INCREASING ACCLIMATION TEMPERATURES AND PERSISTENT SUB - LETHAL CHLORINE EXPOSURE INFLUENCE THE ACTIVITIES OF KEY METABOLIC ENZYMES IN *LABEO ROHITA* (HAMILTON, 1822) FINGERLINGS

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Abstract: The effects of increasing temperatures and sub-lethal chlorine exposure on the metabolic responses of *Labeo rohita* were investigated. *L. rohita* fingerlings were acclimated to four different temperatures (26, 31, 33 and 36°C) and maintained for 30 days in two different groups (control and experimental). In addition to exposure to temperatures, the fish in the experimental groups were treated with chlorine (0.1 mg L^{-1}) for 30 days. Selected enzymes of different biochemical pathways viz. protein metabolism, phosphorus metabolism and energy metabolism were estimated in different tissues at 15 and 30 days acclimation period. Results showed that with increasing temperatures *L. rohita* exhibited metabolic readjustments to provide the extra energy needed to meet the energy demands of the cell. However, the combined effect of increasing temperatures and chlorine adversely affected the protein metabolism and phosphorus metabolism leading to energy-limited condition in the cell. Further, changes in energy metabolism signify initial stages of cellular damage. The results indicate that, increasing temperatures increases the toxicity of chlorine to *L. rohita*, thus eliciting the stress responses in the fish.
Key words: *Labeo rohita*, Thermal acclimation, Temperature, Chlorine, Enzymes.

INTRODUCTION

The aquatic environmental temperature is changing globally. Besides many natural factors, the water temperature is known to be tremendously affected by human activities. Anthropogenic point-source thermal discharges are directly affecting aquatic thermal regimes,

extensively altering the structure of aquatic communities (Caissie 2006). One of the most extreme examples of thermally altered environments is thermal effluents associated with power plants (Encina et al. 2008). For the efficient operation of a nuclear power plant, uninterrupted supply of cooling water into the condensers is pre-requisite (Neitzel et al. 1984), hence, they are built near river banks or sea coasts. Nuclear power plants require, on an average, about $3m^3$ cooling water per minute per megawatt (MW) of electricity produced (Schubel and Marcy 1978). Heated effluents from such power stations are being continuously discharged into these water bodies. Temperature being a very important ecological parameter that affects almost every aspect of aquatic life (Schubel et al. 1978), the discharged warm water therefore, may have an impact on the ecology of the receiving water body (Saravanan et al. 2008). Apart from elevated temperature, the discharged water contains chemical stress factors such as chlorine, which are added to control biofouling on cooling system components (Choi et al., 2002; Saravanan et al. 2006). Chlorine hydrolyses very rapidly in water (U.S. EPA 1989) and is highly toxic to aquatic organisms (Emmanuel et al. 2004). Thus, condenser effluents from thermal power plants have the potential to impart both thermal and chemical stress and therefore, may pose environmental problems to the receiving water body (Krishnakumar et al. 1991).

Aquatic organisms such as fish are, in most cases, exposed to multitudes of stressors that are either naturally or anthropogenically introduced into the environment (Hallare et al. 2005). Laboratory and field studies have documented the effects of chlorinated cooling waters on various fishes (Langford 1990; Chen and Kuo 2005; Verma et al. 2007a, b). However, to our knowledge, so far, there have been no investigations on the concerted effects of increasing temperatures and chlorine on the Indian major carps (*Labeo rohita*, *Catla catla* and *Cirrhinus mrigala*), the main aquaculture species in India.

L. rohita is widely cultured throughout India owing to its high commercial value (Das et al. 2005). Hence, it becomes imperative, especially from aquaculture and toxicological perspectives to investigate the combined effects of thermal and chemical (chlorine) stress on this fish, as elevated temperatures influence the toxicity of chlorine to fish (Cairns et al. 1975). Therefore, the aim of this study is to determine the combined effect of increasing temperatures and sub-lethal chlorine on *Labeo rohita* by the analysis of key enzymes of different biochemical pathways.

