IJPSR (2015), Vol. 6, Issue 3 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 22 July, 2014; received in revised form, 29 September, 2014; accepted, 01 December, 2014; published 01 March, 2015

IMPACT OF PHYSICO-CHEMICAL PARAMETERS ON GILLS OF FRESHWATER CRAB *BARYTELPHUSA CUNICULARIS* (WESTWOOD, 1836)

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Keywords:

Physico-chemical parameter, *Barytelphusa cunicularis*, gill structure, Histology

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ABSTRACT: Environmental pollution is one of the undesirable side effects of industrialization and found responsible factor for environmental degradation. Water samples and crabs *Barytelphusa cunicularis* were collected from Gadhinglaj tahsil and analyzed for Physico- chemical parameters and heavy metals respectively. The results showed high range of pH and high values of alkalinity, hardness, chloride, sulphate, TDS, COD. The heavy, metal contamination was in the order of sodium > iron >potassium. The results indicate altered physico-chemical parameters and heavy metals resulting in massive destruction of gill architecture which was concentration and time dependent. Fluctuations in physico-chemical parameters has influenced gill lammelles of crab *Barytelphusa cunicularis.* Histopathological studies revealed noticeable changes in the gills like clumping of cells, detachment of epithelial surface in primary lamella and destruction of mucosal cells. The gills showed vacuolization in the gill stem, ruptured gill lamellae, connective tissue cells was damaged, destructed with congestion of haemocytes in the gill lamellae are observed. The results obtained were discussed in relation to physico-chemical parameter over respiratory mechanism in crab *Barytelphusa cunicularis*.

INTRODUCTION: Respiration is vital process of life for the derivation of energy in the form of ATP to perform different biological and physiological functions likes locomotion, feeding, reproduction, muscule contraction etc. Metabolic processes are the most sensitive for environmental stress as all enzymatic reactions on the substances and physiological responses were incorporated in a unique manner. The heavy metals concentrate in animal body including human through the food chain. Heavy metals have property and tendency to bioaccumulate in organism, leading to adverse effect at different exposure.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(3).1248-57

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(3).1248-57

Oxygen consumption found to be sensitive physiological process. Any changes in respiratory activity used as indicator of stress. In general gills of aquatic organism found primary target for disturbance by any pollutants as they it directly comes in contact with the external media to perform exchange of gases.

The importance of water as resources found tied to its availability and quantity, and supports the aquatic and terrestrial lives. The qualities of water therefore are explained by its physico-chemical; and biological properties [1, 2]. Physical parameters including light penetration, temperature and ionic strength, while chemical parameters include dissolved gases, major cations, anions and nutrients viz. nitrogen, phosphorus, potassium and other major and minor nutrients has significant role in maintaining the quality of water bodies [3]. Direct discharge of industrial waste into rivers, ponds and lakes has resulted to contamination of water bodies.

Colloidal organic matter and soluble inorganic salts will increase turbidity of this water. All these lead to a large increase of COD and BOD values of that water body stated by [4] and make that water unfit to use by aquatic animals and plants. These effluents causes toxic effects on aquatic animals by depleting the dissolved oxygen, altering the pH, salinity, CO_2 content, and thereby directly and indirectly affects the life cycles as well as the metabolic activities of the aquatic animals.

By the scientific way histological studies provides a way for understanding the pathological conditions of the animal and helping in diagnosing the abnormalities or damages of the tissues exposed to any toxic stressful chemicals [5, 6, 7]. Histotological changes in tissues are biomarkers of effect and exposure that integrate responses to contaminants at the cellular level [8]. Histological changes provide an early indication of pollution hazard, data of nature and degree of damage at cells and tissue levels [9]. According to [10], histological changes in gills of the crab, *Barytelphusa cunicularis* was recorded by cadmium toxicity and observed ruptured gill lamellae. [11] observed histological alterations in gill tissue of *Barytelphusa cunicularis* after exposure to sublethal concentration of mercury. [12], observed toxic effect of copper on the gill histology of a freshwater fish, *Labeo rohita.* [13] noted changes in the gill tissue of freshwater prawns, *Macrobrachium idea* after exposure to mercury.

