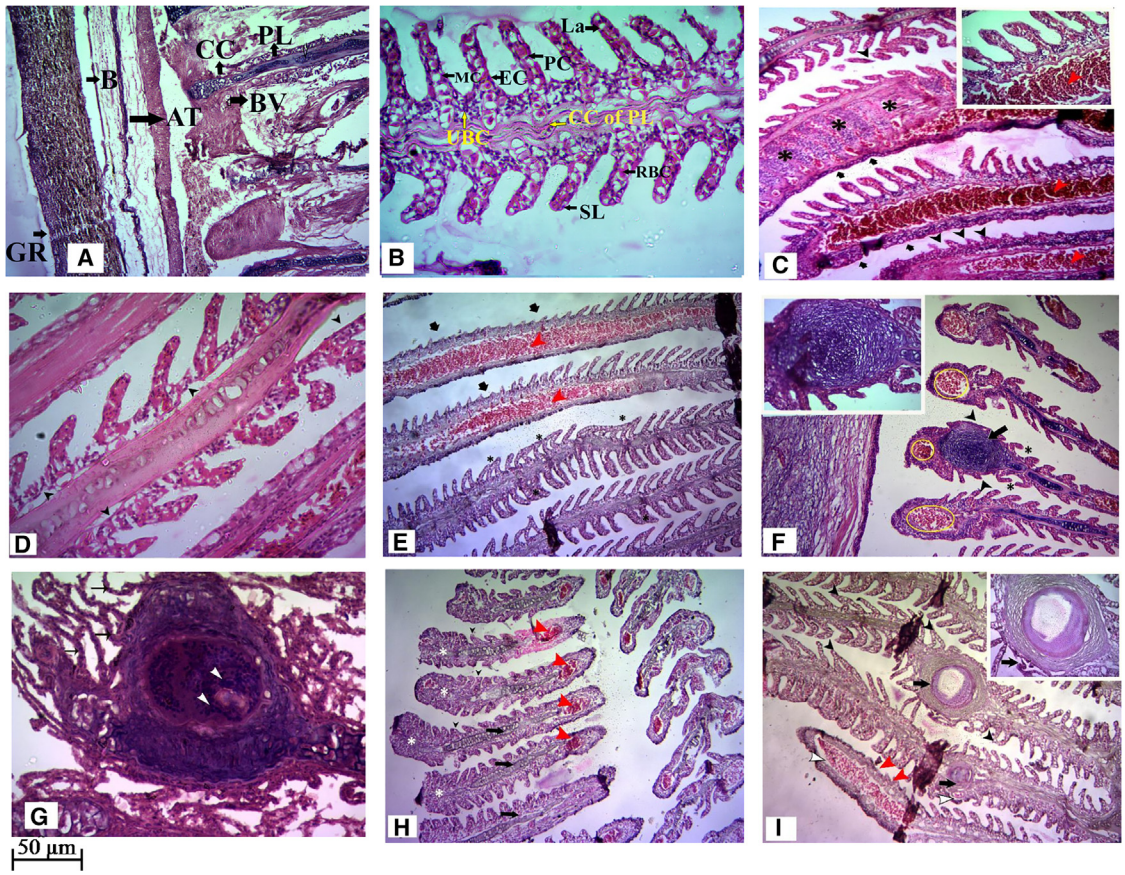

Figure 2. Histopathological photomicrograph of gill from different categories of fish (hematoxylin and eosin staining) under light microscope with 10x magnification (40x in case of inset)

CONT (A and B): (A) different parts of gill from outside: gill rakers (GR), bone (B), adipose tissue (AT), blood vessels (BV), primary lamella (PL) with cartilage core (CC); (B) detailed structure of primary lamella (PL) and secondary lamella (SL), mucous cell (MC), epithelial cell (EC), pillar cell (PC), undifferentiated basal cells (UBC), lacuna (La), red blood corpuscles (RBC). PRNI (C–E): (C) fusion of secondary lamella (*); degeneration of secondary lamella (black arrow head); loss of secondary lamella (black arrow); congestion of blood in primary lamella (red arrow head); (D) epithelial lifting and detachment of secondary lamella from primary lamella (black arrow head); (E) presence of two types of primary lamella, one with blood congestion, another without blood congestion; congestion of blood in primary lamella (red arrow head); degeneration of secondary lamella (black arrow head); fusion of secondary lamella (star). PRI (F and G): (F) hypertrophy in primary lamella (black arrow); 40x magnification of hypertrophy (inset); curling of secondary lamella (star); degeneration of secondary lamella (arrow head); epithelial lifting and blood congestion. (G) Egg of the parasite; disorganized secondary lamella. CI (H and I): (H) fusion of secondary lamella at distal end (white star); degeneration (arrow head) and epithelial lifting (black arrow); blood congestion (red arrow head); (I) hypertrophy caused by parasite (black arrow) with 40x magnification as inset; curling and fusion of secondary lamella (black arrow head); degeneration of secondary lamella (red arrow head); blood congestion (white arrow head). Scale bar, 50 µm.

primary lamellae were composed of two different forms: one with the normal cartilaginous core and the other with a large central venous sinus. In the latter type of gill filament, a large number of erythrocytes was accumulated to form blood congestions, and the secondary lamellae were found to be degenerated either from one side or from both sides of the primary lamella. Abnormal curving and lamellar fusion of the distal end of the secondary lamellae were observed. Their epithelium also showed wrinkling of different degrees. The number of pillar cells decreased, leading to enlargement of the inter-lacunar space (Figures 2C–2E). In polluted river infected (PRI) fish group, different portions of the gills were partially destroyed or damaged. Cellular hypertrophy was observed within the central cartilaginous core of primary lamella and secondary lamella due to the aggregation of adult parasites, their eggs, and other immature stages of their life cycle. The distal ends of the neighboring lamellae were found to be fused in some areas. The infected areas were characterized by aneurism, uplifting of the epithelial layer, curling of secondary lamella, mucous cell proliferation, damaged pillar cells, and migrating eosinophilic granular cells (EGCs) (Figures 2F and 2G). The histopathological alterations were less discernible in the artificially infected fish (CI) (Figures 2H and 2I) compared to the PRI and PRNI fish. A section of the gill filament showed hyperplasia and hypertrophy. Primary lamella remained mostly intact and undamaged except the parasite-laden area. Infiltration of immunological cells and migration of EGCs were also noticed in the parasite-lodged areas. Secondary lamellae near the distal end of primary lamella became

fused, and the rest of the secondary lamellae remained intact and undamaged. Pillar cell, mucosa cell, and epithelial cell occurred in their normal position.

In both groups of fish (PRI and PRNI), blood supply to the gill filament was increased due to accumulation of parasites and hypoxic condition of water, which led to blood congestion in the core of cartilage of primary lamella (marked by black arrow in Figure 2E). In CI fish, the histopathological changes were relatively less pronounced compared to other categories. Parasitic infection inflicted by only a few days' exposure was effective to bring about such changes. Exposure to pollutants is responsible for alterations of gill histopathology.[36] Monogenean "gill" parasites are responsible for the histopathological alteration of gill and secretion of excessive mucous that adversely affect respiration of the host fish.[37] Cell proliferation in gill filament leads to respiratory dysfunction.[38] Parasite infection resulted in diminution of the gill surface area, which reduced gaseous exchange potential. Swelling of secondary lamella was very common due to the accumulation of environmental pollutants and biogenic toxin released by parasites. Aneurism was manifested as a result of dilation of the blood capillaries.[39] Synergetic effects of the multiple stressors represented by the unfavorable physicochemical factors of water and parasitic infections were manifested in diverse histopathological alterations of the fish gill.

Histopathology of liver

In comparison to CONT fish, more intense histopathological changes were observed in the liver and hepatopancreas of PRNI fish. The number of glycogen vacuoles and fat-deposited areas in PRNI fish was less than that of CONT fish. Hepatic cells were shrunken, and no gap was identified between nucleus and plasma membrane (Figure 3D). Yellow ceroid pigments were scattered throughout the liver parenchyma (Figure 3E). Walls of the hepatopancreatic system were separated from liver tissues and became damaged due to the migration of pancreatic cells. Congestion of blood and stagnation of bile were observed in many areas of hepatic parenchyma. Melanomacrophages (MMs) containing dark yellowish granules were scattered within liver tissue. Many melanomacrophage centers (MMCs) of varied shapes were developed as a result of aggregation of melanomacrophage cells near the hepatopancreatic area (Figure 3F).