MATERIALS AND METHODS

Experimental fish and maintenance

L. rohita fingerlings procured from Aarey Fish Farm, Mumbai, Maharashtra, India were transported with proper oxygenation to the laboratory of the Central Institute of Fisheries Education, Mumbai, India. They were acclimatized to the laboratory conditions for 30 days before being used for experiment. During this period they were fed (*ad libitum*) daily with supplementary feed (35% crude protein). Water (chlorine free freshwater) exchange was done daily to maintain water quality.

Experimental variables

Temperature

Carps can survive extreme temperatures (4–39°C) and low dissolved oxygen for several days (Reynolds 1987). However, the optimal temperature for carp growth has been found to be around 25°C (Billiard 2001). Therefore, the acclimation temperatures selected for the present study were 26, 31, 33 and 36°C (Das et al. 2004).

Chlorine

Chlorine level in the immediate locality of thermal power plants (near discharge canal) is about 0.1 mg L^{-1} (personal communication from power plant operators). Therefore, a concentration of 0.1 mg L^{-1} of chlorine was selected for the present study.

Chlorine dosage and analysis

A preliminary trial was conducted to assess the evaporation rate of chlorine at different temperatures (26, 31, 33 and 36°C). Based on the evaporation rate, chlorine was supplemented at every 8 h interval to maintain the level at 0.1 ± 0.02 mg L^{-1} in the experimental tanks. Sodium hypochlorite solution (Merck Ltd. Mumbai, India) was used as the chlorine source. Chlorine levels were constantly monitored by the dipropyl-p-phenylenediamine (DPD) method using Spectroquant chlorine test kit (E-Merck, Germany) with an accuracy of 0.01 mg L^{-1}.

Experimental Design

A total of 120 fish (10 ± 0.52 g) were distributed in eight different treatments (T_1 to T_8), in plastic tanks of 75-L water capacity. Of the eight treatments, four treatments served as the control group (T_1 to T_4) and the remaining four treatments as the experimental group (T_5 to T_8). Each tank was fitted with a heater with temperature controller and sensor (Selectron Process

Controls Pvt. Ltd., Mumbai, India) for maintaining the desired water temperature. The fish in both the control and experimental groups were subjected to an increase in temperature at the rate of 1°C per day over ambient temperature (26°C) to reach the test temperatures of 26, 31, 33 and 36°C (i.e. Δ5, Δ7 and Δ10 over the ambient temperature). A lag period of 3 and 5 days was maintained to increase the temperatures from 26 to 33°C and 26 to 31°C, respectively, so as to reach the prescribed temperatures on the same day. The fish were maintained at these desired temperatures for a period of 30 days. The acclimation period followed in this study was based on our previous investigations in Indian major carps (Chatterjee et al. 2004; Das et al. 2004). Therefore, we assumed that the fish were completely acclimatized to the respective test temperatures at the end of the acclimation period. Once the desired temperatures were reached, in the experimental group a uniform concentration of chlorine as sodium hypochlorite (0.1 mg L^{-1}) was added and maintained throughout the acclimation period. As chlorine evaporates rapidly due to aeration and temperature, its concentration was monitored and supplemented thrice daily to maintain uniform levels of chlorine during the experimental period. Feeding was withheld for 24 h before the sampling (15 and 30 days).

Sample preparation

Fish were sampled at two different acclimation periods, 15 days (short-term acclimation) and 30 days (long-term acclimation), from all the control and experimental tanks and anaesthetized with clove oil (50 µL L^{-1}). Pre-weighed tissues viz., gill (0.5 g), liver (0.1 g) and muscle (0.5 g) were homogenized in chilled sucrose solution (0.25 M) by a mechanical tissue homogenizer. The homogenates were centrifuged (5000 x *g* at 4°C for 10 min), supernatants collected and stored at -20°C for subsequent enzyme assays.

Enzymes assays

Aspartate amino transferase (AST) (L-aspartate-2-oxaloglutarate aminotransferase, E.C.2.6.1.1) was assayed using 200 mM DL-aspartic acid and 2 mM α-ketoglutarate as the substrates in 50 mM phosphate buffer (pH 7.4) and estimated at 540 nm (Wotton 1964). Alanine amino transferase (ALT) (L-alanine-2-oxaloglutarate aminotransferase; E.C.2.6.1.2) activity was measured by the same procedure as for AST except for the substrate, 200 mM DL-alanine instead of aspartic acid (Wotton 1964).

