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K Sobha
Department of Biotechnology, (Currently Department of Mathematics & Humanities), RVR & JC College of Engineering, Guntur, Andhra Pradesh, India

N Yamini Sarada
Department of Biotechnology (Currently Department of Mathematics & Humanities), RVR & JC College of Engineering, Guntur, Andhra Pradesh, India

T Anita Susan
Department of Zoology, Andhra Christian College, Guntur, Andhra Pradesh, India

Correspondence
K Sobha
Department of Biotechnology (Currently Department of Mathematics & Humanities), RVR & JC College of Engineering, Guntur, Andhra Pradesh, India

An evaluation of the alterations in protein content, total free amino acids and protein profiles of some major tissues of the edible carp, *Labeo rohita* (Hamilton) exposed to nitrogenous compounds

K Sobha, N Yamini Sarada and T Anita Susan

Abstract
Anthropogenic discharges containing compounds of nitrogen, especially the excess use of fertilizers in agriculture, effect eutrophication in the water bodies resulting in mass mortality of aquatic fauna. As fish are a highly valued human protein food, the present study is attempted to evaluate the changes that occur in the protein constituents of different tissues of the edible carp, *Labeo rohita*, on exposure to sub lethal concentrations of ammonium, nitrite and nitrate species. Estimation of proteins in the tissues gill, liver, brain and muscle of the control and the exposed fish indicated a very significant drop in the protein levels in ammonia exposed tissues as compared to NO_2^- and NO_3^- exposures. Brain and liver tissues of nitrate exposed fish had higher levels of proteins indicating the operation of damage repair mechanisms in these tissues. The significant increase in the total free amino acid (FAA) levels in fish exposed to the toxicants is suggestive of high protein turn over in them. Fish cultured in fresh water with low doses of nitrogen compounds had more amino acids, in general, than the fish cultured in pure fresh water.
Electrophoretic studies demonstrated several distinct changes in protein patterns of the vital tissues studied, suggestive of a greater vulnerability of high molecular weight proteins of structural and physiological importance, including metabolic enzymes.

Keywords: nitrogenous compounds, protein profiles, free amino acids, electrophoresis, *Labeo rohita*

1. Introduction
Nitrogen cycle operating naturally in ecosystems involves the reactions causing the inter-conversions among the three nitrogenous compounds viz. ammonia, nitrite and nitrate and any imbalance would lead to higher levels of one or more of these compounds posing the problem of toxicity to animals [1-3]. Unionized ammonia is highly toxic to aquatic organisms, particularly fish, against the less toxic/non-toxic ionized ammonia [4-10]. Inhibition of nitrification process because of unionized NH_3 toxicity to nitrifying bacteria can result in increased accumulation of NH_4^+ and NH3 in aquatic environment intensifying the toxicity to bacteria and aquatic animals [9]. While nitrates do not form unionized species, HNO_2 (unionized form of nitrite) is relatively less in concentrations in the pH range 7.5-8.5 and non-toxic [5, 6, 8, 9].
High concentrations of nitrogenous compounds like nitrite and nitrate species in aquaculture facilities with water re-circulating systems were reported to cause severe physiological disturbances and/or mass mortality of fish. Physiological disruptions include ion regulatory, respiratory, cardiovascular, endocrine, excretory, immunological processes etc. and the major effect of nitrates and nitrites is the oxidation of haemoglobin to methaemoglobin / ferrihaemoglobin (caused by NO_2^- sps.), impairing its oxygen carrying capacity [3, 11, 12]. It has also been reported that NH_4^+ ions could contribute to NH_3 toxicity by reducing internal Na^+ to fatally low levels affecting the survival of aquatic animals [4, 7, 9, 13]. NO_3^- must be converted to NO_2^- inside the body to become toxic. The relatively low toxicity of NO_3^- ions is due to their low branchial permeability [14-16]. Field data suggest that nitrogen-based fertilizers such as ammonium nitrate, potassium nitrate and sodium nitrate may be contributing to the decline of sensitive species in aquatic ecosystems like fish [17] and amphibians [15, 18].
The concentration of nitrite in water under normal conditions is in μM range, but gets elevated due to the entry of nitrogenous effluents, particularly under hypoxic conditions, causing intensive

damage to cultured commercial fish species and ornamental fish [12]. Freshwater fish acquire ions through diet and also by mechanism associated with chloride cells of the gills to maintain hyper-osmotic internal milieu [19]. Chloride cells are actively involved in the uptake of Na^+ ions in exchange for NH_4^+ /H^+ ions and Cl^- ions for HCO_3^- ions. NO_2^- has an affinity for the branchial Cl^- uptake through Cl^-/HCO_3^- exchanger and hence the presence of NO_2^- in water causes a shift of Cl^- uptake, in part, for NO_2^- uptake [2]. Nitrite toxicity in fish is alleviated to a certain extent by its possible oxidation to nitrate by the enzymes like catalase and cytochrome oxidase [20, 21]. The rise of extracellular K^+ adversely affects the excitable tissues like heart and liver and the decrease in intracellular K^+ concentration affects muscular metabolism & function [2]. Considering the indiscriminate use of nitrogenous fertilizers in the agricultural fields for high productivity, the present study was aimed at evaluation of toxicity of these nitrogenous compounds in terms of LC_{50} values to the cultured edible carps like *Labeo rohita* and to know the variations, if any, in the protein content, total free amino acid composition and protein profile of the control and exposed tissues.

