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Toxicological and Biochemical Alterations of Cypermethrin (Synthetic Pyrethroids) Against Freshwater Teleost Fish *Colisa fasciatus* at Different Season

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Abstract: The cypermethrin (synthetic pyrethroids) has shows strong piscicidal activity in freshwater teleost fish *Colisa fasciatus* for all the exposure periods (24h or 96h) in time as well as dose dependent manner. The LC_{50} values decreases from 0.009 (24h) to 0.006 (96h) in winter season (water temp. 16°C) and 0.06 (24h) to 0.02 (96h) in summer season (water temp. 28°C). Sub-lethal doses (40 and 60% of LC_{50}) of cypermethrin after 96h was also significantly alter the levels of total protein, total free amino acid, in muscle and liver tissues, nucleic acids (DNA and RNA) in gonadal tissues and the activity of enzyme acetylcholinesterase (AChE), lactic dehydrogenase (LDH) and succinic dehydrogenase (SDH) in nervous tissue of the freshwater teleost fish *C. fasciatus* in time and dose dependent manner.

Key words: *Colisa fasciatus* · Cypermethrin · DNA and RNA · Metabolism

INTRODUCTION

The use of pesticides has increased considerably to reduce the change caused by pests to standing crops. Among these pesticides the synthetic pyrethroids are commonly used because of their rapid biodegradability and non-persistent nature. These compounds, which frequently enter the aquatic ecosystem through agricultural run-off and spraying operations adversely, affect non-target animals such as fish [1-3].

Pyrethroids are used preferably over organochlorine, organophosphorous and carbamate due to their high effectiveness, low toxicity to birds and mammals and easy biodegradability [4]. Cypermethrin is highly potent and broad-spectrum pyrethroids insecticides [5], used extensively for pest control. Although, it has non-persistence in the environment, the excess use of this, pyrethroids may enter into natural waters through agricultural run-off and ultimately cause damage to non-target organism. Fish are particularly highly sensitive to very low concentration of cypermethrin in the range of 0.4 to 2.2 µg/L [6-8]. Malla Reddy and Harold Philip [9] reported that the acute exposure of fish to cypermethrin induced decrease in some enzymatic activities. During sub-acute exposure to cypermethrin, the total protein, soluble protein and structural protein contents decreased in all the tissues. They further reported the decrement of all protein fractions was maximum in liver. Bradbury *et al.* [10] reported adverse effect of pyrethroids toxicity on fish gill structure.

C. fasciatus is the common larvivorous fish of South-East Asia [11]. This fish is also used for biological control of mosquito larvae in freshwater. This fish is also eaten by poor people, especially in villages. So, the aim of this study was to examine the toxicity of cypermethrin to the freshwater teleost fish *C. fasciatus* and evaluate the effect of sub-lethal doses of this pesticide on biochemical profiles of the fish.

MATERIALS AND METHODS

Fish: Adult freshwater teleost fish *C. fasciatus* of uniform size range (length 6.3±0.86 cm; width 3.6±0.49 cm; weight 2.4±0.24 g) were collected from different water bodies of Gorakhpur district of Uttar Pradesh, India and kept in glass aquaria containing 50L of de-chlorinated tap water for 7 days to acclimatize them to laboratory conditions. Water quality was measured according to the method of APHA [12]. The temperature of the experimental water was 23±0.7°C, pH was 7.3±0.2 dissolved oxygen was 7.2±0.3 mg/L, free carbon dioxide was 5.9±0.9 mg/L and alkalinity was 107. 7 .8 mg/L. Water was changed every

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day. Dead fish were removed as soon as possible to avoid water fouling. Fishes were fed daily on commercial fish food manufactured by Tokyu, Japan.

Pesticide: Technical grade synthetic pyrethroid (cypermethrin) 10% EC/active ingredient manufactured by Ira Chem. Ltd. Jyotiba Phule Nagpur (U.P.), India was used in the present toxicological and biochemical experiments.