Taking account of effects of environmental parameters and their hazardous effects on the aquatic organisms particularly on invertebrates, the present work was undertaken to study the respiratory aspect in relation to physicochemical characteristics and heavy metals. In present study highly economic important species of fresh water crab was used as experimental animal study also enlights histopathological alterations in the gill of crab.

MATERIAL AND METHODS:

Study area:

Gadhinglaj is important tahsil of Kolhapur district, state Maharashtra. Geographically has latitude 16°13´26"N and longitude 74°26´9" E. River Hiranyakeshi **(Fig. 1)** is one of the important flowing in two states of India (Maharashtra and Karnataka). The River originates at Amboli hill station at Sindhudurg district of Maharashtra, and enters into district Kolhapur. Continue to district Belgaum of Karnataka, covering 140 Km distance and finally meets to River Ghataprabha. Geographically the area is flat except some part of Sawantwadi and Ajara Tahsil.

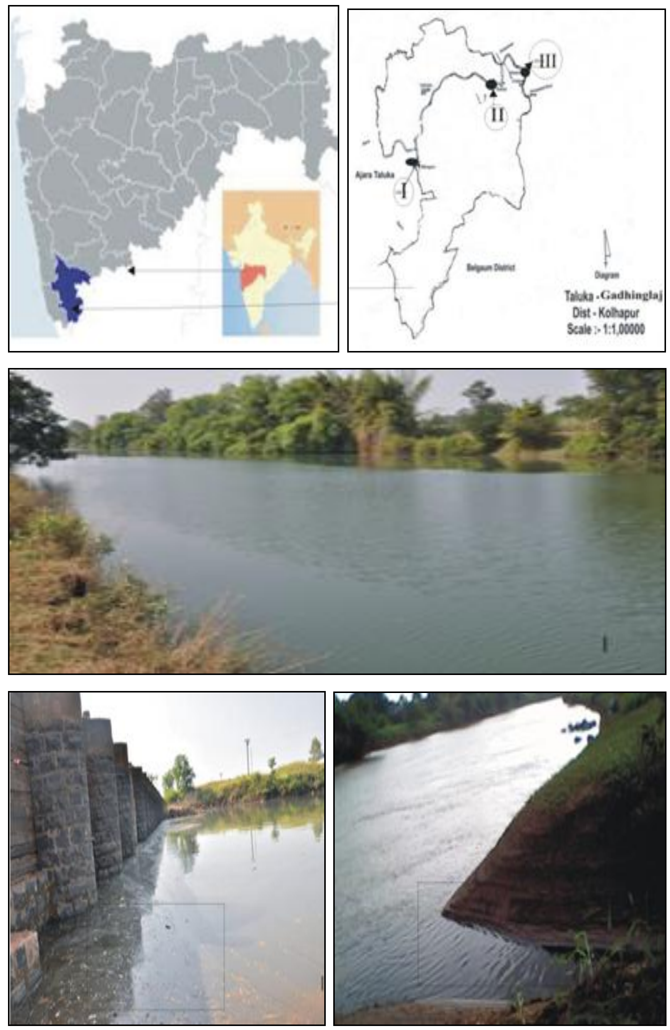

FIG. 1: SAMPLING SITES OF STUDY AREA, SITE I: MAHAGOAN RIVER, II: NILGI RIVER, III: NANGNUR RIVER

Major area of the basin of river is under agricultural practice, whereas remaining is forest covered. The quantity and quality of water from this river is affected by municipal, industrial as well as agricultural discharge. So, for the present study three sites were selected from Hiranyakeshi River. Site I (Mahagoan spot N 16°9'54" E 74°19'58"). Site II (Nilgi spot N 16° 14´ 16" E 74° 25´ 41") where domestic and agricultural waste added continuously. Site III (Nangnur spot N 16°14’3” E 74° 29’46”).

Water Sampling and animal collection:
For the present study, water samples were collected from collection sites. Samples were brought to the laboratory by using plastic containers for analysis of different physico-chemical parameters applying standard methods [14, 15]. Base level heavy metal analysis was carried out by using Atomic Absorption Spectrophotometer (AAS) (Kemito company- 201). During study Physico-chemical parameters like temperature, pH, dissolved oxygen and free CO_2 etc. were determined at the sampling sites respectively. For histological study, animals were collected from selected study sites. Animal were dissected and targeted tissues were fixed in Bouin’s fixative for 24 hr and washed, dehydrated and processed for routine microtechnique. Sections at 5-6µm thickness were taken and stained with hematoxylene and eosin.