Several histopathological alterations were observed in PRI fish (Figure 3). Glycogen accumulation and fatty deposition were absent from liver parenchyma, and therefore, several degenerated hepatocytes were observed. Hepatocytes manifests characteristic pathological symptoms such as swelling and necrosis, which paved way to an aggregation of inflammatory cells among them. Due to the partial degeneration and derangement of the hepatic cord, sinusoid became wider, producing a huge gap between the cords of hepatocytes. The irregular-shaped nuclei and nuclear hypertrophy were also found. The structure of hepatopancreas associated with the bile duct, vein, and arterioles was observed to undergo radical disintegration. Pancreatic cells from the hepatopancreas were found to be migrated and scattered within the liver parenchyma as marked with black arrow head in Figure 3H. Bile stagnation and melanomacrophage aggregation were identified as a brownish-yellow or dark yellow area of various shape and size near the bile duct, blood vessels, and hepatopancreatic area. MMCs and MMs were found in several areas of liver parenchyma. The total number and size of MMCs increased much compared to PRNI.

Less pronounced yet significant alterations were encountered in CI fish, characterized by shrunken and swollen types of hepatic cells. Glycogen vacuoles were observed but less in number compared to that of CONT fish. Although the arrangement of hepatopancreas and pancreatic cells was found to be near normal, the hepatopancreas showed early phase degeneration and consequent migration of pancreatic cells via blood vessels along with the red blood corpuscles (RBCs). The appearance of MMC near hepatopancreatic area was also observed (Figures 3K and 3L).

The fish liver is a vital organ and a digestive gland, which controls the metabolism of biomolecules such as carbohydrate, protein, and fat. It acts as a storage center for biomolecules.[40] Other important functions of the liver include synthesis of plasma protein, bile production, and detoxification.[41] The study of histological changes in PRNI fish (Figures 3D–3F) indicates that in polluted water the food sources were very limited along with high energy requirement for overcoming multiple stress situations; therefore, storage of glycogen and fat deposition was less than that of CONT fish (Figures 3A and 3B). The toxic pollutants from water entered into the body of fish, and histopathological alteration is owing to detoxification of pollutants.[42] Aggregation of macrophage and presence of MMCs (marked by yellow circle in Figure 3I) in the fish liver are important biomarkers of degraded water quality. Under environmental stress conditions, the size of MMCs and the number of melanomacrophage increase.[43] The histopathological alterations of PRI fish were intensified due to increased water uptake resulted from alterations in membrane permeability.[44] Being a part of the immune system of fish, MMCs play important roles in phagocytosis, antigen processing and destruction, detoxification, and recycling of endogenous and exogenous materials.[45] Besides, MMCs serve as center for aggregation and reservoir of accumulated metabolites and pigments such as melanin and lipofuscin. The color of pigment within MMCs was yellowish in non-infected fish due to the deposition of lipofuscin pigment. The pigments are derived from cell membrane disintegration and disruption of lipid metabolism.[46] The size and number of MMCs and MMs were greater in PRI fish than that of PRNI fish. MMCs are associated with the defense mechanism of fish. Within MMCs, antigens are being taken up by MMs, and they react with the lymphocyte.[47] In infected fish, the color of MMCs was dark brown due to the deposition of melanin pigment. In fish, the accumulation of melanin pigments is common at the site of tissue injury. In MMC, melanin pigment acts as a scavenger for free radicals.[48] For this reason, the number of MM was found to be much higher in PRI fish than that in PRNI fish. Histopathological alteration in CI fish (which was not exposed to polluted water but infected artificially in the laboratory by ectoparasites for 2 to 4 weeks) showed migration of the pancreocytes through blood vessels (shown by black arrow head in (Figures 3K and 3L), which marked the onset and continuation of disorganization of hepatopancreas. The appearance of yellowish MMC near hepatopancreatic tissue was observed. Thus, the study indicates that the synergetic effects of degraded water quality resulted in the

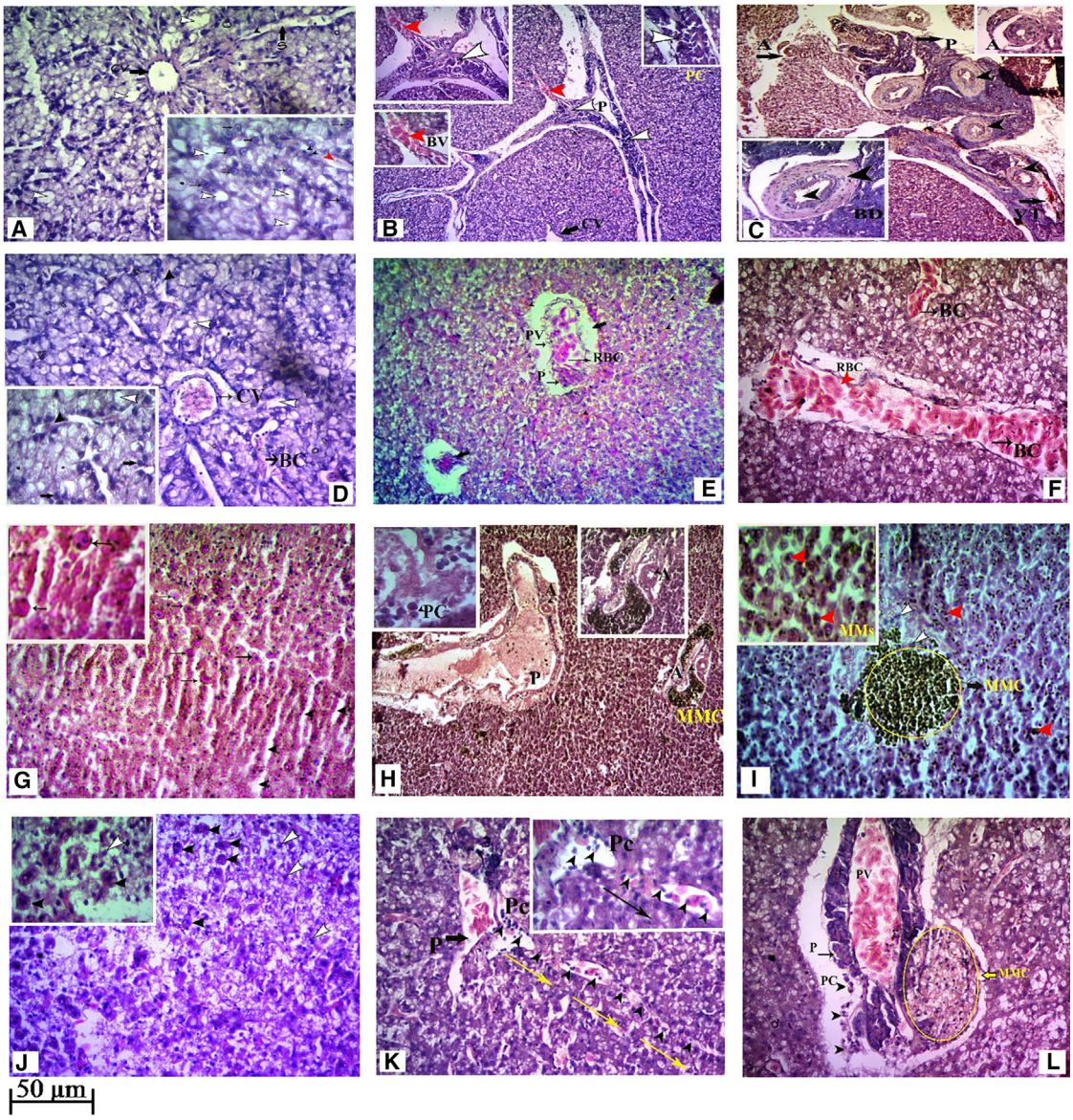

Figure 3. Histopathological photomicrograph of liver from different categories of fish (hematoxylin and eosin staining)