Toxicity Experiments: Ten fishes were kept in glass aquaria containing 25L of de-chlorinated tap water. Fishes were exposed to four different concentrations of cypermethrin which were 0.005, 0.006, 0.007 and 0.008 mg/L at winter season and 0.02, 0.03, 0.04 and 0.05 mg/L at summer season. Synthetic pesticides cypermethrin were given as the final concentration (w/v) of aquatic ingredient in the test aquaria. Control fishes were kept in de-chlorinated tap water only. Each set of experiment was replicated six times. Mortality was recorded every 24h during the observation period of 96h. The LC values (LC_{10}, LC_{50} and LC_{90}), upper and lower confidence limits (UCL, LCL at 95% confidence limits), slope values, 't' ratio and heterogeneity were calculated by POLO computer programme [13]. The regression coefficient was determined between exposure time and different values of LC_{50} [14].

Biochemical Experiment

Total Protein: Total Protein levels were estimated according to the method of Lowry *et al.* [15], using bovine serum albumin as standard. Homogenates (5 mg/mL, w/v) were prepared in 10% Tri Chloro Acetic acid (TCA).

Total Free Amino Acids: Estimation of total free amino acid was made according to the method of Spices [16]. Homogenates (10 mg/mL, w/v) were prepared in 95% ethanol, centrifuged at 6000 xg and used for amino acid estimation.

Nucleic Acids (DNA and RNA): Estimation of nucleic acid (DNA and RNA) was performed, by the methods of Schneider [17] using diphenylamine and orcinol reagents, respectively. Homogenates (1 mg/mL, w/v) were prepared in 5% TCA at 90°C, centrifuged at 5000 xg for 20 min and the supernatant was used for the estimation of nucleic acids.

Acetylcholinesterase Activity: Acetylcholinesterase inhibition was measured in the nervous tissue of fish 96h to 40 and 60% of LC_{50} of the cypermethrin. Controls were kept in water alone. After 96h the acetylcholinesterase (AchE) activity was measured in the nervous tissue of *C. fasciatus* by the method of Ellman *et al.* [18], as modified by Singh and Agarwal [19], for this fish. Pooled nervous tissue (50mg) dissected from around the buccal mass was homogenised in 1.0 ml of 0.1M phosphate buffer pH 8.0 for 5 min. in an ice bath and centrifuged at 1000 g for 30 min at-4°C.

The enzyme containing supernatant (0.05 ml) was pipetted to a cuvette. To this was added $5x10^{-4}$M of freshly prepared acetylcholine iodide solution in distilled water as substrate, 1.45 ml of buffer (pH 8.0) and 0.05 ml of chromogenic agents, 5:5 dithio-bis-nitrobenzoate (DTNB). The change in optical density at 412 nm caused by the enzymatic reaction, was monitored for 3 min at 25°C.

Lactic Dehydrogenase Activity (LDH): Lactic dehydrogenase (LDH) activity was measured according to the method of Anon [20]. Homogenates (50 mg/L, w/v) were performed in 0.1 M phosphate buffer (pH 7.5) for 5 min at 4°C and supernatant was taken as enzyme source. Taken 0.038 mL M pyruvate substrates added 0.01 mL of supernatant, 0.5 mL phosphate buffer and 0.01 $NADH_2$ and incubate the mixture at 37°C for 45 min. Add 0.5 mL of 2, 4-dinitrophenyl hydrazine and mixture kept at room temperature for 20 min then added 0.5 mL of 0.4 M NaOH mixed and kept the mixture at room temperature for 30 min. Optical density was measured at 540 nm which converted to LDH units, by standard curve. Enzyme activity has been expressed as nano mole of pyruvate reduced/min/mg protein.

Succinic Dehydrogenase Activity (SDH): Succinic dehydrogenase (SDH) activity was measured by the methods of Arrigoni and Singer [21]. Homogenate (50 mg/L, w/v) was prepared in 0.5 M potassium phosphate buffer (pH 7.6) for 5 min in an ice bath and centrifuged at 10,000 g for 30 min at 4°C. Mixed 50 µ mole of succinate in supernatant and kept it pre-incubated at 37°C for 7 min. Then pre-incubated kept on ice and 0.05 mL aliquots for enzyme assay. Now in 2.9 mL cocktail containing 100 µ moles of phosphate buffer (pH 7.6) mix 30 µ mole of KCN and 0.80 µ mole of $CaCl_2$ and 0.04 µ mole of 2, 6-dichlorophenol indophenol (DCIP), 5-µ mole of succinate and 0.05% mL of 2% phenazine methosulphate (PMS) and finally mixed 0.05 mL of pre-incubated enzyme. Decrease in absorbance at 600 nm was monitored for 3 min. Enzyme activity has been expressed as µ mole dye reduced/min/mg protein.