2. Materials and Methods

Labeo rohita is a fresh water edible fish, extensively cultured in ponds, particularly in Guntur district of Andhra Pradesh, India.

2.1 Experiments for determination of LC_{50}

Fish with mean length of 7 centimeter and weight of 5.5 gram were brought from local fish farm of Nandivelugu, Guntur District, Andhra Pradesh, India and acclimated to the laboratory conditions at 28 – 30ºC for a period of 7 days. If, in any batch of fish, mortality exceeded 5% during acclimation, that batch of fish was discarded. Feeding was stopped 24h before the commencement of experiments and through the period of experimentation. APHA [22] protocols were employed for preparation of stock solutions of ammonia, nitrite and nitrate (sources were ammonium chloride, sodium nitrite and sodium nitrate) and the concentrations of the three 'N' species were estimated spectrophotometrically before conducting toxicity tests. NH_3-N was determined by Nessler's reagent, NO_2-N by Griess reaction using sulphanilic acid and NO_3-N by reaction with sodium salicylate in an acidic environment. Experimental tubs, each containing 10L water (without or with toxicant) and 10 fish of uniform size and weight were used to determine the LC_{50} concentrations for the three toxicants. From the wide range of test concentrations studied, five concentrations were taken that resulted in a mortality range from 10 - 90% for 24 hours in static system. All the tests were done in triplicate and the mean values determined. Finney's [23] probit analysis as recommended by Roberts and Boyce [24] was followed to calculate the median lethal concentration LC_{50} values. The chemical parameters of the tap water used for the toxicity experiments were: Turbidity – Nil; pH 7.6; Total hardness – 660 mg/l; Carbonate hardness as CO_3^{2-} – 660mg/l; Chloride as Cl^- – 340 mg/L; Calcium – 140 mg/L; Magnesium – 520 mg/L; Phenolphthalein alkalinity – Nil; Dissolved oxygen – 7 mg/L; TDS – 1326 mg/L; Electrical conductivity at 25^{oC}: 0.201 S/m; Total alkalinity – 3.996 mmol/L.

2.2 Protein Estimation

Fish exposed to sub lethal concentrations (1/10th of 24h LC_{50}) of ammonia, nitrite and nitrate for ten days were sacrificed and vital tissues viz. Gill, brain, muscle and liver were pooled for proteomic studies. 5% homogenate of gill and brain and 2% homogenates of muscle, liver and kidney were prepared in ice cold 5% trichloroacetic acid (TCA) and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and the resulting protein pellet was dissolved in 1 ml of 1N NaOH. Protein concentration in each of the tissues was estimated by modified method of Lowry *et al*. [25] and expressed as mg of protein per gram of the weight of the tissue using the standard graph plotted with Bovine Serum Albumin (BSA – 1mg/ml) purchased from Sigma Aldrich, USA. Student's 'T' test was conducted to know whether there were any significant differences between the control and the exposed tissues.

2.3. Determination of Molecular weight

SDS-PAGE was performed as described by Laemmli *et al*. [26] using 10% resolving gel. Electrophoresis was performed at a constant voltage of 200 volts when the samples were in stacking gel and increased to 245 volts when in the resolving gel. After that, resolving gel was stained using coommassie brilliant blue and molecular weights of proteins were determined by comparing them with standard protein markers.

2.4. Estimation of Total Free amino acid content

1 gm of muscle tissue was taken from the fish exposed to sub-lethal concentrations of ammonia, nitrite and nitrate for a period of 10 days, mashed in a homogenizer with 8 ml of phosphate buffer pH 7, centrifuged at 3000 rpm for 20 minutes at 4ºC. The supernatant was stored at 4ºC while the deposit was dissolved in 5 ml of 1N NaOH and centrifuged at 3500 rpm for 20 minutes at 4ºC. The supernatant was collected and stored at 4ºC while the deposit was subjected to protein hydrolysis by dissolving in 6 ml of 6N HCl and keeping in incubator shaker at a temperature of 60ºC for a period of 24 hrs. Then the tubes were centrifuged at 3500 rpm for 15 minutes. The supernatant was filtered and neutralized with 1N NaOH. Finally the filtrate was pooled with other supernatants collected during the earlier steps and was analyzed for amino acid content by HPLC.