40x magnification (100x in case of inset) under light microscope. CONT (A, B, and C): (A) central vein (CV), sinusoid (S), glycogen vacuoles (white arrow head), Kupffer cells (black arrow head), hepatocytes (black arrow), RBC (red arrow head); (B) blood vessels (BV) red arrow head; pancreatic tissue within liver parenchyma known as hepatopancreas (P) white arrow head; pancreocyte (PC); (C) pancreatic-venous-biliary-arteriolar tract (P-VAT), arteriole (A), biliary duct (BD), pancreatic tissue (P), venous tract (VT). PRNI (D–F): (D) hepatocyte shrinkage (black arrow); blood congestion (BC); (E) disorganized hepatopancreas (black arrow), portal vein (PV), yellow ceroid pigments (black arrow head); (F) blood congestion (BC). PRI (G, H, and I): (G) acute cellular swelling (black arrow), wide space between hepatic cords (black arrow head); (H) scattered pancreocyte, disorganized hepatopancreas, melanomacrophage center (MMC); (I) hepatocyte with scattered melanomacrophage (red arrow head). CI (J–L): (J) hepatocyte hypertrophy (black arrow head), hepatocyte shrinkage (white arrow head); (K) movement of pancreocyte from hepatopancreas through blood vessels; (L) shedding of pancreocyte from hepatopancreas and appearances of melanomacrophage center near hepatopancreas. Scale bar, 50 µm.

deterioration of fish health, and the fish became more susceptible to biotoxins released by parasites. Therefore, the multiple stress situation induces the severe damage of the liver characterized by a suite of pathological symptoms such as hypertrophy, hyperplasia, disorganized hepatopancreas, MMCs, and MMs as revealed by the histopathological study of the liver.

Histopathology of kidney

PRNI fish showed the degeneration of glomerulus and enlargement of Bowman's space, showing some sign of peripheral degeneration. Occlusions and fusion of renal tubules were observed; therefore, lumen space was not defined. Other accompanied alterations observed were characterized by

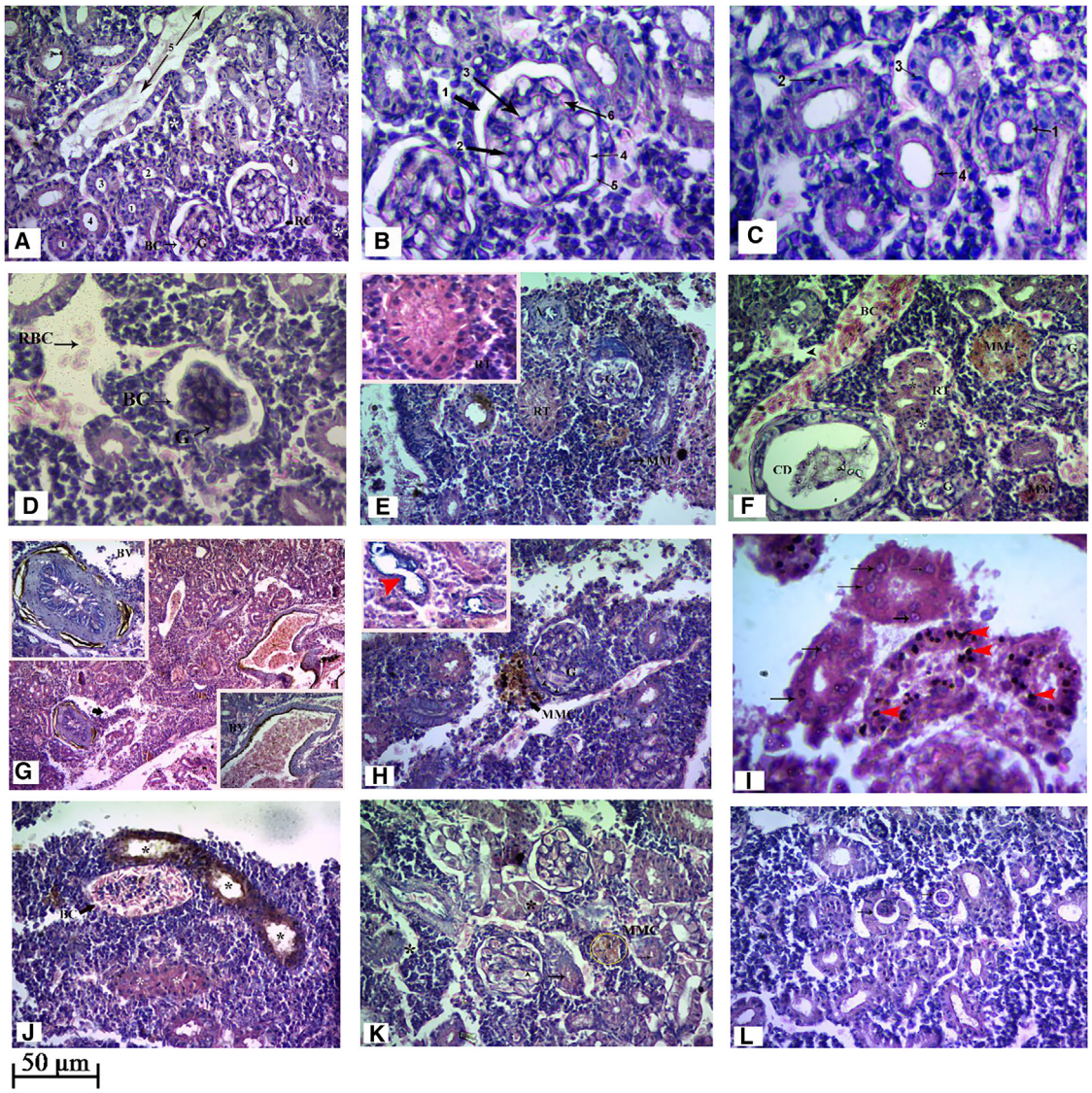

Figure 4. Histopathological photomicrograph of kidney from different categories of fish (hematoxylin and eosin staining)

40x magnification (100x in case of inset) under light microscope. CONT (A–C): (A) renal corpuscles (RC), glomerulus (G), Bowman's capsule (BC). (1) First part of PCT; (2) second part of PCT; (3) intermediate part of tubules; (4) DCT; (5) large collecting duct. Hematopoitic tissue (white star). (B) (1) Bowman's space; (2) endothelial cell; (3) mesangial cell; (4) visceral epithelium of renal capsule; (5) parietal epithelium of the renal capsule; (6) RBC in capillary. (C) (1) Low columnar epithelium; (2) intercalating cell; (3) rodlet cell; (4) brush border. PRNI (D–G): (D) dilation of glomerular capillary; accumulation of RBC. (E) Occlusion of renal tubule. (F) Shrinkage of renal corpuscles (G); fusion of renal tubules (star); melanomacrophage aggregation (MM); blood congestion and rupturing the wall (black arrow head); dilation of blood vessels (black arrow) 40x in inset; rupturing of blood vessels and blood leakage or hypermia (40x in inset). PRI (H–J): (H) dilation of glomerulus (G); melanomacrophage center (MMC) near renal corpuscles (black arrow); necrosis due to parasitic infection (red arrow head). (I) Nuclear hypertrophy and cellular hypertrophy in renal tubules (black arrow) with melanin pigment (red arrow head); (J) cloudy swelling; dilation of tubular lumen (white star); degenerating renal tubules (black star); blood congestions (BC). CI (K and L): (K) dilation of glomerular capillary (star): renal tubules with narrow lumen (black arrow): appearance of melanomacrophage center (yellow circle); (L) deformation of renal tubules architecture (black arrow). Scale bar, 50 μm.

aggregation of MMs, congestion of blood, rupturing of blood vessels, and unusual blood losses (Figures 4D–4G).