Sub-lethal (40 and 60% of LC_{50} 24h) concentrations of cypermethrin pesticides used for biochemical experiments [22]

		Sub-lethal Concentrations (mg/L)	
Pesticides	Season	40% of 24h LC_{50}	60% of 24h LC_{50}
Cypermethrin	In winter	0.004	0.005
	In summer	0.024	0.036

RESULTS AND DISCUSSION

The mortality of freshwater teleost fish *C. fasciatus* due to exposure to four different concentrations of cypermethrin (synthetic pyrethroids) for 24, 48, 72 and 96h in different water temperature (16°C and 28°C) are represented in (Tables 1 and 2). The LC_{50} values of cypermethrin for *C. fasciatus at* 24, 48, 72 and 96h were 0.009, 0.008, 0.007 and 0.006 mg/L, respectively at 16°C water temperature (Table 1). However, this values were 0.06, 0.04, 0.03 and 0.02 mg/L for 24, 48, 72 and 96h at 28°C water temperature, respectively (Table 2). Cypermethrin was more toxic in winter season than the comparison of summer season.

The slope values were steep and the results were found to be within the 95% confidence limits of LC values. The 't' ratio was greater than 1.96 and the heterogeneity factor was less than 1.0. The 'g' value was less than 0.5 at all probability levels (Tables 1 and 2).

Cypermethrin was used as synthetic pesticides for biochemical studies. Tables 3 and 4 indicated a significant ($P<0.05$) dose dependent decrease in total protein level in both muscle and liver tissue of fish *C. fasciatus* exposed for 40 and 60% of 24h LC_{50} of cypermethrin. Protein depletion in both muscle and liver tissue was observed at both water temperatures. Exposure to 40 and 60% of the 24h LC_{50} of cypermethrin resulted in a decrease of protein levels in muscle of *C. fasciatus* to 78 and 69% at 16°C and 82 and 74% of control at 28°C, respectively. In case of liver the decrease amounted to 70 and 65% at 16°C and 78 and 70% of control values, respectively (Tables 3 and 4). The maximum reduction in protein level (65% of control) was observed in the liver and muscle of fish treated with 60% of 24h LC_{50} of cypermethrin at 16°C (Table 3).

Changes induced by the sub-lethal concentration of cypermethrin in the level of free amino acid in muscle and liver of fish *C. fasciatus* have been presented in Tables 3 and 4 at water temperature 16°C and 28°C, respectively. Exposure to 40 and 60% of 24h LC_{50} of cypermethrin upto 96h resulted in an increase of total free amino acids in muscle of *C. fasciatus* to value of 120 and 142% at 16°C water temperature and 116 and 137% of controls at 28°C, respectively. In liver increase was upto 125 and 156% at 16°C and 120 and 143% at 28°C on exposure to 40 and 60% of 24h LC_{50}, respectively (Tables 3 and 4). The maximum enhancement was observed (156% of control) in liver on exposure to 60% of 24h LC_{50} at 16°C (Table 3).

Sub-lethal concentration of cypermethrin resulted in a significant decline in the nucleic acids level (DNA and RNA) in gonadal tissue of fish *C. fasciatus* (Tables 3 and 4). In case of DNA, 96h exposure to 40 and 60% of 24h LC_{50} of cypermethrin resulted in a decrease of DNA level in gonadal tissue to value of 54 and 31% at 16°C and 58 and 36% of the controls at 28°C water temperature respectively. For RNA the decrease noted to 55 and 31% at 16°C and 58 and 35% of the level of control at 28°C water temperature respectively (Tables 3 and 4).

Tables 3 and 4 show that the treatment of fish with sub-lethal doses for 96h, caused significant ($P<0.05$) inhibition of AChE activity in the nervous tissue of *C. fasciatus*. Thus treatment with 40 and 60% of 24h, LC_{50} of cypermethrin reduced the AchE activity. Exposure to 40 and 60% of the 24h LC_{50} of cypermethrin resulted in a decrease of AchE activity in nervous tissue of *C. fasciatus* to 67 and 56% at 16°C and 72 and 59% of control at 28°C, respectively (Tables 3 and 4). Analysis of variance demonstrated that the inhibition of AChE was both time and dose dependent ($P<0.05$).