Alkaline phosphatase (ALP) (Orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) was estimated using 0.2 M bicarbonate buffer, 0.1 M $MgCl_2$ and 0.1 M para-nitrophenyl phosphate as reaction mixture and incubated at 37°C for 15 min. The reaction was terminated using 0.1 N NaOH and the absorbance was recorded at 410 nm (Garen and Levinthal, 1960). Adenosine triphosphatase (ATPase) (Adenosine triphosphate phosphohydrolase, E.C.3.6.1.3) activity was determined using an assay mixture comprising of 100 mM Tris-HCl buffer (pH 7.8), 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$ and 5 mM ATP. The assay mixture was incubated for 15 min and the reaction was arrested by the addition of 10% trichloroacetic acid (Post and Sen 1967). The inorganic phosphate liberated in the reaction was read at an absorbance of 660 nm (Fiske and Subbarow 1925). Total protein content was analyzed from the supernatant (Lowry et al. 1951) for calculating enzyme activities. All the colorimetric assays were carried out using UV–ViS spectrophotometer (E-Merck, Germany).

Statistical analysis

Data analyses for significant differences among different treatments were performed using one-way analysis of variance. Duncan's multiple range tests were used to determine the differences among treatment means at $P < 0.05$. Student's t-test was used to compare the differences between the acclimation periods (15 and 30 days). All the statistical analyses were done using SPSS version 15.0.

RESULTS

Enzymes of protein metabolism

The AST activity in the liver and muscle increased significantly ($P<0.05$) with increasing acclimation temperatures both in the control and experimental groups after 15 and 30 days of acclimation period (Table 1). However, AST activity in the liver decreased significantly ($P<0.05$) in chlorine treated experimental groups as compared to the enzyme activity in the control groups. In contrast, the muscle AST activity increased significantly ($P<0.05$) in the experimental groups when compared to their respective controls at both 15 and 30 days of exposure period. Student's t-test comparison revealed a significant increase in the liver and muscle AST activities both in the control and experimental groups at the end of 30 days of acclimation compared to that of 15 days acclimation period.

The ALT activity both in the liver and muscle demonstrated a similar trend like the AST activity in the liver and muscle at both the acclimation periods of 15 and 30 days (Table 1).

Enzymes of phosphate metabolism

After 15 and 30 days acclimation, the liver and muscle ALP activities increased significantly ($P < 0.05$) with increasing temperatures both in the control and experimental groups (Table 2). However, due to chlorine treatment, the ALP activity was comparatively lower over the control groups. In the control groups, the ALP activity increased with increase in the acclimation period. In contrast, a decreasing trend with increasing exposure period was observed in the experimental groups.

Enzymes of energy metabolism

An increasing trend in the gill, liver and muscle ATPase activities was observed with increasing temperatures in both the control and experimental groups after 15 and 30 days of acclimation (Table 2). Presence of chlorine increased the ATPase activity over their respective control groups. Comparison of the ATPase activity between the two exposure periods (15 and 30 days) demonstrated a significant decrease in the enzyme activity in most of the control and experimental groups at the end of 30 days acclimation over 15 days.

DISCUSSION

Temperature is a major determinant of cellular physiology for ectotherms, as it affects physiological processes by a direct effect on the rates of biochemical reactions. In the present study, the activities of transaminase enzymes both in the liver and muscle increased with increasing acclimation temperatures and period. This suggests that high temperature creates higher free amino acid mobilization (Das 2002), which in turn might have produced glucose to cope up with the stress, via the process of higher gluconeogenesis (Das et al., 2006). Similar observation was recorded in *Cyprinus carpio* after being exposed to higher acclimation temperature (Verma et al. 2007b). The results also showed decreased activity of AST and ALT in the liver in the experimental groups (i.e., in the presence of chlorine) as compared to the control groups, which might be due to the inhibition of *de novo* synthesis of protein as indicated by Verma et al. (2007b) in *C. carpio* exposed to increasing acclimation temperatures and chlorine. In contrast, the increase in the muscle transaminase activity in the chlorine-treated groups over their respective controls may be due to the direct entry of keto acids into the Krebs

cycle for energy yielding process as suggested by the above authors in *C. carpio*. The increase in the liver and muscle transaminase activities with increasing acclimation period in the chlorine-treated groups were in accordance with the findings of Arshad et al. (2007) in endosulfan intoxicated mice. The observed effect in the present study suggest a shift towards catabolic pathways of protein metabolism, possibly favoring mobilization of energy reserves to supply the extra energy needed to combat stress (Scott and Sloman, 2004).