In PRI fish, Bowman's space was reduced due to the dilation of the glomerulus. Aggregation of MMs near the glomerulus was observed. Necrosis and hyperplasia were observed within and between renal tubules. Several alterations found inside renal tubules comprise cellular and nuclear hypertrophy, presence of aggregated and scattered MMs, degeneration of epithelial cells, and congestion of blood (Figures 4G–4I).

Histopathological changes were at the initial stage in CI fish. In this group of fish, two types of renal tubules were observed, one with completely blocked, very few, and the other with a partial block with a very narrow lumen. Negligible changes were found within the glomerulus except for some wide space in glomerular

capillaries. Few MMCs were observed between renal tubules (Figures 4J–4L).

Histopathological alterations in PRNI fish were due to degraded water quality. The pollutants of water caused damage to the kidney and its internal parts. Hyperemia (too much blood accumulation) is usually accompanied by inflammation due to localized release of inflammatory mediators.[49] Aggregation of MMs or presence of MMCs in kidney of fish is a good indicator of general stress that resulted from multiple factors occurring in a system and that cannot be attributed to a particular factor.[50] Like liver, the number, size, and pigmentation in MMCs depend on the abundance of parasite and amounts of pollutants in water. Most common histopathological alterations in fish kidney are caused by water pollution.[7] Renal necrosis caused by some microbial activities and metabolic disorders resulted in inflammatory response in active tissue around the necrotic area (marked with * in Figure 4F).[51] In PRI fish, the necrosis categorized as liquefactive necrosis resulted in complete disintegration of the renal tissue culminated into a liquid of varying consistency. The liquefaction was caused by the toxic substances released by pathogen.[52] Normal histological features were not discernible at the neighboring parts of the necrotic area of the kidney as characterized by glomerular destruction, hemorrhagic, and leucocytic infiltration. Like liver, accumulation of melanin pigment in kidney is common at the site of tissue injury. Some chronic inflammatory areas were observed in histological study of kidney in PRI fish. Inflammation is an early warning signal that triggers the protective process that helps in healing and repairing of the damaged tissues.[53] It involves macrophages that provide phagocytic and killing activity and the other cell types such as lymphocyte and plasma cells in such immune response. The kidney of the fish can be affected by the co-occurring multiple stressors such as water pollutants and biotoxins released by ecto- or/and endoparasites and transmitted via blood.[54]

Therefore, the study indicates that lack of oxygen and presence of pollutants in water are weakening the overall body's defense in fish, making it more vulnerable to be affected by the parasitic infestation. The ailing and weak fish with intense histopathological alterations amplifies the chance of parasitic infections that intensify further damages and aggravate histological damage and endanger immune defense under multiple stress situation. Hence, a vicious cycle continues to affect gradually deteriorating fish health culminating in precipitous fallouts. Thus, the qualitative and quantitative matric of the histopathological damage inflicted by the ectoparasite and endoparasite harboring fish gill and other organs can be presented as potential stress biomarkers of fish health.

Serum biochemical profile

The descriptive statistics of serum biochemical parameters of the four groups of fish (CONT, CI, PRNI, and PRI) are presented in Table 3.

Total protein, albumin, and globulin

Serum biochemical parameters of fish are considered as potential biochemical monitoring tools for detecting the adverse effects of environmental pollution and parasitic infection.[55] The mean concentration of TP was the highest (1.65 ± 0.1 gdL^{-1}) in CONT group, which showed significant difference from both the infected groups as revealed by KWANOVA (between CONT and CI, $p < 0.05$; between CONT and PRI, $p < 0.001$). The concentration of TP showed significant reduction among the other three groups tested (PRI = 60%, CI = 50.30%, PRNI = 37.76%) compared to CONT due to negative impact of pollution and/or parasitic infection acting discretely or synergistically.[56] The concentration of TP in PRNI fish was 120.45% higher than that in PRI group, which indicated that the protein concentration was more affected by the long-term energetic cost of parasitic infestation. Since parasite utilized the host's nutrition and altered metabolism,[57] protein concentration became lower in both the infected fish groups (CI and PRI). Deamination activity by aminotransferase enzyme is reduced due to histopathological alterations.[58] Subject to environmental stressors of pollution and/or parasitic infection, the concentration of albumin showed a declining pattern in the following order: PRI (77.91%) > CI (61.63%) > PRNI (41.86%) (LSD test; $p < 0.001$). Significant differences in mean globulin concentration was discernible among the tested fish groups in the following order of variation: CONT (0.78 ± 0.1 gdL^{-1}) > CI (0.49 ± 0.01 gdL^{-1}) > PRNI (0.56 ± 0.07 gdL^{-1}) > PRI (0.47 ± 0.05 gdL^{-1}) as revealed by one-way ANOVA ($F_{3,\ 16} = 3.940$, $p < 0.05$) followed by LSD test, $p < 0.05$. The variation in mean Alb:Glo ratio was also satisfied by one-way ANOVA ($F_{3,\ 16} = 3.745$, $p < 0.05$). Both the infected groups (CI = 0.66 ± 0.05; PRI = 0.44 ± 0.10) exhibited significant difference (LSD, $p < 0.05$ and 0.01, respectively) in Alb:Glo ratio from that of CONT (1.23 ± 0.32). Globulin is produced by many parts of the body, whereas albumin is produced by the liver.[59] The normal value of Alb:Glo remains between 0.8 and 2.0, whereas the change in this ratio serves as an important index for tracking the variations of serum protein.[60] In this study, the Alb:Glo value of CONT stood >1 (1.27 ± 0.32), which indicated that the concentration of albumin was greater than that of globulin. In the case of PRNI fish, the value was almost =1 (0.97 ± 0.15). On the other hand, the value was <1 for both the infected groups (CI and PRI), which showed that the amount of globulin was greater than albumin due to a strong immune response elicited against parasitic infection. The toxic substances produced by the parasite induce the production of globulin protein because γ globulin is one of the most important components of immunoglobulin.[61] In PRI group, the Alb:Glo value fell below 0.5 and in CI group the value remained between 0.5 and 1, which indicated a worse pathological situation in PRI and onset of parasitic infection in CI groups. Albumin globulin ratio is an important manifestation for serum or plasma protein.[62] Histopathological alterations in the internal organs are indicated by lower concentration of albumin. Serum albumin plays an important role in energy metabolism and maintenance of colloid osmotic pressure of blood.[63] In polluted water, the xenobiotic substances accumulate in the liver and other internal organs and cause toxicity in those target organs. Fish blood utilized more albumin to meet the energy demand for the removal of xenobiotic substances or its transformed product from the body. The liver acts as the key metabolic center for xenobiotic transformation and albumin production.[64] The concentration of serum albumin in PRI fish was found to be the lowest due to the combined effect of the

Table 3. Mean (±SE) minimum (min) and maximum (max) values of serum biochemical parameters of four groups of fish studied

Biochemical parameters of blood		Fish group			
		CONT	CI	PRNI	PRI
TP (gdL^{-1})	Mean ± SE	1.65 ± 0.1	0.82 ± 0.03	1.06 ± 0.07	0.66 ± 0.03
	Min	1.30	0.74	0.85	0.55
	Max	1.90	0.93	1.26	0.75
Albumin (gdL^{-1})	Mean ± SE	0.86 ± 0.06	0.33 ± 0.02	0.50 ± 0.02	0.19 ± 0.03
	Min	0.64	0.29	0.43	0.1
	Max	1.00	0.43	0.57	0.29
Globulin (gdL^{-1})	Mean ± SE	0.78 ± 0.1	0.49 ± 0.01	0.56 ± 0.07	0.47 ± 0.05
	Min	0.37	0.45	0.35	0.39
	Max	1.00	0.55	0.75	0.66
Alb:Glo	Mean ± SE	1.23 ± 0.32	0.66 ± 0.05	0.97 ± 0.15	0.44 ± 0.10
	Min	0.74	0.55	0.68	0.5
	Max	2.51	0.86	1.43	0.74
Cholesterol ($mgdL^{-1}$)	Mean ± SE	126.8 ± 14	28.5 ± 1.07	53 ± 7.4	32.2 ± 1.4
	Min	77	26	34	28
	Max	159	32	72	36
Triglyceride ($mgdL^{-1}$)	Mean ± SE	36 ± 1	16.2 ± 0.86	18.4 ± 2.29	14.6 ± 1.02
	Min	33	17	12	11
	Max	39	19	25	17
HDL ($mgdL^{-1}$)	Mean ± SE	52.9 ± 4.3	17.2 ± 1.65	40.4 ± 5.14	15.4 ± 1.50
	Min	40	13	22	10
	Max	67	22	52	19
LDL ($mgdL^{-1}$)	(Mean ± SE)	57.8 ± 8.6	9.2 ± 0.86	17.4 ± 2.18	16.36 ± 1.19
	Min	31	7	10	13
	Max	84	12	23	19
VLDL ($mgdL^{-1}$)	Mean ± SE	6.96 ± 0.32	3.4 ± 0.43	3.70 ± 0.53	2.94 ± 0.30
	Min	6	2.5	2	2.2
	Max	7.8	5	5	4
GLF ($mgdL^{-1}$)	Mean ± SE	6 ± 0.2	4.9 ± 0.33	6.4 ± 0.43	5.0 ± 0.31
	Min	5.6	4	5	4
	Max	6.7	6	7.5	6
GLPP ($mgdL^{-1}$)	Mean ± SE	21.36 ± 0.52	11.20 ± 0.86	20.18 ± 0.66	19.20 ± 0.86
	Min	19.9	9	18	17
	Max	23	14	22	22