Pre-column OPA (orthopthalaldehyde) derivatization was done for HPLC (High Performance Liquid Chromatography) analysis of amino acids [27]. The Principle is based on the reaction of primary amines with OPA in the presence of mercaptoethanol to form 1-thio substituted 2-alkyl isoindoles. The reaction is rapid with high sensitivity and the isoindoles so obtained were subjected to HPLC separation. Mobile phase A was 20mM sodium acetate with 0.018% triethylamine. Mobile phase B consisted of 20% of 100 mM sodium acetate, 40% methanol and 40% acetonitrile. pH of both the mobile phases A and B was adjusted to 7.2±0.05 with 2% acetic acid. OPA and standard amino acids' mixture of different concentrations (1nm, 250 pm, 100 pm, 25pm and 10 pm) provided by Hewlett Packard were used as standards. The instrument Agilent 1100 HP – HPLC (Eclipse AAA columns ZORBAX) and chemstation software were used in the present study.

First, the instrument was calibrated using 1.0 nm, 500 pm and 250 pm standards individually. 10 µl of the standard was mixed with 60 µl borate buffer and 10 µl of OPA reagent in dilution vial and cyclomixed. From this mixture, 50 µl was injected in the HPLC using Hamilton syringes. Each standard is individually run in the gradient program and the chromatogram obtained. The two consecutive runs that had the same retention time were taken and the average of them

was used for plotting the calibration curve. Then 50 µl of the sample mixture was injected in HPLC using Hamilton syringes and from the chromatogram obtained, the area of the peaks was recorded, calibrated along with the standards and used for calculation. Reaction temperature was maintained at 40°C, the flow rate of the sample was kept at 0.5 ml/min and the detection wavelength (VWD) was 338 nm. The limit of detection (LOD) and the limit of quantification (LOQ) were determined based on the residual standard deviation of the response and the slope of the linearity plot [28].

Percentage composition of the essential amino acids in relation to the measured total FAA concentrations was calculated by the expression, quantity of essential amino acids/quantity of total amino acids X 100. A/E ratio percentages of essential amino acids was calculated using the formula, Quantity of essential amino acid/Total quantity of essential amino acids X 100 [29].

3. Results and Discussion

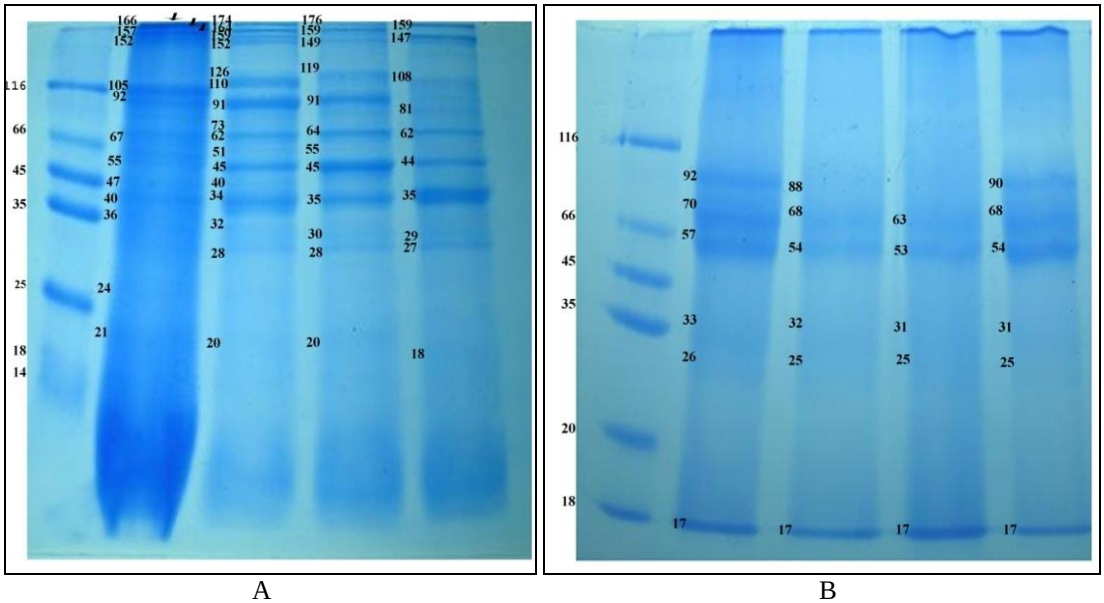
Nitrogenous compounds Ammonia, Nitrite and Nitrate were tested on fresh water fish *Labeo rohita* and 24h LC_{50} values for ammonia, nitrite and nitrate were determined to be 0.06 mg/L, 9.09 mg/L and 145.1 mg/L respectively. $1/10^{th}$ of the 24h LC_{50} values obtained (0.006 mg/L, 0.909 mg/L and 14.51 mg/L for ammonia, nitrite and nitrate respectively) were used for further experiments on evaluation of the effects of sub-lethal exposure to the three nitrogenous metabolic toxicants. Sub-lethal exposure for 10 days was done by adding the $1/10^{th}$ of 24h LC50 concentration everyday to the experimental tubs at a fixed period of time in the day.