ALP, a zinc-containing metallo-enzyme, plays a key role in phosphorus metabolism. The increased ALP activity in the tested tissues, liver and muscle, with increasing acclimation temperatures and period could be due to the hydrolysis of high-energy phosphate bonds to liberate phosphate ions to combat stressful conditions or high metabolic rate (Verma et al. 2007b). Similar results were reported for *Cirrhinus mrigala* subjected to increasing acclimation temperatures (Das 2004). The inhibition in the liver and muscle ALP activity in the chlorine treated groups and a further inhibition of the enzyme with increasing acclimation period could be due to the effect of chlorine in inhibiting protein synthesis. Similar inhibition of ALP activity was reported for mice, *C. punctatus* and *C. gachua* (Sharma 1990; Arshad et al. 2007; Sarma et al. 2009) due to chemical exposure.

In the present study, increase in the ATPase activity with increasing acclimation temperatures in the control groups indicated that there was no depletion of ATP from 26 to 36°C, which demonstrated the role of ATPase in the regulation of energy balance in fish (Love 1980). Results also showed that sub-lethal chlorine exposure increased the ATPase activity in all the tested tissues (gill, liver and muscle) of *L. rohita*. This might be due to high energy demand to cope with stress, as suggested by Sarma et al. (2009) in *C. punctatus*. Additionally, the ATPase activity showed a decreasing trend with increase in the acclimation period from 15 to 30 days. A similar trend of initial increase and subsequent decrease in the enzyme activity was reported by Verma et al. (2007b) in *C. carpio* exposed to increasing temperatures and chlorine.

CONCLUSION

In essence, overall results suggest that the combined effect of increasing temperatures and chlorine lead to stress in fish, thereby affected the protein, phosphorus and energy metabolism. The findings of the present work clearly delineate the effects of increasing temperatures and persistent chlorine toxicity on the metabolic profile of the Indian major carp, *L.*

rohita. Therefore, this study may serve as a base-line data for field investigations which may provide a better insight in to the health status of fish exposed to thermal pollutants at the source and at various points of its discharge into the environment.

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Table 1: Activities of the enzymes, aspartate amino transferase (AST) and alanine amino transferase (ALT) in *L. rohita* exposed to increasing temperatures and 0.1 mg L^{-1} chlorine for 15 and 30 days.

Enzyme	Acclimation period (days)	Temperature (°C) (Control group)				Temperature(°C) + 0.1 mg L^{-1} Chlorine (Experimental group)			
		26 (T$_1$)	31 (T$_2$)	33 (T$_3$)	36 (T$_4$)	26 (T$_5$)	31 (T$_6$)	33 (T$_7$)	36 (T$_8$)
AST (Liver)	15	84.13±1.43 [b,***]	104.29±2.63 [c,*]	110.38±2.91 [c,*]	159.36±3.65 [f,***]	62.35±1.61 [a,*]	90.76±1.87 [b,**]	121.19±2.45 [d,**]	137.92±2.08 [e,*]
	30	93.24±1.16 [b]	129.37±2.98 [d]	145.13±1.42 [e]	174.26±0.72 [g]	78.70±1.33 [a]	102.72±0.90 [c]	133.92±2.62 [d]	158.51±2.40 [f]
AST (Muscle)	15	50.04±0.88 [a,*]	58.70±1.37 [b,*]	67.53±1.83 [c,**]	80.27±1.58 [d,*]	61.80±1.81 [bc,*]	89.61±2.10 [e,**]	108.79±2.51 [f,***]	117.31±3.17 [g,***]
	30	61.33±1.00 [a]	65.66±1.12 [a]	83.86±1.75 [b]	99.53±0.85 [c]	94.44±1.40 [c]	105.53±2.79 [d]	121.24±2.55 [e]	135.80±2.22 [f]
ALT (Liver)	15	32.55±1.33 [b]	48.51±0.83 [c,**]	54.75±1.23 [d,**]	67.52±2.14 [f,**]	23.83±0.66 [a,*]	34.93±1.29 [b,*]	47.29±0.78 [c,*]	59.35±1.28 [e,**]
	30	38.54±1.99 [b]	60.12±1.20 [d]	73.40±2.50 [f]	88.64±1.17 [g]	31.30±0.87 [a]	53.21±2.23 [c]	66.75±1.12 [e]	74.49±2.81 [f]
ALT (Muscle)	15	14.52±0.36 [a,*]	15.42±0.43 [a,*]	26.53±1.11 [c,*]	29.72±0.34 [d,*]	19.79±0.32 [b,**]	25.20±0.67 [c,*]	32.03±0.89 [d,*]	41.50±1.50 [e,**]
	30	19.24±0.33 [a]	32.32±0.66 [c]	43.18±1.05 [d]	51.51±0.54 [f]	25.36±0.73 [b]	42.12±1.26 [d]	48.34±0.36 [e]	65.23±2.37 [g]