damage of the liver caused by the parasite infection and the decline in body's defense in the face of xenobiotic assault.[65]

Lipid

Among five lipid profile parameters, the mean concentration of cholesterol and low-density lipoprotein (LDL) were statistically significant by Levene's test (cholesterol: $F_{3,\ 16} = 4.849$, $p < 0.02$; LDL: $F_{3,\ 16} = 3.891$, $p < 0.05$). The mean concentration of cholesterol was much higher (126.8 ± 14 $mgdL^{-1}$) in CONT and differed significantly from both the infected groups (PRI = 32.2 ± 1.4 $mgdL^{-1}$; CI = 28.6 ± 1.07) by KWANOVA (between CONT and CI, $p < 0.001$; between CONT and PRI, $p < 0.05$). The mean concentration of LDL was highest (57.8 ± 8.6 $mgdL^{-1}$) in CONT, which differed significantly from CI (9.2 ± 0.86 $mgdL^{-1}$) by KWANOVA ($p < 0.001$). The mean triglyceride concentration in CONT (36 ± 1 $mgdL^{-1}$) differed significantly (ANOVA, $F_{3,\ 16} = 48.86$; $p > 0.001$; LSD, $p < 0.001$) from other three groups (PRNI = 18.4 ± 2.29 $mgdL^{-1}$; CI = 16.2 ± 0.86 $mgdL^{-1}$; PRI = 14.6 ± 1.02 $mgdL^{-1}$) and was satisfied by Levene's test ($F_{3,\ 16} = 3.108$; $p = 0.056$). Mean HDL concentration of CONT (52.9 ± 4.3 $mgdL^{-1}$) and PRNI (40.4 ± 5.14 $mgdL^{-1}$) exhibited significant difference from both the infected groups (CI = 17.2 ± 1.65 $mgdL^{-1}$; PRI = 15.4 ± 1.50 $mgdL^{-1}$) by LSD test, $p < 0.001$, whereas no significant difference was observed between CI and PRI group by LSD test, $p = 0.725$. The mean concentration of very low-density lipoprotein (VLDL) among different fish groups exhibited distinct pattern from HDL. Cholesterol is LDL-rich lipid while triglyceride contains more VLDL.[66] Therefore, LDL and VLDL followed characteristic pattern similar to cholesterol and triglyceride. On the other hand, HDL

Table 4. Mean (±SE) minimum (min) and maximum (max) values of stress and metabolic enzyme of four groups of fish studied

Enzymes		Fish Group			
		CONT	CI	PRNI	PRI
CAT (KUL^{-1})	(Mean ± SE)	13.42 ± 1.61	29.18 ± 2.04	18.82 ± 1.18	42.80 ± 1.49
	Min	9.5	24.5	15.8	38.6
	Max	18.5	35.3	22.3	46.6
LDH (UL^{-1})	(Mean ± SE)	453.6 ± 39.09	966.4 ± 15.73	416.6 ± 25.30	654.2 ± 20.69
	Min	345	916	364	613
	Max	578	998	501	705
SGOT (UL^{-1})	(Mean ± SE)	49.40 ± 5.6	74.6 ± 2.03	201.4 ± 3.77	273.4 ± 3.32
	Min	35	70	195	216
	Max	69	82	265	285
SGPT (UL^{-1})	(Mean ± SE)	10.4 ± 0.36	16.8 ± 0.86	13.4 ± 0.92	17.2 ± 0.86
	Min	9.5	14	11	15
	Max	11.5	19	16	20

concentration exhibited different pattern from that of other lipids. HDL is considered as good cholesterol, because it plays an important role in transport of reserved cholesterol.[67] Besides, it has some important defensive and antioxidative properties such as anti-inflammatory, anti-oxidant, anti-thrombotic, and anti-apoptotic, etc.[68] Higher amount of HDL indicates sound health status in CONT. In contrast lower HDL values in other polluted and/or infected groups (CI and PRI) reflect inferior health status. Fish need more energy to overcome the stress related to water pollution and parasite infection. Constant energy demand is responsible for mobilization of lipid.[69] In both the infected fish, parasites used host's lipid to satisfy for their energy demand source. On the other hand, poor water quality is responsible for stressful physiological condition affecting in the fish health. Depending on the duration, intensity and co-occurrence of stressors, fish adopt different adaptive metabolic strategies and energy allocation patterns.[70] Both triglyceride and VLDL are related to endogenous lipoprotein pathway, therefore monitoring of serum triglyceride and VLDL concentration is a useful tool for diagnosis of liver dysfunction.[71] Damage in the liver cell, bile duct, hepatopancreatic system, kidney and gill declines triglyceride concentration, because membrane biogenesis requires triglyceride. In immune system of the parasite-infected fish requires more VLDL to meet its increasing demand for eliciting body's defense against emergent assault of parasitic/pathogenic infections.[72]

The concentration of serum glucose was measured in two different conditions, postprandial (SGPP) and fasting (SGF). In both the conditions significant variation was observed among four groups of fish by one-way ANOVA ($F_{3,16} = 5.0$; $p < 0.01$ for fasting and $F_{3,16} = 38.60$ $p > 0.001$ for consuming). The mean concentration of SGPP was lower (11.2 ± 0.86 $mgdL^{-1}$) in CI and differed significantly (LSD test; $p < 0.001$) from other three groups (CONT = 21.36 ± 0.52 $mgdL^{-1}$; PRNI = 20.18 ± 0.66 $mgdL^{-1}$; PRI = 19.2 ± 0.86 $mgdL^{-1}$). While the mean SGF of both the infected (CI = 4.9 ± 0.33; PRI = 5.0 ± 0.31) groups exhibited significant difference (LSD test; $p < 0.05$) from non-infected group. Serum glucose concentration has been widely used as stress biomarkers.[73] The mean concentration of serum glucose was lower in CI group, while it was higher (70%–80%) in both the groups of polluted river fish (PRNI and PRI) compared to CI group. In infected fish, glucose is used as a preferred energy source for both host and parasite. Liver histopathology showed that glycogen accumulation was totally absent in hepatic cells in PRI group. This might happen due to continuous exploitation of glucose by the parasite from the fish host leaving a little scope for storage of carbohydrate (glucose) in the liver as glycogen. Glucose concentration in serum is maintained by glycogen breakdown from liver parenchymal cells also by high glucose turnover due to parasitic infestation.[74] On the other hand, serum glucose concentration was reduced in CI group. The reduction might be resulted for the utilization of serum glucose under contained laboratory condition constrained with food resource. Although concentration of glucose showed comparatively less variations among the groups tested compared to other serum biochemical parameters.

Stress and metabolic enzymes

The descriptive statistic of stress and metabolic enzyme from four groups of fish (CONT, CI, PRNI, and PRI) are presented in Table 4.