Protein levels in different tissues of the experimental fish *Labeo rohita* are presented in Table 1. Highest levels of protein were present in the control muscle tissue followed by liver, gill and brain. In all the tissues of the fish exposed to ammonia, there is a drastic reduction in the protein content suggesting the highly inhibitory effect of the toxicant ammonia. In nitrite exposed fish, similar trend was found except that in muscle tissue, there was a marginal increase in protein level while nitrate exposed fish, gill and muscle tissues had lowered protein levels in comparison with the control. But brain and liver tissues of the fish exposed to nitrate showed an increase in the protein content, suggesting the role of these tissues in damage repair mechanisms. Student's 'T' test indicated that in general there was a highly significant decrease in the protein levels in the exposed tissues as compared to control ($P<0.001$) except in the case of muscle and liver in which the differences were in terms of increase between the control and the nitrite/nitrate exposed and were significant ($P<0.05$).

Table 1: Protein levels in different tissues of the control and the exposed fish, *Labeo rohita* to nitrogenous compounds.

S. No.	Amount of protein in mg/g of the wet weight of the tissue expressed as mean±S.D. (n=5)			
	Control	Ammonia	Nitrite	Nitrate
Gill	144.40±13.3	1.61±0.15	59.40±6.12	106.24±13.4
Brain	115.81±5.34	2.52±0.24	85.50±3.43	186.74±14.45
Muscle	232.00±20.5	9.20±0.4	246.00±31.9	221.80±37.5
Liver	227.00±19.42	10.60±1.36	131.80±13.32	256.40±29.28

Protein banding pattern in gill, brain, muscle and liver of *Labeo rohita* exposed to the three nitrogenous compounds was studied in comparison to the healthy, control fish. Several distinct changes in the proteins of these tissues were found in the present study. Marker protein has six protein sub unit bands with molecular weights 116 kDa, 66 kDa, 45 kDa, 35 kDa, 25 kDa and 18 kDa. Among the control tissues, muscle has the highest number of protein sub unit bands (24) followed by liver (16), brain (12) and gill (6) in the order of decrease (Fig. 1A-1D).



A B

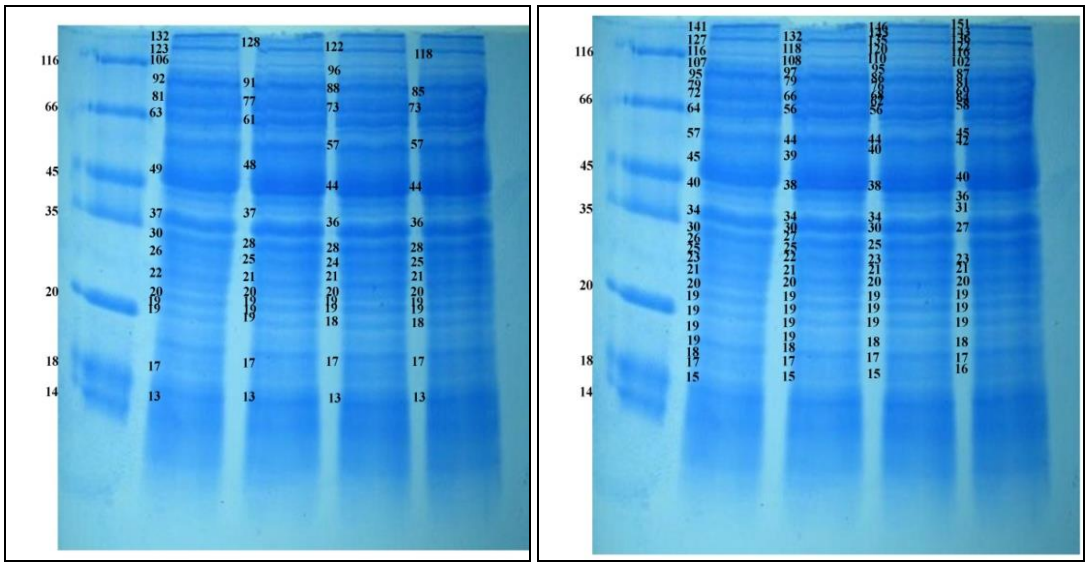


Fig 1: Protein fractions of tissues of *Labeo rohita* exposed to various nitrogenous compounds. Lane 1: Marker; Lane 2: Control; Lane 3: Ammonia exposed; Lane 4: Nitrite exposed; Lane 5: Nitrate exposed. Molecular weights are indicated in kDa. A: Brain; B: Gill; C: Liver; D: Muscle.