RESULTS AND DISCUSSION:

Physico-chemical parameters:
Physico-chemical parameters were analyzed at sampling sites. The results obtained were as follows,

Temperature:
During the experiment we found that, surface water temperature ranged between 23°C to 26.25°C. It was recorded minimum during rainy and maximum in summer. On an average the maximum range of temperature was seen at site I (26.25ºC) while minimum at site III (23 ºC) throughout the year (Table 1, 3 and Fig. 2, 3).

pH:
The pH is an important factor for determining productivity of ecosystem. pH of surface water was alkaline and fluctuated between 6.15 and 7.65. It was well within the limits (ICMR Standard). The alkaline pH was seen at all sites.

Alkalinity:
The average range of maximum value of alkalinity was (125 mg/l) at site III in summer, at site II (62.75 mg/l) while minimum (28 mg/l) at site I in rainy (Table 1, 2, 3 and Fig. 1, 2, 3). Results indicated that, in river water carbonate and bicarbonate ions were less dissolved.

Hardness:
Hardness results from the presence of divalent cations of which Ca^{++} and Mg^{++} which are most abundant in groundwater. The average range of total hardness was maximum at site III (100.5mg/l) in summer season due to higher concentration of metallic ion, at site II (68.75 mg/l) while minimum recorded at site I (47.5mg/l) in rainy season (Table 1, 2, 3 and Fig. 1, 2, 3). Recorded reading indicated that, in river water metallic ions are less dissolved.

Chloride:
The most important source of chloride in natural water is discharge of sewage. The minimum value of chloride is 11.49 mg/l at site I in rainy, whereas maximum 152 mg/l in winter season at site III and (51.83mg/l) at site II. Water analysis from site I indicated that it was contaminated due to organic content of sewage, agricultural wastes inducing fertilizers etc. with discharge by surrounding areas (Table 1, 2, 3 and Fig. 1, 2, 3).

Sodium:
The maximum range of sodium seen at site III (59mg/l) in summer season at site II (42.25mg/l) while minimum at site I (5mg/l) in winter (Table 1, 2, 3 and Fig. 1, 2, 3). Maximum sewage discharge was found at site III leading to its pollution as compared to others.

Potassium:
The high concentration of potassium content was noted at site III (33.75mg/l) in rainy season, at site II it was (3.75mg/l) while minimum at site I (1.5 mg/l) in rainy (Table 1, 2, 3 and Fig. 1, 2, 3).

Iron:
Iron is the vital element of life. It is a natural component of soil and its concentration can be influenced by industrialization. Iron content of water was high at site III (1.617mg/l) in winter at site II (1.313mg/l) while minimum at site I (0.02 mg/l) in winter season (Table 1, 2, 3 and Fig. 1, 2, 3).

Chemical Oxygen Demand: Chemical Oxygen Demand found reliable parameter for analysis of water pollution. The maximum COD was found at site III (240 mg/l) in winter season at site II (92.5mg/l), while at site I (2.75 mg/l) in rainy season (Table 1, 2, 3 and Fig. 1, 2, 3).

TABLE 1: SEASONAL VARIATION OF PHYSICO-CHEMICAL PARAMETER FROM SITE I

Parameter	Season		
	Summer	Rainy	Winter
Temperature	26.25±2.061	23.5±2.516	24.75±1.5
pH	7.41±0.321	7.3875±0.278	7.5±0.36
Alkalinity	40.5±6.806	28±14.76482	39±12.463
Hardness	65.5±28.734	47.5±13.0767	65±9.695
Chloride	21.3±8.829	11.4925±1.894	19.0075±0.849
Sodium	8±3.16	7.25±2.75	5±0.816
Potassium	1.3±0.577	1.25±0.5	1.5±0.57
Iron	0.291±0.213	0.6675±0.653	0.02±0
COD	5.625±4.269	2.75±0.957	9±10
TS	63±10.893	60±19.866	76.5±18.64
TDS	58±13.165	52.5±13.279	68±12.75
SS	5±3.464	7.5±7.852	8.5±6.608
DO	5.87±1.314	8.75±0.5	6.525±1.147
Free co_2	8.4±1.870	7.425±2.781	7.925±3.781
Nitrate	0.09025±0.014	0.0815±0.007	0.0725±0.007
phosphate	0.08325±0.006	0.04±0.014142	0.049±0.0257