After 96h exposure to 40 and 60% of LC_{50} of cypermethrin, lactic dehydrogenase (LDH) activity was increased to 130 and 154% at 16°C water temperature and 121 and 150% of controls at 28°C, respectively (Tables 3 and 4). The maximum enhancement was observed (154% of control) in nervous tissue on exposure to 60% of 24h LC_{50} at 16°C (Table 3).

After 96h exposure to 40 and 60% of LC_{50} of cypermethrin, succinic dehydrogenase (SDH) activity was decreased to 62 and 54% at 16°C water temperature and 67 and 58% of controls at 28°C, respectively (Tables 3 and 4). The maximum decrease in (54% of control) in nervous tissue on exposure to 60% of 24h LC_{50} at 16°C, respectively (Table 3).

Data of biochemical section of the results clearly indicates that the sub-lethal exposure (40 and 60% of LC_{50}) of pesticides after 96h, significantly decrease the level of total protein, nucleic acids levels and enzyme activity acetylcholinesterase (AChE), succinic dehydrogenase (SDH) and significantly increase in total free amino acids level and enzyme lactic dehydrogenase (LDH) in muscle, liver, gonadal and nervous tissue of the freshwater teleost fish *C. fasciatus* at both the water temperature. The rate of depletion in total protein and nucleic acids level or enhancement in free amino acids level, were significantly ($P<0.05$) dose dependent.

Table 1: Toxicity (LC_{10}, LC_{50} and LC_{90}) of cypermethrin (synthetic pyrethroids) against freshwater teleost fish *C. fasciatus* at different time intervals in winter season (water temp. 16°C)

		Limits (mg/L)					
Exposure periods	Effective doses (mg/L)	LCL	UCL	Slope value	'g' factor	't' ratio	Heterog-eneity
24h	LC_{10}= 0.005						
	LC_{50}=0.009	0.008	0.010	6.41±1.34	0.17	4.54	0.20
	LC_{90}=0.014						
48h	LC_{10}=0.004						
	LC_{50}=0.008	0.007	0.010	4.90±1.16	0.21	4.06	0.12
	LC_{90}=0.014						
72h	LC_{10}=0.003						
	LC_{50}=0.007	0.006	0.008	4.32±1.10	0.25	3.86	0.14
	LC_{90}=0.013						
96h	LC_{10}=0.003						
	LC_{50}=0.006	0.005	0.006	5.08±1.13	0.19	4.61	0.22
	LC_{90}=0.010						

- There was no mortality in control groups.
- Water temperature was 16°C during the experiment.
- Batches of ten fishes were exposed to four different concentrations of cypermethrin.
- Concentrations given are the final concentrations (w/v) in aquarium water.
- Regression coefficient showed that there was significant ($P<0.05$) negative correlation between exposure time and different LC values.
- LCL = Lower confidence limit; UCL = Upper confidence limit.

Table 2: Toxicity (LC_{10}, LC_{50} and LC_{90}) of cypermethrin (synthetic pyrethroids) against freshwater teleost fish *C. fasciatus* at different time intervals in summer season (water temp. 28°C)

		Limits (mg/L)					
Exposure periods	Effective doses (mg/L)	LCL	UCL	Slope value	'g' factor	't' ratio	Heterog-eneity
24h	LC_{10}= 0.02						
	LC_{50}=0.06	0.04	0.08	3.43±0.70	0.16	4.85	0.23
	LC_{90}=0.13						
48h	LC_{10}=0.01						
	LC_{50}=0.04	0.04	0.07	2.37±0.59	0.23	4.01	0.16
	LC_{90}=0.11						
72h	LC_{10}=0.009						
	LC_{50}=0.03	0.03	0.05	2.12±0.56	0.27	3.76	0.12
	LC_{90}=0.09						
96h	LC_{10}=0.006						
	LC_{50}=0.02	0.01	0.02	2.20±0.57	0.25	3.85	0.31
	LC_{90}=0.08						

Details are as given in Table 1.