Neural proteins include structural, metabolic, signal transducing and oxidative stress response proteins which get altered on exposure to various inorganic and organic toxicants [30]. In the present study, significant variations in the brain protein profiles (Fig. 1A) revealed the acute toxic effects of nitrogenous compounds to aquatic organisms. Further proteomic studies might help in identifying potential protein biomarkers for aquatic environmental monitoring [30]. In ammonia exposed brain tissues, 14 new bands with molecular weights of ~20, 28, 32, 34, 45, 51, 62, 73, 91, 110, 126, 159, 164 and 174 kDa were found indicating the enormous strain imposed on brain metabolism by the toxicant. In nitrite and nitrate exposed brain tissues, the changes were relatively less with three (30, 55, 140 kDa) and seven (18, 27, 29, 44, 81, 108, 147 kDa) new bands respectively. The proteins identified in the brain tissue appear to correspond to the enzymes enolase, dihydropyrinidinase like 2, ATPase, pyruvate kinase fragment, carbonic anhydrase, creatine kinase, brain apolipoprotein A.

The gills (Fig. 1B) representing an extremely large relative surface area are the first to get exposed to the external medium, owing to their respiratory function and hence are rapidly affected by any change in environmental water quality [31]. Control fish showed six protein bands corresponding to molecular weights 17, 26, 33, 57, 70 and 92 kDa. In ammonia exposed fish, the total number of proteins bands obtained were six, out of which four bands corresponding to 32, 54, 68 and 88 kDa were new. Likewise, nitrite exposed gills showed three new bands with molecular weights 31, 53, and 63 kDa; nitrate exposed fish demonstrated four new bands corresponding to 31, 54, 68 and 90 kDa proteins. Changes in gill epithelium and intracellular turnover, chloride cell turnover, mucus glycoprotein and collagen of the musculoskeletal structures of the gill filaments appear to be affected by the toxicants [31].

Protein banding pattern in the liver (Fig. 1C) of control and exposed fish could be corroborated to the standard structural (keratin and keratin like proteins), oxidative stress response (superoxide dismutase (SOD), signal transducing (annexin-4), and metabolic proteins like methionine adenosyl transferase, adenosyl homocysteinase and pyruvate dehydrogenase [32]. Protein expression in the liver of fish exposed to ammonia is significantly different with reference to control as well as nitrite and nitrate exposed tissues. Protein bands of molecular weights 61, 77 and 128 kDa were exclusively noticed under ammonia exposure. The occurrence of approximately 16 kDa protein that possibly corresponds to superoxide dismutase in the liver of ammonia exposed fish indicates the heavy oxidative stress induced by the toxicant. The predominantly similar protein profiles in liver of fish exposed to nitrite and nitrate in low molecular weight range is suggestive of the fact that the toxicity induced by the two toxicants, nitrite and nitrate, at the level of metabolism and oxidative stress were similar. A 44 kDa protein band found in nitrite and nitrate exposed liver, absent in control, might be specifically associated with the above two toxicants.

The major group of enzymes of the sarcoplasm are the enzymes associated with energy producing metabolism like glycolytic activity and ATP hydrolysis, in addition to structural proteins like myosin, tropomyosin and actin. Other proteins include those responsible for cellular activity such as ribosomal protein, transcription factor - zinc finger protein and signal transduction proteins [33-35]. Electrophoresis of sarcoplasmic proteins of the control and the exposed fish showed protein bands (Fig. 1D) corresponding to the major constituent structural proteins like myosin, tropomyosin, fimbrin; enzymes of glycolysis and transcription factors. Significant differences were noticed in high molecular weight proteins, while the low molecular weight proteins appeared less affected. In the ammonia exposed muscle tissue, 8 new bands corresponding to molecular weights 38, 44, 56, 66, 97, 108, 118 & 132 kDa appeared. Similarly nitrite and nitrate exposed muscle tissue revealed 8 & 10 new bands respectively, suggesting the vulnerability of muscle protein expression in the toxicant induced fish.

The lowest LOD and LOQ values (Table 2) were obtained for tyrosine (21.65 and 65.62nmoles/ml respectively) and the highest for arginine (211 and 639.65 nmoles/ml respectively). Changes in free amino acid contents of tissues are reported to provide useful information regarding the levels of stress at the tissue level. Increase in FAA levels is attributed to reasons like proteolysis and/or increased synthesis of FAA by transaminase

reaction [36]. To meet the energy demands induced by stress, proteins may undergo breakdown and release amino acids which in turn get oxidized to furnish energy for body function [37]. Studies on the effect of toxicants on protein metabolism of the fish *Labeo rohita* demonstrated a decrease in the total protein and an increase in the free amino acid levels [38-40].

Table 2: LOD and LOQ for the fifteen standard amino acids calculated from the details of calibration curve.