The mean concentration of CAT, LDH, SGOT, and SGPT in blood of host fish (*Channa punctatus*) are presented in Table 4. The assumption of homogeneity of variances was confirmed by Levene's test for CAT ($F_{3,16} = 1.153$; $p = 0.358$), LDH ($F_{3,16} = 1.153$; $p = 0.358$), SGOT ($F_{3,16} = 0.961$; $p = 0.435$), and SGPT ($F_{3,16} = 1.227$; $p = 0.332$); therefore, the null hypothesis was accepted for equality of variances for all the four enzyme concentrations.

Living organisms are affected by stress, and they respond to stress that is reflected at different hierarchical levels such as individual organism to systemic levels, cellular, molecular, and biochemical levels. Both pollution in aquatic environment and parasitic infection are responsible for stress in aquatic organisms. Both the situations, i.e., pollution and parasitic infection, can cause oxidative stress in aquatic animals including fish by catalyzing the formation of ROS like hydrogen peroxide (H_2O_2), hydroxyl radicle (OH^-), superoxide anion (O_2^-), etc.[75] Survival

of aquatic organisms is reduced by multiple stress situation such as parasitic infestation in polluted conditions. Antioxidant defense enzymes have been established as stress biomarkers of water pollution and parasitic infection.[18]

The mean concentration of CAT differed significantly among four groups of fish with the following order of variation: PRI (42.8 ± 1.49 KUL^{-1}) > CI (29.18 ± 2.04 KUL^{-1}) > PRNI (18.82 ± 1.18 KUL^{-1}) > CONT (13.42 ± 1.61 KUL^{-1}) as validated by one-way ANOVA ($F_{3, 16} = 63.95$; $p > 0.001$), followed by LSD test, $p < 0.001$. CAT is considered as an antioxidant enzyme. It is involved in the degradation of H_2O_2 into H_2O and O_2. It protects the cell from pollutants by neutralizing the toxic effect of ROS.[76] The mean concentration of CAT was raised in all the three groups of fish compared to CONT. CAT activity was much higher in both the parasite infected fish, which indicated high peroxidase concentration in both infected fish. In PRNI and CI group, only a single stressor was responsible for oxidative stress but in PRI group multiple stress situation such as water pollution and parasitic infestation caused synergistic effect, resulting in amplified oxidative stress. Host immune system employs macrophages at the site of infection for elimination of parasites by producing ROS.[77] So increased CAT activity reduces the toxic effect of H_2O_2 in stress situation.

The mean LDH concentration was highest (966.4 ± 15.7 UL^{-1}) in CI group and exhibited significant difference (ANOVA, $F_{3, 16} = 89.14$, $p > 0.001$) from other three groups (PRI = 654.2 ± 20.69 UL^{-1}; CI = 453.6 ± 39.09 UL^{-1}; PRNI = 416.6 ± 25.3 UL^{-1}). LDH is a tetrameric molecule available in the five isoenzyme forms in fish and acts as a terminal enzyme for anaerobic glycolysis.[78] Activity of this enzyme has been recognized as a potential marker for oxidative stress and histopathological alterations.[79] In the present study, LDH activity was elevated in both the infected groups (CI = 113.05% and PRI = 44.22%) compared to CONT. Concentration of glycogen and LDH activity became high in infected group as LDH is related to carbohydrate metabolism. In CI group, the maximum elevation of LDH activity was due to sudden parasite infection. Within the host body gill, liver and kidney were damaged by parasitic infection, and anaerobic glycolysis was the source of energy for parasites. Therefore, LDH activity was found to be more in both the infected (CI and PRI) groups. In PR-2 of polluted river, the concentration of DO was very low (1.84 ± 0.34 mgL^{-1}). On the other hand, gill suffocation occurred due to parasite infection (*Gyrodactylus* sp. and *Trichodina* sp. both are gill parasites). Both situations were responsible for hypoxic condition. LDH converts lactate to pyruvate in the presence of NADH. In the presence of optimum amount of oxygen, pyruvate enters in tricarboxylic acid (TCA) cycle, but under anaerobic condition pyruvate is converted to lactate. The elevation of serum LDH activity indicates that toxicity is produced through persistent anaerobic or oxygen-starved conditions. LDH is present in the cells of different body tissue, and as a result of some damage, it is released from cells to the blood.[80] Consequently, the concentration of LDH was much higher in the infected fish than in CONT and PRNI group.

The mean concentration of SGOT followed a pattern different from other three enzymes tested. In both the groups of polluted river (PRNI and PRI), the mean SGOT concentration was much higher (PRNI = 201.4 ± 3.77 UL^{-1}; PRI = 273.4 ± 3.33 UL^{-1}) and differed significantly from CI (74.6 ± 2.03 UL^{-1}) and CONT (49.4 ± 5.6 UL^{-1}) groups as revealed by one-way ANOVA ($F_{3, 16} = 734.37$, $p > 0.001$) followed by LSD test, $p < 0.001$.

The mean SGPT concentrations in all the four groups of fish were much lower than that of SGOT. It was higher in both the infected groups (PRI = 17.2 ± 0.86 UL^{-1}; CI = 16.8 ± 0.86 UL^{-1}) and differed significantly from CONT (10.4 ± 0.36 UL^{-1}) and PRNI (13.4 ± 0.42 UL^{-1}) groups as validated by one-way ANOVA ($F_{3, 16} = 16.44$, $p > 0.05$), whereas the difference was not significant between the infected groups (CI and PRI) as verified by LSD test, $p = 0.724$.

SGOT and SGPT are two important enzymes associated with metabolism and energy production. They also play an important defensive role against tissue damages and stress.[81] Different internal organs such as liver, kidney, and muscle contain SGOT and SGPT. They are released from the cell to blood during tissue injury or organ dysfunction.[82] Alanine is converted into pyruvate by SGPT, and aspartate is converted into oxaloacetate by SGOT. In all of the tested groups, the mean concentration of SGOT was much higher than that of SGPT, indicating formation of more oxaloacetate than pyruvate by protein metabolism. Prominent histopathological alterations were observed in the gill, liver, and kidney of both the PRI and PRNI fish. The elevation of SGOT level might be due to the histopathological alterations.[83] Conversely, the elevation of SGOT level was significantly higher in fish from all conditions—polluted non-infected (PRNI), infected (PRI), and polluted-infected compared to CONT. However, while comparing with the reference system, the levels of significance in SGOT concentration were much higher in PRNI (307%) and PRI (453.4%) (ANOVA; LSD test; $p < 0.001$) than that of CI (51%) ($p = 0.002$). PRI fish experienced chronic co-exposure of pollutants and parasite infection for a long period of time but controlled infected fish were artificially infected in laboratory for 20 to 30 days. The histological alterations resulted from artificial and sudden exposure to parasitic infection over a short term, which elevated the concentration of SGOT and SGPT level slightly. Organic loading and parasitic infection synergistically induced severe stress on fish in general and liver in particular as the principal target, which was reflected in elevated concentrations of SGOT and SGPT in blood. These two enzymes help the host fish to overcome stress resulting from cellular and tissue damage in fish subject to multiple stress situations such as aquatic pollution and parasitic infestation.[84] Fish exposed to pollutants showed the elevated activity of SGOT and SGPT in blood, which can be presented as sensitive indicators for cellular and tissue damages as reported by several studies using different fish, such as Tilak et al.[85] in *Channa punctatus*, Saravanan et al.[86] in *Labeo rohita*, and Al-Ghanim et al.[87] in zebrafish (*Danio rerio*). Different life-cycle stages such as egg, larva, adult, and cyst of ecto- and endoparasite and their toxic substances are responsible for damages of internal organs such as the liver, kidney, and intestine. Elevated level of SGOT and SGPT in parasitized fish indicates malfunction and extensive damages of liver. These types of study have been reported in fish by several researchers demonstrating damages of liver by *Eustrongylides* sp. in *Channa punctatus*,[87] in *Clarias gariepinus* infected by *Trichodina* sp.[88] in marine fish *Trachinotus ovatus* infected by *Cryptocaryon irritans*.[89]