TABLE 2: SEASONAL VARIATION OF PHYSICO-CHEMICAL PARAMETER FROM SITE II

Parameter	Seasons		
	Summer	Rainy	Winter
Temperature	26±1.63	23.5±1.73	23.75±0.5
pH	7.51±0.463	7.65±0.759	7.3±0.391
Alkalinity	62.25±19.60	44.5±22.985	62.75±11.44
Hardness	68.75±14.77	60±13.564	61.5±14.271
Chloride	51.83±35.95	19.02±2.699	52.87±17.74
Sodium	42.25±19.36	28±14.165	19.75±2.87
Potassium	3.75±2.872	3.3±0.57	4.25±2.629
Iron	0.885±0.876	1.31±0.604	0.228±0.075
COD	25.5±5.196	8±6.164	92.5±62.55
TS	229±149.759	84±51.562	175.5±111.407
TDS	192.25±99.63	75.75±51.771	157.5±115.243
SS	36.75±58.35	8.25±0.5	11.25±4.991
DO	5.75±2.061	7±0.816	5.75±2.217
Free co_2	23.675±5.550	11.81±3.936	17.55±6.378
Nitrate	0.4225±0.043	0.365±0.259	0.183±0.129
Phosphate	0.073±0.007	0.07±0.062	0.124±0.053

TABLE 3: SEASONAL VARIATION OF PHYSICO-CHEMICAL PARAMETER FROM SITE III

Parameter	Seasons		
	Summer	Rainy	Winter
Temperature	25.75±1.5	23±2.58	23.5±1.29
pH	6.8±0.39	7.55±0.655	6.15±0.58
Alkalinity	125±112.80	36±6.055	54±24.276
Hardness	100.25±24.198	51.25±22.5	71.25±12.996
Chloride	76.6875±41.430	51.85±55.466	152.39±20.804
Sodium	59±15.705	36±16.753	57±16.451
Potassium	11.5±3.872	33.5±10.847	33.75±9.604
Iron	0.66±0.716	1.56±0.569	1.617±1.0933
COD	97.5±90.559	14.5±5.744	240±233.978
TS	545±266.755	134.5±106.61	677.75±649.965
TDS	461±341.582	117.75±96.347	646.25±645.043
SS	9±4.760	16.75±10.5	31.5±9.574
DO	4.75±1.5	6±1.4142	6.25±2.217
Free co_2	18.425±2.710	16.82±4.707	17.055±4.534
Nitrate	1.92±0.0546	1.88±0.071	1.055±0.526
Phosphate	2.57±0.105	0.022±0.0090	0.945±1.255

Total solids:
Water as a universal solvent it can dissolve different type of materials as compare to other solvents. The average range of total solid content was maximum at site III (677 mg/l) in winter season at site II (229mg/l), while minimum at site I (60 mg/l) in rainy season (Table 1, 2, 3 and Fig. B, C, D).

Total dissolved solids:
In the present investigation the concentration of total dissolved solid found high at site III (646 mg/l) in winter season which has decreased potability and reduced utility of water for drinking, irrigation and industrial purposes. Minimum range of total dissolved solid was seen at site I (52.5 mg/l) in rainy season (Table 1, 2 and Fig. B, C).

Total suspended solids:
The higher concentration of total suspended solids was found due to insoluble organic matter in sewage. The average range of suspended solids was maximum at site II (36 mg/l) in winter at site III (31mg/l) while minimum at site I (5 mg/l) in summer season (Table 1, 2, 3 and Fig. B, C, D). Results Indicated enrichment of suspended solids at site II and III found unfit for drinking, irrigation and also for industrial purpose.

Dissolved oxygen:
Oxygen is an index of the physical, chemical and biological processes. It also acts as an indicator of trophic status and magnitude of eutrophication in freshwater ecosystem. The concentration of dissolved oxygen ranged between 4.75 mg/l to 8.75 mg/l. It was minimum during summer at site III (4.75 mg/l) and maximum (8.75 mg/l) during rainy at site I (Table 1, 3 and Fig. B, D).