S. No	Standard amino acid	Residual Std. deviation (SD)	Slope (s)	LOD (nmoles/ml) 3.3*(SD/s)	LOQ (nmoles/ml) 10*(SD/s)	R^2
1	Aspartic acid	91.789	5.63967	53.7	162.75	0.98957
2	Glutamic acid	266.4	5.26397	167.0	506.08	0.96750
3	Serine	207.44	5.25317	130.3	394.8	0.98822
4	Histidine	613.829	12.80086	158.2	479.5	0.97068
5	Glycine	39.5289	4.66498	27.9	84.7	0.99714
6	Threonine	162.437	4.85340	110.4	334.6	0.95800
7	Alanine	158.935	6.01367	87.2	264.2	0.99081
8	Arginine	468.202	7.31958	211.0	639.65	0.97292
9	Tyrosine	56.356	8.58790	21.65	65.62	0.99943
10	Valine	70.563	5.45813	42.6	129.2	0.99778
11	Methionine	163.0	5.79821	92.7	281.1	0.98963
12	Phenyl alanine	85.808	5.32506	53.17	161.1	0.99656
13	Isoleucine	56.83	5.44076	34.4	104.4	0.99855
14	Leucine	142.84	5.71352	82.5	250.0	0.99177
15	Lysine	91.94	8.69824	34.8	105.6	0.99851

Table 3: Measured concentrations of the chosen fifteen amino acids present in the muscle tissue of the fresh water fish *Labeo rohita* exposed to the three nitrogenous compounds, as analyzed by High Performance Liquid Chromatography (HPLC).

S. No.	Name of the amino acid	Measured concentrations of amino acids in nmoles/ml			
		Control	Ammonia	Nitrite	Nitrate
1	Aspartic acid	21.5	39.3	11.8	4
2	Glutamic acid	97.2	112.1	92.6	74.5
3	Serine	160.1	300.1	81.2	76.9
4	Histidine	6.3	26.1	18.8	17.1
5	Glycine	361	622.4	718.7	919.5
6	*Threonine	42.8	73.3	73.8	94.9
7	Alanine	342.2	383	653.6	541.6
8	Arginine	26.9	71.7	79.6	88.8
9	Tyrosine	11.1	67.5	48.1	18.9
10	Valine	67.3	39.9	35.1	77.4
11	*Methionine	20.1	45.8	28.2	45.1
12	Phenyl alanine	6.2	22.7	7.2	19.2
13	Isoleucine	16.6	37.2	17.2	30.4
14	Leucine	32.4	47.5	31	48.7
15	*Lysine	36.3	103.5	146	121.9
Total		1248.0	1992.0	2042.9	2178.9

*Amino acids often limiting are lysine, threonine, tryptophan and sulfur-containing amino acids (Methionine and Cysteine).

From the data presented in Fig.s 2A to 2D and Table 3, it is evident that fish cultured in fresh waters with low doses of nitrogen compounds would have more amino acids in general than the fish cultured in pure fresh water. This could be taken advantage in preparing amino acid supplements from processed fish cultured in nitrogen rich water.