Values are expressed as mean ± SE ($n = 6$). Different superscripts (a, b, c, d, e, f, g, h) in the same row indicate significant difference amongst different treatments (Duncan's multiple range test, $P < 0.05$). Different superscripts (*$P < 0.001$, **$P < 0.01$, ***$P < 0.05$) in the same column indicate significant difference between two different acclimation periods (Student's *t*-test). Units: nanomole oxaloacetate formed/mg protein/min at 37°C (AST), nanomole pyruvate formed/mg protein/min at 37°C (ALT).

Table 2: Activities of the enzymes, alkaline phosphatase (ALP) and adenosine triphosphatase (ATPase) in *Labeo rohita* exposed to increasing temperatures and 0.1 mg L^{-1} chlorine for 15 and 30 days.

Enzyme	Acclimation period (days)	Temperature (°C) (Control group)				Temperature(°C) + 0.1 mg L^{-1} Chlorine (Experimental group)			
		26(T₁)	31(T₂)	33(T₃)	36(T₄)	26(T₅)	31(T₆)	33(T₇)	36(T₈)
ALP (Liver)	15	31.54±1.10 [a,**]	49.86±1.37 [c,***]	65.23±1.47 [e,**]	77.69±2.31 [f,***]	29.31±0.78 [a,**]	37.10±1.50 [b,***]	56.41±2.26 [d]	69.54±1.52 [e,***]
	30	37.20±0.77 [c]	57.18±2.32 [e]	75.37±1.69 [f]	85.56±0.52 [g]	21.89±1.03 [a]	30.23 ± 1.75 [b]	49.24±1.28 [d]	58.77±2.11 [e]
ALP (Muscle)	15	29.11±0.80 [b,***]	36.64±1.41 [c]	41.00±1.84 [d,**]	51.93±1.73 [f,**]	21.10±0.63 [a]	30.01±1.71 [b,***]	35.34±1.27 [c]	46.46±1.69 [e,**]
	30	32.64±0.71 [c]	40.32±0.78 [e]	48.61±0.36 [f]	64.96±1.56 [g]	18.56±0.93 [a]	23.05 ±1.26 [b]	31.23±1.51 [c]	36.24±2.00 [d]
ATPase (Gill)	15	0.345±0.032 [a]	0.577±0.025 [c,**]	0.625±0.022 [c,***]	0.805±0.006 [e,*]	0.478±0.026 [b]	0.697±0.031 [d,**]	0.798±0.009 [e,**]	0.910±0.013 [f,*]
	30	0.322±0.007 [a]	0.427±0.015 [b]	0.512±0.021 [c]	0.628±0.011 [d]	0.435±0.013 [b]	0.528±0.017 [c]	0.692±0.010 [e]	0.783±0.016 [f]
ATPase (Liver)	15	0.267±0.008 [a]	0.332±0.009 [b,**]	0.398±0.008 [c,**]	0.490±0.006 [d,**]	0.457±0.015 [d,**]	0.538±0.014 [e]	0.653±0.020 [f,***]	0.722±0.005 [g,***]
	30	0.248±0.011 [a]	0.270±0.013 [a]	0.345±0.007 [b]	0.427±0.015 [c]	0.375±0.008 [b]	0.495±0.016 [d]	0.565±0.019 [e]	0.640±0.021 [f]
ATPase (Muscle)	15	0.570±0.008 [a]	0.720±0.026 [b]	0.917±0.010 [d,**]	1.077±0.016 [f,**]	0.795±0.009 [c,***]	0.970±0.012 [e,*]	1.207±0.025 [g,**]	1.445±0.028 [h,**]
	30	0.553±0.011 [a]	0.633±0.031 [b]	0.757±0.023 [c]	0.920±0.012 [e]	0.645±0.037 [b]	0.833±0.008 [d]	1.010±0.014 [f]	1.278±0.008 [g]