Free CO2:
Free CO_2 concentration in water indicates the presence of decomposable organic matter, bacterial action on organic matter and physiological activities of biotic components. The concentration of carbon dioxide values ranged between 7.425 mg/l to 18.425 mg/l. It was minimum during rainy at site I (7.425 mg/l) and maximum (18.425 mg/l) during summer at site III (Table 1, 2 and Fig. B, C).

Nitrate: The nitrate concentration values ranged from 0.0725mg/l to 1.92 mg/l. It was maximum at site III in summer season (1.92 mg/l) and was minimum (0.0725mg/l) at site I during winter season (Table 1, 2 and Fig. B, D).

Phosphate:
The phosphate concentration (Table 1, 2 and Fig. B, D) ranged from 0.04 mg/l to 2.57 mg/l. It was maximum at site III in summer season and minimum at site I during rainy season. Thus, Physico-chemical parameters in the present at study sites (I, II and III) were found at wide variations shown in (Table 1, 2, 3 and Fig. B, C, D) with the mean, standard deviation and their percentage composition.

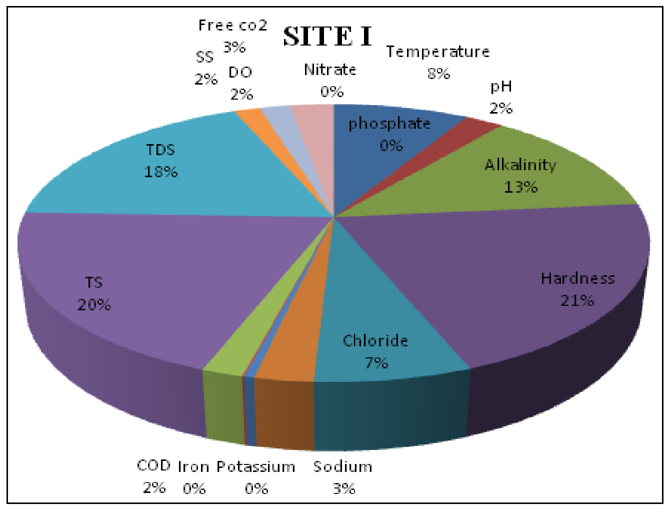

FIG. 2: PERCENTAGE COMPOSITION OF PHYSICO-CHEMICAL PARAMETER FROM SITE I

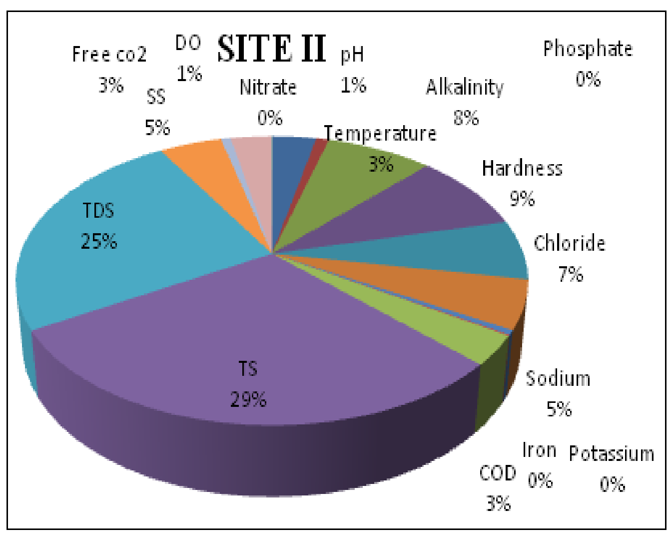

FIG.3: PERCENTAGE COMPOSITION OF PHYSICO-CHEMICAL PARAMETER FROM SITE II

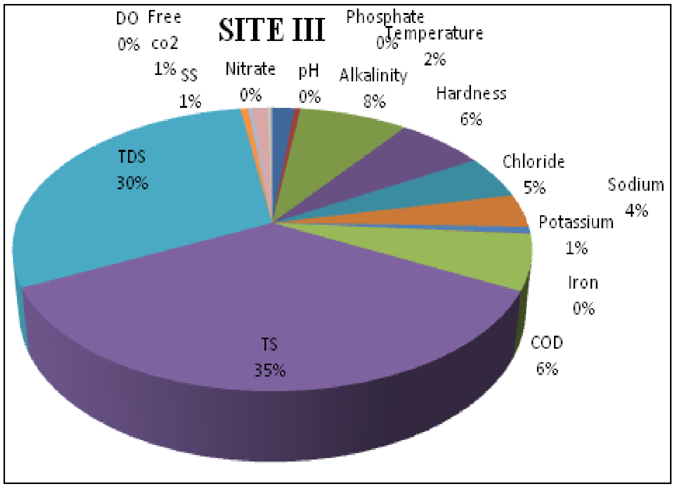

FIG. 4: PERCENTAGE COMPOSITION OF PHYSICO-CHEMICAL PARAMETER FROM SITE III

Histological alterations in the gills of crab from site I.

The respiratory organs were located in the gill chamber in the antero-ventral region of thoracic region. Gills were located laterally on either side of the hepatopancreas and alimentary canal deep brown in colour and paired with crescent shaped structure.