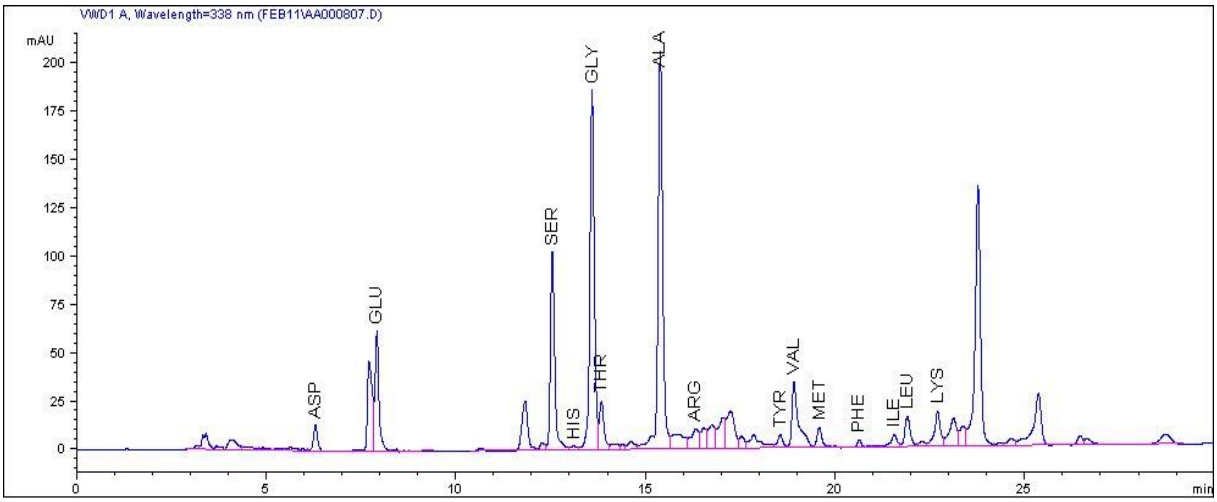


Fig 2A: Concentrations of total free amino acids in control *L. rohita*

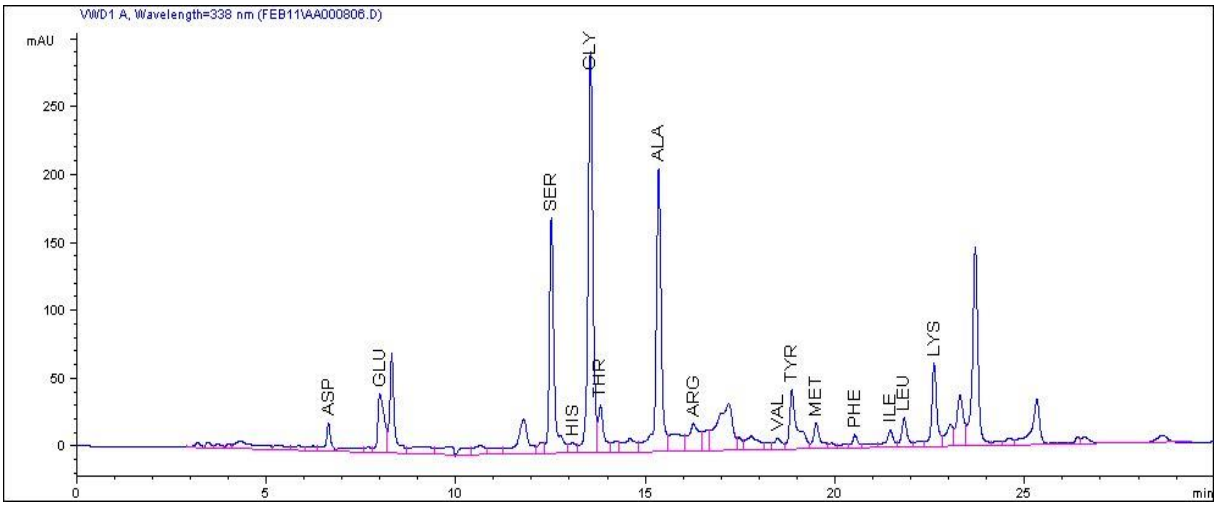


Fig 2B: Concentrations of total free amino acids in Ammonia exposed ***L. rohita***