Values are expressed as mean ± SE (*n* = 6). Different superscripts (a, b, c, d, e, f, g, h) in the same row indicate significant difference amongst different treatments (Duncan's multiple range test, α = 0.05). Different superscripts (*P < 0.001, **P < 0.01, ***P < 0.05) in the same column indicate significant difference between two different acclimation periods (Student's *t*-test). Units: nanomoles *p*-nitrophenol released/mg protein/minute at 37°C (ALP), microgram phosphorus released/mg protein/minute at 37°C (ATPase).

REFERENCES

[1] Arshad N, Shabbir G, Aleem S, Arshad M (2007) Effects of α-tocopherol on liver biochemistry of endosulfan intoxicated mice: A preliminary study. Asian J Exp Sci 21(2):239-246.

[2] Billiard R (2001) Carp: Biology and Culture. Springer-Verlag, New York.

[3] Cairns J, Heath AG and Parker BC (1975) The effects of temperature upon the toxicity of chemicals to aquatic organisms. Hydrobiolgia 47: 135-171.

[4] Caissie D (2006) The thermal regime of rivers: a review. Freshw Biol 51:1389-1406.

[5] Chatterjee N, Pal AK, Manush SM, Das T, Mukherjee SC (2004) Thermal tolerance and oxygen consumption of *Labeo rohita* and *Cyprinus carpio* early fingerlings acclimated to three different temperatures. J Therm Biol 29: 265-270.

[6] Chen SM and Kuo CM (2005) Behavioral and physiological responses of white spotted snapper juveniles, *Lutjanus stellatus* under the stress of temperature and residual chlorine. J Fish Soc Taiwan 32(2): 129-147.

[7] Choi DH, Park JS, Hwang CY, Huh SH, Cho BC (2002) Effects of thermal effluents from a power station on bacteria and heterotrophic nanoflagellates in coastal waters. Mar Ecol Prog Ser 229: 1-10.

[8] Das D (2002) Metabolism of proteins. In: Biochemistry (Ed. D. Das). Academic Publishers. pp. 463-504.

[9] Das T (2004) Biochemical and molecular characterization of Indian major carps in relation to thermal tolerance. PhD Thesis, Vidyasagar University, West Bengal, India.

[10] Das T, Pal AK, Chakraborty SK, Manush SM, Sahu NP, Mukherjee SC (2005) Thermal tolerance, growth and oxygen consumption of *Labeo rohita* fry (Hamilton, 1822) acclimated to four temperatures. J Therm Biol 30: 378-383.

[11] Das T, Pal AK, Chakraborty SK, Manush SM, Chatterjee N, Apte SK (2006) Metabolic elasticity and induction of heat shock protein 70 in *Labeo rohita* acclimated to three temperatures. Asian-Aust J Anim Sci 19(7):1033-1039.

[12] Das T, Pal AK, Chakraborty SK, Manush SM, Chatterjee N, Mukherjee SC (2004) Thermal tolerance and oxygen consumption of Indian Major Carps acclimated to four temperatures. J Therm Biol 29: 157-163.

[13] Emmanuel E, Keck G, Blanchard JM, Vermande P, Perrodin Y (2004) Toxicological effects of disinfections using sodium hypochlorite on aquatic organisms and its contribution to AOX formation in hospital wastewater. Environ Int 30: 891-900.

[14] Encina L, Rodrıguez-Ruiz A, Granado-Lorencio C (2008) Distribution of common carp in a Spanish reservoir in relation to thermal loading from a nuclear power plant. J Therm Biol 33: 444-450.