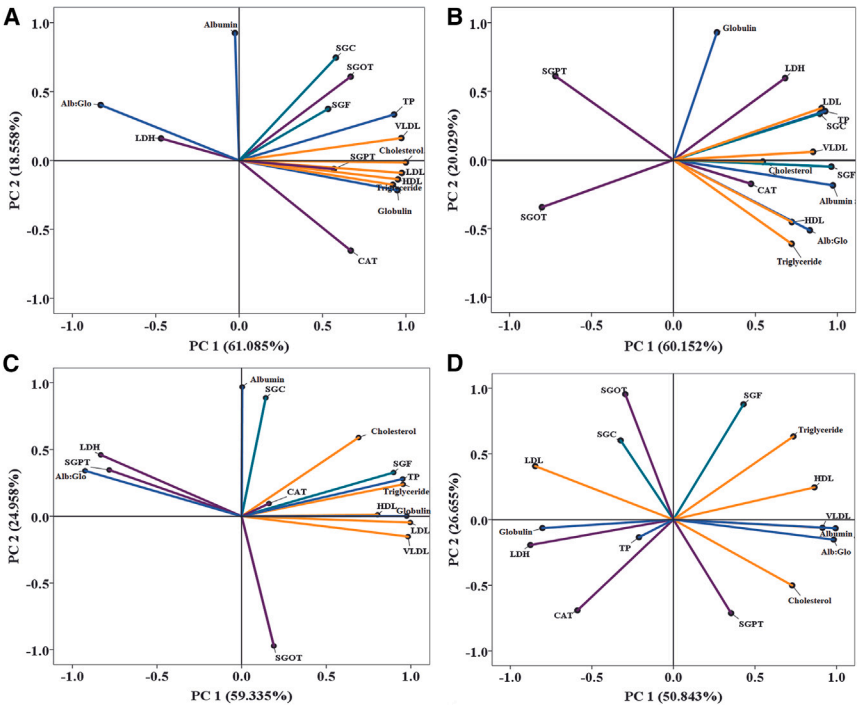

Figure 5. Principal-component analysis (PCA) of serum biochemical parameters, stress, and metabolic enzyme of fish

Two principal components (PC 1 and PC 2) explained 79.64% for CONT, 80.18% for CI, 84.29% for PRNI, and 75.80% for PRI of the total variation between serum biochemical parameters, stress, and metabolic enzyme. Alterations of biochemical parameters and enzyme are related to parasitic infestation and water pollution. (A) CONT; (B) CI; (C) PRNI, and (D) PRI.

Principal-component analysis of serum biochemical profile

To understand the overall serum biochemical alteration, data were subjected to ordination by principal-component analysis (PCA). The PCA bi-plot (Figure 5) explains the percentage of variation among concentration of protein, lipid profile, and activity of stress and metabolic enzymes in four groups of fish. The PCs were obtained from the comparison based on Eigen values. In CONT group, two PCs explained 79.64% (PC-1 = 61.085% and PC-2 = 18.59%) of the total variation. Concentration of TP and activity of SGOT were positively associated with both the PCs but negatively associated with globulin concentration (PC-2). All the parameters of lipid profile and CAT activity were positively associated with PC-1 except VLDL (PC-1 and PC-2). Concentration of glucose was positively associated with both the PCs but negatively associated with LDH activity (PC-1). In CI group, 80.24% of the total variation was exhibited by first two PCs (PC-1 = 60.15% and PC-2 = 20.09%). Concentrations of TP and globulin were positively related to both the PCs but negatively related to SGOT (PC-1; PC-2) and SGPT (PC-1) activity. The alteration in activity of both enzymes was caused by parasitic infection. The pattern of lipid profile was similar to CONT except the concentration of LDL (PC-1; PC-2). Concentrations of glucose and LDH activity were positively associated with both the PCs. Parasitic infestation and starvation under laboratory conditions altered the normal relationship between glucose concentration and LDH activity. In PRNI group PC-1 = 59.335% and PC-2 = 24.958%, total 84.293% variation was exhibited by both the PCs. The relationship between protein concentration and metabolic enzyme was similar to CI group. The concentration of most of the lipid profile parameters (cholesterol, triglyceride, and HDL) and CAT activity were positively associated with both the PCs. This alteration was due to lack of sufficient nutrition in polluted water. In PRI group, two PCs explained 77.498% (PC-1 = 50.843% and PC-2 = 26.655%) of the total variation. Remarkable variations were observed in this fish group. The activity of SGOT and SGPT was positively associated with PC-2 and PC-1, respectively, whereas the concentration of TP and globulin was negatively associated with them. The concentrations of cholesterol and VLDL were positively related to PC-1, whereas the concentrations of triglyceride, LDL, and HDL were positively related to PC-2. The activity of CAT was negatively associated (PC-1; PC-2) with them. Activity of CAT is indirectly related to lipid profile because H_2O_2 enhances lipogenesis and accumulation of lipid.[90] In PRI group, relation between CAT concentration and lipid profile was found to be different from that of CONT group due to high CAT build up under multiple stress situation. Overall, the PCA bi-plot indicated that the glucose concentration and LDH activity were related to parasitic infestation and starvation. In CI, activity of LDH was highest for onset of histopathological alterations due to parasitic infection. Intensity of CAT, SGOT, and SGPT activity of fish was implicated for the physiological adaptation to cope with the pollution of the ambient water.[91,92] The altered relationship between concentration of protein and SGOT activity as well as lipid profile and CAT activity in PRI were due to the combined adverse effects of multiple stressors, i.e., water pollution and parasitic infection. The PCA outputs have significant connotations for the biochemical profile of fish blood as conditioned by the physicochemical attributes of their aquatic habitat, parasitic insult inflicted upon them, and their synergistic effects.

Conclusions

The results show that the river Saraswati in under severe stress of environmental quality degradation due to organic loading in particular, which is reflected from the physicochemical regimes prevailing at both the spots studies (PR-1 and PR-2). The parasitic load was found to be a function of a suite of physicochemical factors comprising temperature, EC, free CO_2, BOD, COD, and DO. The parasitic load increased under degraded water

quality and low temperature regime. The parasitic loads of the ciliate *Trichodina* sp. and the monogenean *Gyrodactylus* sp. were directly influenced by the physicochemical stressors operating in the ambient system. Contrarily, the population of endoparasite *Eustrongylides* sp. was unaffected by ambient water quality for two reasons: (1) they are comparatively less exposed to environmental stressors; (2) their life cycle involves two hosts: larval stages thrive on fish, whereas adults rely on wading birds. Fish gills, liver, and kidneys act as exposure windows, metabolic transformers, and biological filters for pollutants, respectively. The fish in the polluted aquatic environment faces the spiraling double trouble in a vicious cycle: (1) The health of the fish becomes weak in the polluted environment while they require more energy to cope with the polluted environment (ii) infected fish's health gets further compromised to satisfy the nutrients and energy demand of the parasites. The concentrations of biomolecules (protein, lipid, and glucose) were found to be decreased in multiple stress situation, whereas activities of stress and metabolic enzymes (CAT, LDH, SGOT) amplified owing to combined assault of parasitic infection and water pollution, which can serve as biochemical stress markers. The histopathological alterations and variation in serum biochemical profile in response to degrading water qualities at PR-2 reflect deteriorated fish health. Being stricken with multiple stressors (e.g., deficiency of dissolved oxygen, increase in free CO_2, and BOD), the fish health deteriorates and becomes vulnerable to parasitic infection. Nonetheless, alterations in histopathological and serum biochemical parameters not only depend on a single factor; rather, it is the outcome of the tripartite interactions among fish, parasite, and pollution, which entail mostly counteracting and antagonistic trade-offs. Since *Channa punctatus*, the selected fish considered as an air-breathing "hard" fish, has high stress tolerance capacity, the multiple stressors functioning in the heavily polluted river Saraswati might elicit more intense stress to "soft" organisms, resulting in poorer health and greater vulnerability to parasite infection, which warrants further investigation.