Table 3: Changes in total protein, total free amino acids, nucleic acid (DNA and RNA) (µg/mg), AChE activity (µ mole 'SH' Hydrolysed/min/mg protein), LDH activity (µ mole pyruvate reduced/min/mg protein) and SDH activity (µ mole dye reduced/min/ mg protein) in different tissues of freshwater fish *C. fasciatus* after exposure to (40% and 60% of 24h LC_{50}) of cypermethrin at winter season for 96h

Parameter	Tissues	Control	40% of LC_{50}	60% of LC_{50}
Protein	Muscle	156.50±1.14 (100)	122.07±1.26$^+$ (78)	107.98±1.36$^+$ (69)
	Liver	118.80±1.15 (100)	83.16±1.35$^+$ (70)	77.22±1.32$^+$ (65)
Amino acid	Muscle	26.50±0.98 (100)	31.80±1.25$^+$ (120)	37.63±1.45$^+$ (142)
	Liver	19.20±1.05 (100)	24.00±0.98$^+$ (125)	29.95±0.96$^+$ (156)
DNA	Gonadal	137.60±1.52 (100)	74.30±1.42$^+$ (54)	42.66±1.08$^+$ (31)
RNA	Gonadal	94.80±0.96 (100)	52.14±0.98$^+$ (55)	28.44±1.04$^+$ (31)
AChE	Nervous	0.098±0.0008 (100)	0.066±0.0008$^+$ (67)	0.055±0.0012$^+$ (56)
LDH	Nervous	0.037±0.0005 (100)	0.048±0.0006$^+$ (130)	0.057±0.0014$^+$ (154)
SDH	Nervous	46.28±1.12 (100)	28.69±1.04$^+$ (62)	24.99±1.06$^+$ (54)

- Values are mean ±SE of six replicates.
- Values in parenthesis are % change with control taken as 100%.
- +, Significant ($P<0.05$) when student's 't' test was applied between control and treated groups.

Table 4: Changes in total protein, total free amino acids, nucleic acid (DNA and RNA) (µg/mg), AChE activity (µ mole 'SH' Hydrolysed/min/mg protein), LDH activity (µ mole pyruvate reduced/min/mg protein) and SDH activity (µ mole dye reduced/min/ mg protein) in different tissues of freshwater fish *C. fasciatus* after exposure to (40% and 60% of 24h LC_{50}) of cypermethrin at summer season for 96h

Parameter	Tissues	Control	40% of LC_{50}	60% of LC_{50}
Protein	Muscle	154.50±1.15 (100)	126.69±1.35^{+} (82)	114.33±1.08^{+} (74)
	Liver	118.50±1.18 (100)	92.43±1.16^{+} (78)	82.95±1.14^{+} (70)
Amino acid	Muscle	27.20±1.08 (100)	31.55±1.20^{+} (116)	37.26±1.08^{+} (137)
	Liver	18.50±1.08 (100)	22.20±0.96^{+} (120)	26.45±0.98^{+} (143)
DNA	Gonadal	138.56±1.64 (100)	80.33±1.02^{+} (58)	49.86±1.18^{+} (36)
RNA	Gonadal	95.50±0.98 (100)	55.39±1.08^{+} (58)	33.42±1.15^{+} (35)
AChE	Nervous	0.096±0.0004 (100)	0.069±0.0012^{+} (72)	0.057±0.0008^{+} (59)
LDH	Nervous	0.038±0.0006 (100)	0.046±0.0008^{+} (121)	0.057±0.0012^{+} (150)
SDH	Nervous	46.56±1.25 (100)	31.20±1.14^{+} (67)	27.00±1.06^{+} (58)

Details are as given in Table 3.

The depletion of protein fraction in various tissues may have been due to their degradation and possible utilization of degraded products for metabolic purposes. Mommensen and Walsh [23] reported that proteins are mainly involved in the architecture of the cell, which is the chief source of nitrogenous metabolism and during chronic period of stress they are also a source of energy. Decreased total protein level was observed in the muscle and liver tissues of the freshwater teleost fish *C. fasciatus* exposed to sub-lethal doses of malathion and carbaryl pesticides [24].

The quantity of protein depends on the rate of protein synthesis or its degradation. It also affected due to impaired incorporation of amino acids into polypeptide chains [25]. The synthesis of RNA plays an important role in protein synthesis. The inhibition of RNA synthesis at transcription level, thus may affect the protein level. In this study, a significant decline in RNA level in exposed freshwater fish was observed. The decrease in the RNA concentration may also have been a cause of protein depletion. Alternatively, the increase in protease activity may be the cause of increased protein degradation.