Histologically the gills of *Barytelphusa cunicularis* are phyllobrachiate type consisting of central stem (axis, raphe) that bears serially the paired plate or leaf like lamellae, which found to be actually flattened sacs. The central axis has afferent and efferent haemal channels on each end. Three types of cells were found: (1) *Connective tissue* located in the gill stem; (2) *Branched arthrocytes* located in the stem and proximal lamellae; (3) *Lamellar cells* located in the epithelium. Each gill plate has outer cuticular layer enclosing a single layer of gill epithelial cells. The gill epithelium stained deep pink. The central axis contained large amount of darkly stained haemocytes. The central axis showed few nephrocytes. The nephrocytes were vacuolated with large cells indicating amount of pale brown material.

Histologically the gills of *Barytelphusa cunicularis* from site I was normal phyllobrachiate type consisting of well developed central stem and gill lamellae arranged in two rows on either side (Fig.-E C, F and I) (axis, raphe) with huge number of hemocytes.

Histological alteration in the gills from site II and III:

The histological study has been carried out to know lesions in gills that had resulted from lethal exposure of the freshwater crab, *Barytelphusa cunicularis* to different physico-chemical parameters. Histologically the gills of *Barytelphusa cunicularis* from site II and III showed disturbed gill stem. Central axis had also showed disturbance in afferent and efferent haemal channels on each end. Only few hemocytes were observed in central axis. Along with (Fig. E) the vacuolization in the gill stem, gill lamellae were found ruptured, connective tissue in the stem damaged, destructed and overcrowding of haemocytes in the gill lamellae was noted.

Thus the structure of the gills observed in the present study was well at site I in accordance with earlier reports on different crabs and it clearly indicated that, histological changes not only give an early indication of pollution hazard, but also provide useful data on nature and degree of damage to cells and tissues of aquatic animals in water bodies.