Limitations of the study

The present study selected *Channa punctatus*, which is considered as an air-breathing "hard" fish, it is speculated that multiple stressors functioning in the heavily polluted river Saraswati might elicit more intense stress to "soft" organisms, resulting in poorer health and greater vulnerability to parasite infection, which warrants further investigation. Further assessments with wide diversity of parasites and fish hosts are required to establish the use of fish parasites as flagship bioindicator superior to other methods while coupled with hematological, histopathological, and serum biochemical parameters for the determination of fish health under multiple stress situations. Several other factors such as seasonal variations, size, genetic attributes, sex, population density, and lack of food supply need to be considered to earn a better insight into the pollution and related stressor-induced hematological alterations in fish as an indicator of fish health. Omics approaches can be adopted to offer comprehensive mechanistic insights into the tripartite interactions among pollutants, hosts, and parasites and their implications for aquatic ecosystem health.

RESOURCE AVAILABILITY

Lead contact

Further information on resources can be directed to the lead contact, Prof. J.K. Biswas (jkbiswas@klyuniv.ac.in).

Materials availability

This study did not generate any unique reagents. Although information on and request for materials may be made to the lead contact person.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- No original code was generated for this study.
- Additional information on the data reported in this paper and their analysis is available from the lead contact on request.

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AUTHOR CONTRIBUTIONS

S.P.: formal analysis, data curation, methodology, investigation, validation, writing—original draft; J.K.B.: conceptualization, resources, methodology, investigation, validation, supervision, writing—original draft, review & editing.

DECLARATION OF INTERESTS

The authors declare that J.K.B. is an Associate Editor of iScience but was not involved in the editorial handling of this article.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Fish (*Channa punctatus*)	Bharti and Rasool,[13]	https://doi.org/10.1016/j.toxrep.2021.02.018
Ectoparasite (*Trichodina* sp.)	Basson and As[93]	https://doi.org/10.1007/BF00015224
Gill Parasite (*Gyrodactylus* sp.)	Bakke et al.[94]	https://doi.org/10.1016/S0020-7519(01)00331-9
Endoparasite (*Eustrongylides* sp.)	Moravec et al.[95]	https://doi.org/10.1186/1756-3305-2-42
Chemicals, peptides, and recombinant proteins		
Fixative formaldehyde solution (10%) Graded alcohols (30%, 50%, 70%, 90% & 100%) Xylene Paraffin DPX	Sonia et al.[96]	https://www.scribd.com/document/226102834/Fish-Histology-Manual-v4
Sodium, potassium phosphate buffer Hydrogen peroxide Ammonium molybdate	Hadwan and Hussein,[97]	https://doi.org/10.1016/j.dib.2015.12.012
Software and algorithms		
SPSS 20	IBM	https://www.ibm.com/products/spss-statistics
Origin Pro 21	OriginLab Corporation	https://www.originlab.com/origin
Other		
Analysis of water quality parameters	APHA[98]	https://www.apha.org

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Fish (*Channa punctatus*)

The fish were collected from river Saraswati (PR-2) by the local fishermen with the help of fishing gears (bamboo fish cage and fish net). Sex and age were not determined for host fish. The collected fishes were grouped under four categories, such as 1) Control group (CONT) i.e. collected from the reference pond, 2) Control infected (CI) group i.e. normal fishes were infected artificially in the laboratory, 3) polluted river noninfected (PRNI) group i.e. polluted river fish without parasitic infection and 4) Polluted river infected fish group (PRI) with parasitic infection.

Fish parasites (*Trichodina* sp., *Gyrodactylus* sp. and *Eustrongylides* sp.)

Host fish were investigated for protozoan and metazoan parasites. *Trichodina* sp. and *Gyrodactylus* sp. representing protozoan and monogenean ectoparasites respectively were collected from the body surface mucous and gill of fish. *Eustrongylides* sp. (nematode) is endoparasite collected from dissected host fish. Year wise host and parasite counts are provided in Table 2 and Figure S1.

Ethical issue

No ethical permission is applicable for our study since no issue of toxic treatment, genetic manipulation or biosafety concern arose during any phase of the experimentation.

METHOD DETAILS

Study area

The study concerns river Saraswati (22° 58' 47.1216'' N and 88° 23' 36.1248'' E), a distributary of the river Ganga (Hooghly River) bearing a historical legacy, which flows through the Hooghly district of the state of West Bengal, India. Two spots designated as PR-1 and PR-2 were selected for the study; the former is the connection point between the two rivers, while the latter lies about 4 km away from PR-1 on the south-western side along the river.

Collection of water samples for physicochemical analyses

The water samples were collected from the selected sampling sites of the reference pond (RP) and two strategic sites of the polluted river (PR 1 and PR 2) once a month from March 2017 to February 2020 for analysis of physicochemical parameters. The RP is located close to the polluted river (2 km to the north) and comparatively free from anthropogenic pollution and other human impacts. Water quality parameters were monitored periodically using a digital water and soil analysis kit {Electronics India (EI); Model – 172} and as per the standard methods.[98]

Collection of host fish and parasites

Altogether 394 fish *Channa punctatus* samples were procured periodically from the second site (PR-2) of the polluted river under study for three consecutive years (March 2017 - February 2020). The length and breadth of the fish (both normal and infected) procured ranged 7–20 cm and 1.2–3 cm, respectively. The fishes were examined two different kinds of parasites comprising two ectoparasites (*Gyrodactylus* sp. and *Trichodina* sp.) and one endoparasite (*Eustrongylides* sp.). The former parasites were harvested from the body surface mucous and gills while the latter were harvested from the abdominal and thoracic cavities as well as surfaces of different internal organs (intestine, liver, ovary, etc.) of the host fish. The harvested parasites were then subjected to standard fixative (10% formaldehyde) solution and examined under light microscope. Collected parasites were taxonomically identified by adapting the works of Basson and As[93] for *Trichodina* sp., Bakke et al.[94] for *Gyrodactylus* sp., and Moravec et al.[95] for *Eustrongylides* sp. Prevalence, mean intensity and abundance for each sampling period were calculated according to Bush et al.[99]

$$\text{Prevalence}\ (\%) = \frac{\text{Number of host infected}}{\text{Number of host examined}} \times 100 \quad \text{(Equation 1)}$$

$$\text{Mean intensity} = \frac{\text{Total number of parasite}}{\text{Number of infected host}} \quad \text{(Equation 2)}$$

$$\text{Relative diversity or Abundance} = \frac{\text{Total number of parasite}}{\text{Number of host examined}} \quad \text{(Equation 3)}$$

Histopathological study

For histopathological study the collected fishes were grouped under four categories, such as 1) Control group (CONT) i.e. collected from the reference pond, 2) Control infected (CI) group i.e. normal fishes were infected artificially in the laboratory, 3) polluted river not infected (PRNI) group i.e. polluted river fish without parasitic infection and 4) Polluted river infected fish group (PRI) with parasitic infection. Gill, kidney and liver were dissected out from the freshly killed fish and were fixed in 10% formaldehyde solution for 24 hours. Fixed tissues were then dehydrated with graded alcohols, cleaned with xylene and then embedded in paraffin. Sections of the tissue (5μm) were cut using a microtome (Rotary Microtome, model-YSI-055) fixed in clean grease free glass slide and stained with haematoxylin and eosin. Slides with stained tissue section were observed under light microscope in different magnification.

Estimation of serum biochemical parameters

Collected blood samples from different groups of fish were centrifuged at 2000 rpm for 5 minutes. The serum was collected for determination of biochemical parameters. Serum protein, albumin, globulin, glucose, lipid profile and SGOT and SGPT activity (UL^{-1}) in serum from different groups of fish were determined by full automated analyser (Erba EM 200 Transasia Bio-Medicals, India). LDH activity (UL^{-1}) in serum from different groups of fish was determined by semi-auto analyser (Erba Chem 5 Plus v2, Erba, India). Catalase activity in serum was assayed according to the Hadwan and Hussein.[97]

Statistical analyses

All data obtained were subjected to appropriate statistical validation. The statistical methods used for the study are one-way ANOVA and KWANOVA, the least significant difference (LSD) test using the statistical software SPSS 20 and Origin Pro 21.