[15] Fiske CH and Subbarow Y (1925) The colorimetric determination of phosphorus. J Bio Chem 66: 375-400.

[16] Garen A and Levinthal CA (1960) Fine-structure genetic and chemical study of the enzyme Alkaline phosphatase of *E. coli*. I. Purification and characterization of Alkaline phosphatase. Biochem Biophys Acta 38: 470.

[17] Hallare AV, Schirling M, Luckenbach T, Kohler HR, Triebskorn R (2005) Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. J Therm Biol 30: 7-17.

[18] Krishnakumar V, Sastry JS, Swamy GN (1991) Implication of thermal discharges into the sea - a review. Indian J Environ Prot 11: 525-527.

[19] Langford TEL (1990) Ecological effects of thermal discharges. Elsevier Applied Science Publishers Ltd. England. pp. 28-103.

[20] Love RM (1980) The chemical physiology of fishes. vol. 2. Academic Press, London.

[21] Lowry OH, Ronebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin Phenol Reagent. J Biol Chem 193: 265-276.

[22] Neitzel DA, Johnson KI, Page TL, Young JS, Aing PM (1984) Correlation of bivalve biological characteristics and service water system design. In: Bivalve fouling of nuclear power plant service water systems. vol.1. U.S. Nuclear Regulatory Commission, Washington. pp. 1-l19.

[23] Post RL and Sen AK (1967) Methods in Enzymology In: Colowick SP, Kaplan NO, editors. 10. New York. Academic Press. pp. 762-8.

[24] Reynolds F (1987) European Carp. Agfact F3.2.3, NSW Fisheries.

[25] Saravanan P, Moushumi Priya A, Sundarakrishnan B, Venugopalan VP, Rao TS, Jayachandran S (2008) Effects of thermal discharge from a nuclear power plant on culturable bacteria at a tropical coastal location in India. J Therm Biol 33: 385-394.

[26] Saravanan P, Nancharaiah YV, Venugopalan VP, Subba rao T, Jayachandran T (2006) Biofilm formation by *Pseudoalteromonas ruthenica* and its removal by chlorine. Biofoul 22: 371-381.

[27] Sarma K, Pal AK, Sahu NP, Ayyappan S, Baruah K (2009) Dietary high protein and vitamin C mitigates endosulfan toxicity in the spotted murrel, *Channa punctatus* (Bloch, 1793). Sci Total Environ 407: 3668–3673.

[28] Schubel JR, and Marcy Jr BC (1978) Power Plant Entrainment-A Biological Assessment. New York, Academic Press Inc.

[29] Schubel JR, Coutant CC, Woodhead PMJ (1978) Power Plant Entrainment - A Biological Assessment.

[30] Scott GR and Sloman KA (2004) The effects of environmental pollutants on complex fish behaviour: integrating behavioral and physiological indicators of toxicity. Aquat Toxicol 68: 369-392.

[31] Sharma RM (1990) Effect of endosulfan on acid and alkaline phosphatase activity in liver, kidney and muscle of *Channa gachua*. Bull Environ Contam Toxicol 44(3): 443-448.

[32] US EPA (United States Environmental Protection Agency), (1989) Drinking water criteria document for chlorine, hypochlorous acid and hypochlorite ion. Cincinnati (OH): Environmental Criteria and Assessment Office.

[33] Verma AK, Pal AK, Manush SM, Das T, Dalvi RS, Chandrachoodan PP, Ravi PM, Apte SK (2007a) Persistent sub-lethal chlorine exposure augments temperature induced immunosuppression in *Cyprinus carpio* advanced fingerlings. Fish Shellfish Immunol 22: 547-555.

[34] Verma AK, Pal AK, Manush SM, Das T, Dalvi RS, Chandrachoodan PP, Ravi PM, Apte SK (2007b) Persistent sub-lethal chlorine exposure elicits the temperature induced stress responses in *Cyprinus carpio* early fingerlings. Pestic Biochem Physiol 87: 229-237.

[35] Wotton IDP (1964) Microanalysis. In: Churchill J, Churchill A, editors. Medical Biochemistry. 4th ed. London; 1964. p. 101-107.