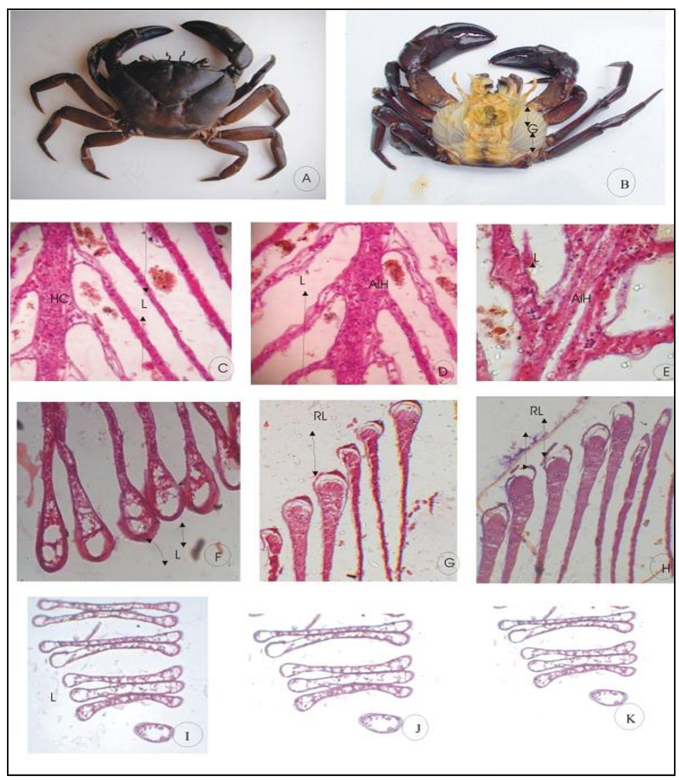

FIG. 5: A: FRESHWATER CRAB, B: POSITION OF CRAB GILL, C, F, I: GILL STRUCTURE FROM SITE I. D, G, AND J: GILL STRUCTURE FROM SITE II. E, H, K: GILL STRUCTURE FROM SITE III

Abbrevations: H- hemocytes, ATH: abnormal infilteration of hemocytes, HC: hemal canal, L: gill lamelliae, RL: ruptured gill lamelliae, SL: swelling in gill lamelliae.

Temperature is an important parameter, and has vital role in chemical and biological activities it alters with changeable environmental factors. In our investigation the moderate range of temperature was observed at site I throught the year. The high value of alkalinity indicated the presence of weak and strong base such as carbonates, bicarbonates and hydroxides in the water body. The high values of alkalinity may also be due to increase in free carbon dioxide in the river, which ultimately showed increased alkalinity of water.

In our investigation the average range of maximum value of alkalinity was (150 mg/l) at site III in winter. So high alkalinity may cause problems if, water used for irrigation purposes, by increasing relative proportion of sodium in soil by precipitating Ca and Mg ions. The values having 40 mg/l and more levels of total alkalinity was considered to be more productive reported by [16].

Total hardness of water is not a polluting parameter, but indicates water quality in terms of Ca^{++} and Mg^{++} cations. We found hardness of the Hiranyakeshi River was within the permissible limit of WHO as below 300 mg/l is considered for potability but, beyond this limits cause gastrointestinal irritation [17]. Normal water hardness does not pose any direct health problems. Due to addition of sewage and large scale human use, it might cause elevation of hardness [18, 19, 20.] The total hardness above 200 mg/l found to be not suitable for domestic use like drinking and cleaning.

Chloride reported to be an indication of pollution when present in higher concentration. As per report of Royal Commission, water having 30 mg/l of chloride was considered fairly clean. Sources of chloride pollution in water include fertilizers, sewage, effluents from drainage, salts and human as well as animal wastes. High chloride content cause high blood pressure in people [21]. Chlorides reported toxic to most of plants so it should be checked for irrigation water. In the present study chloride concentration was high at site II and III which clearly indicated that, the discharge of fertilizers, sewage, effluents from drainage, salts and human as well as animal wastes was high.

Higher values of sodium during summer season and lower during in monsoon was recorded at site II and III as compare to site I which clearly indicates that, domestic sewage discharge was high at site II and III. Sodium when present in high concentration restrictes the biological diversity due to its osmotic stress [22]. Like, sodium potassium was also a naturally occurring element, but the concentrations in freshwater bodies remained quite lower than the sodium and calcium.

In general, concentration of sodium remained quite higher than the potassium in natural water, thus high values being an indicator of pollution by domestic sewage [15]. The concentration of sodium was higher than that of potassium in the present study. Iron found essential element in human nutrition. Potassium has vital role in the metabolism of freshwater organisms [23]. Cell membrane in aquatic animals continually pumps the potassium and sodium, which requires the expenditure of large amount of energy [24]. [25] showed that dissolved oxygen decreases with increased concentration of iron in water. Similar results were found at sites II and III in our investigation.

Chemical oxygen demand (COD) is a measure of the oxygen equivalent of the organic matter content of water which found susceptible to oxidation by a strong chemical oxidant. Thus, COD was a reliable parameter for judging the extent of pollution in water [26]. The COD of water increases with increasing concentration of organic matter [27].

In our investigation COD concentration was comparatively high at site III due addition of chemical waste from Municipal waste of Sankeshwar city was added into river water. [28] stated that, total dissolved solids (TDS) in water found mainly in the form of sodium, potassium, calcium, magnesium, carbonates, bicarbonates, chlorides, nitrates and sulphates. They influence the taste, hardness and corrosive property of water.