Cypermethrin was also significantly decreased the level of nucleic acids in the various tissues of the fish *C. fasciatus*. Several reports are available on the reduction in DNA and RNA level on exposure to different pesticides [26,27]. Mahendru [28] suggested that the anti-AChE compounds attack many enzymes responsible for normal metabolism pathway.

The increase in free amino acid level suggests tissues damage probably due to the increased proteolytic activity under toxic stress. However, the elevated levels of free amino acid can be utilised for energy production by feeding them in to the TCA cycle through aminotransferase reaction. The increase in the levels of free amino acid can also be attributed to the synthesis of amino acids in addition to their elevation by protein hydrolysis. A third possibility for increased amino acid level might be their increase due to transamination and deamination of keto acids [29,30].

The enzyme acetylcholinesterase (AChE) occurs in the outer basal lamina of synapses [31], neuromuscular junction [32] and in some other tissues. AChE is responsible for the termination of cholinergic impulse by the hydrolysis of acetylcholine (ACh) to choline and acetic acid [33-38].

Acetylcholine (ACh) is the most important neurotransmitter in most animals. It is resulted by a stimulated nerve cell into the synapse, or neuromotor junction with another nerve cell. Once ACh has been secreted into the synapse it binds to receptor sites on the next nerve cell, causing the latter to propagate the nerve impulse. Before the transmission of second impulse through the synapse, ACh secreted after the first impulse must be hydrolised by the AChE in the junction.

Inhibition of AChE resulted in abnormal accumulation of acetylcholine, which cause, eventual paralysis of the muscle. Death occurs as a result of asphyxia caused by the paralysis of respiratory muscle [39]. Several studies on the mode of action and inhibition of acetylcholinesterase have been carried out for the last few decades. Indeed, inhibition of this enzyme is the focal target for most of the current synthetic pesticides. It has been established that the AChE enzyme unit consists of a negative sub-site, which attracts the quarternary group of choline through both coulombic and hydrophobic forces and an esteratic sub-site, where nucleophilic attack occurs on the acyl carbon of the substrate [40]. The catalytic mechanism resembles that of other serine esterases (e.g. alkaline phosphatases), where a serine hydroxyl group is rendered highly nucleophilic through a charge-relay system involving the close apposition of an imidazole group and, presumably a carboxyl group on the enzyme.

Lactic dehydrogenase (LDH) forms the centre for a delicately balanced equilibrium between catabolism and anabolism of carbohydrates [41] and also associated with cellular metabolic activity [42]. Lactic dehydrogenase (LDH) catalyzed the inter-conversions of lactic acid and pyruvic acid during anaerobic conditions. Inhibition of LDH and SDH activity indicates that cypermethrin pesticides significantly inhibits aerobic, as well as anaerobic metabolism in exposed animals. Succinic dehydrogenase (SDH) is one of the active regulatory enzymes of the TCA cycle. The reasons for an increase SDH level after exposure to pesticides are not clear. A similar situation was observed increase of SDH level in malathion exposed, irradiated rats. Ganathy *et al.* [43] observed LDH activity in the gill of *Channa punctatus* exposed to hexachlorocyclohexane.

Succinic dehydrogenase (SDH) activity was decreased in the body tissues of *Lymnaea acuminata* exposed to sub-lethal doses of rogohit, sevin and stop. Similar decrement in the SDH activity was also observed by the various workers in different organism exposed to the toxicants [44]. Decreased SDH activity was observed in the hepatopancreas and ovotestis tissues of the freshwater snail *Lymnaea acuminata* exposed to sub-lethal doses of dimethoate and carbaryl pesticides [45]. The general decrease in SDH activity during pesticides stress was associated with the inhibition of mitochondrial respiratory mechanism [46], or derangement in ultra structure, architectural integrity and permeability of mitochondria [47].

In conclusion, the cypermethrin is toxic to the freshwater teleost fish *C. fasciatus*. The sub-lethal doses of cypermethrin significantly altered the total protein, total free amino acids, nucleic acids (DNA and RNA) and certain enzyme AChE, LDH and SDH in the fish. Fish with less nutritional value are not good for human consumption.

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