We found, total dissolved solids content was high at site II and III and was unfit. The dissolved oxygen concentration was higher during the monsoon season at site I was due to the monsoon floods. The dissolved oxygen was found to decrease with increasing COD at site II and III. The carbon dioxide content of water depends upon the water temperature, depth, rate of respiration, decomposition of organic matter, chemical nature of the bottom and geographical features of the surrounding water body was reported by [29] similar results were coinside with present study.

Higher nitrate content was recorded at site II in summers, which clearly indicate that, as site II found as polluted station with the direct discharges of organic wastes, in addition to the discharge of industrial wastes and munciple waste. Most of the nitrate might have been derived from the decomposition of organic wastes [30].

The importance of nitrate and phosphate content in water bodies showed a direct relationship with phytoplankton. Content of nitrate and phosphate also favored good growth of blue green algae. Some of the parameters were also act as indicators of sewage pollution such as chloride, phosphate and nitrate. [31], showed increased concentration of chloride, nitrate and phosphate during summer and winter by which planktonic population found to be increased which also act as bioindicator of pollution. The total phosphate content in water was due to mixing up of agricultural runoff. Observations showed that at site II and III the agricultural activities along with industrial discharge resulted contamination of water in high concentration as that of site I. .

Freshwater forms very important media for the production of protein-rich fishes, prawns and crabs. But day by day it get contaminated due to discharge of industrial effluents [32]. The disposal of industrial effluents in the aquatic environment is toxic to animals living in it. Abnormal physico-chemical characteristics of industrial effluents were responsible for mortality of aquatic animals [33, 34]. Histological studies have a way for understanding the pathological conditions of the animal by helping in diagnosing the abnormalities or damages of the tissues exposed to toxic stress of metals [5, 6, 7]. Aauatic animals are relatively sensitive to changes in their surrounding environment including an increase in pollution. Health of aquatic organism may thus reflect, and provide a good indication of the health status of aquatic ecosystem. Histological analysis appears to be a very sensitive parameter and is fundamental in determining cellular changes that may occur in target organs, such as the gills, liver and kidney [35].

A histological investigation may therefore prove to be a gainful tool to determine the health of aquatic populations, hence reflects health of an entire aquatic ecosystem in the bio-monitoring process. Micro-anatomy (histology) of the gill tissue constitutes an important diagnostic tool to show histological changes caused by various pollutant at site II and III. Results showed abnormal infilteration of hemocytes (AIH) in the hemocoelic space of gill lamellae **(Fig.5)** which indicated recruitment of hemocytes in host defence reaction against increased COD content, the tissue inflammation was also observed.

Toxic substances damage the gill tissue first, thereby reducing the oxygen consumption and also disturbs the osmoregulatory function in crustaceans [36,37] observed structural changes including the swelling and fusion of the lamellae; abnormal gill tips; and necrotic lamellae in gills of a giant freshwater prawn, *Macrobrachium rosenbergii* exposed to waterborne copper. Thus, many researchers have also reported the degenerative changes in selected tissues of the animals in response to pollution by various toxicants [38,39,40,41,42].

CONCLUSION: The experimental animals collected from site I showed normal gill structure **(Fig. 5).** Which can be concluded that respiration of crustaceans get controlled by increased (temperature, depth, pH and alkalinity) with biotic factors including food availability, predation, alternation in oxygen consumption and the site is still unpolluted.

While histological study on gills showed that at site III organs were progressively damaged and degenerated and indicating the progressive contamination of water i.e. extent of tissue damage increases with the increasing pollution. Damage of gills in contaminated water lead to decline in oxygen carrying capacity. Injury of gills resulted in the disturbance of overall metabolism and several physiological processes of crab. Histopathological changes can be taken as biomarker for indication of pollution at site II and III.

ACKNOWLEDGEMENT: Authors are thankful to the Head, Department of Zoology, Shivaji University Kolhapur for providing facilities in the progress of work.

CONFLICT OF INTEREST: The authors report no conflict of interest.

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How to cite this article:
Sakhare SS and Kamble NA: Impact of Physico-Chemical Parameters on Gills of Freshwater Crab *Barytelphusa Cunicularis* (Westwood, 1836). Int J Pharm Sci Res 2015; 6(3): 1248-57.doi: 10.13040/IJPSR.0975-8232.6(3).1248-57.