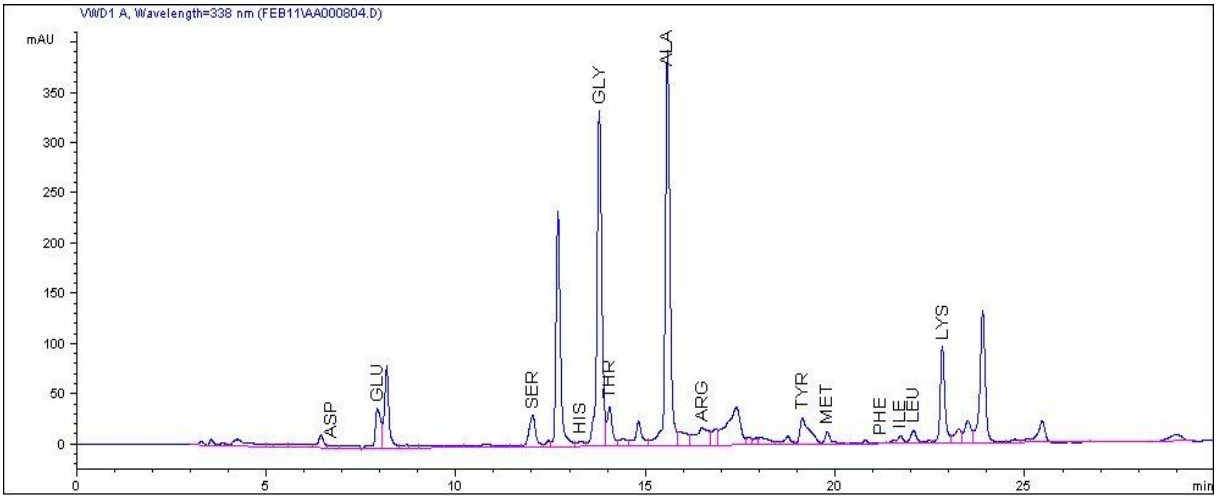


Fig 2C: Concentrations of total free amino acids in Nitrite exposed *L. rohita*

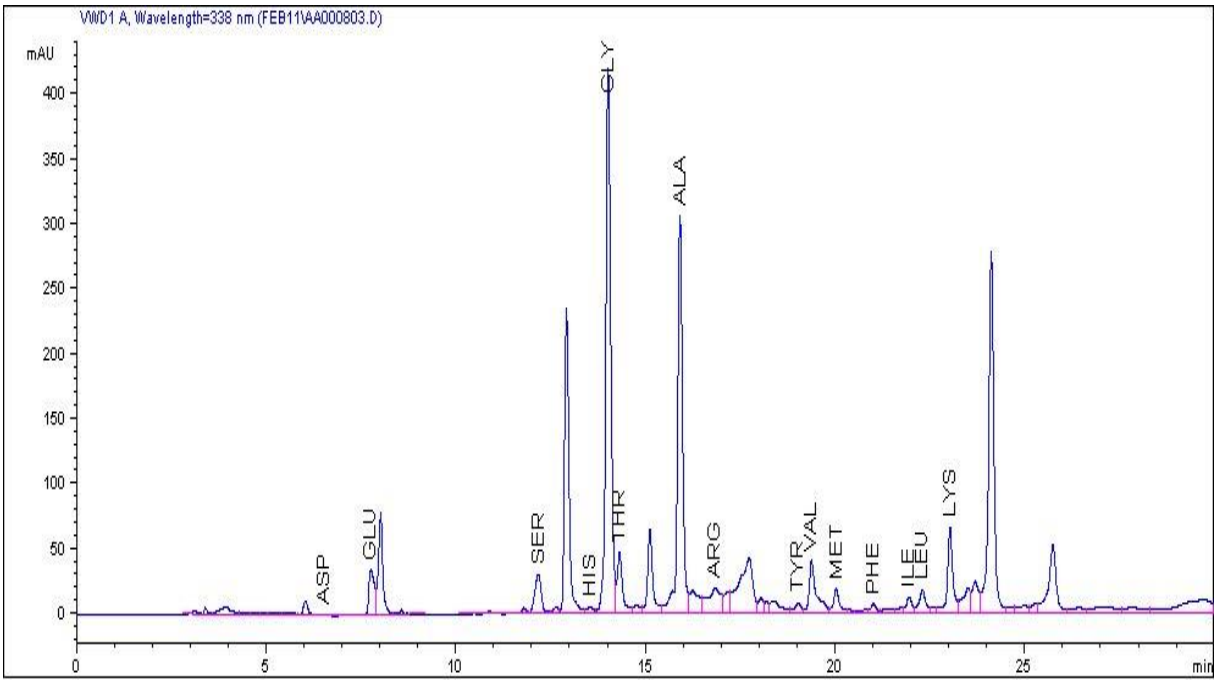


Fig 2D: Concentrations of total free amino acids in Nitrate exposed *L. rohita*

Percentage composition of essential amino acids in relation to the total measured FAA composition (A/E) of the muscle tissue in control, ammonia, nitrite and nitrate exposed fish were 20.4, 23.5, 21.4 and 24.9 respectively (Fig. 3). In general, FAA profiles of the exposed fish were significantly higher than the control.

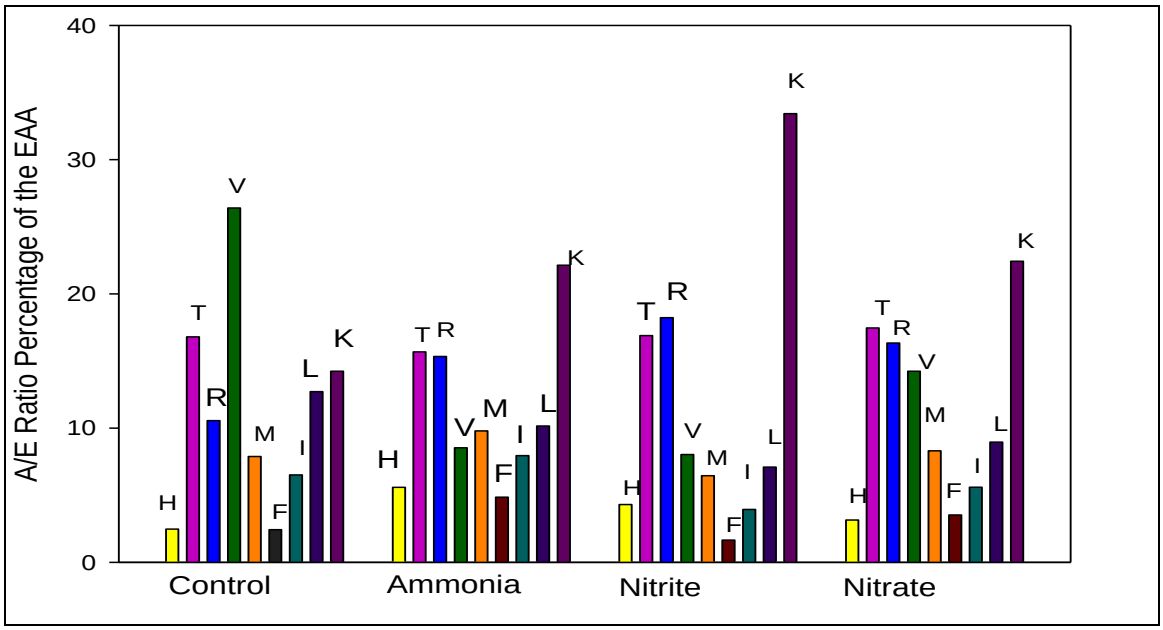


Fig 3: Essential amino acids (A/E) ratio percentages in the muscle tissue of fish *Labeo rohita* exposed to the three nitrogenous compounds.

Essential amino acids Histidine, methionine, phenylalanine and isoleucine were highest (28.2%) in ammonia exposed fish; Lysine was the most abundant (33.2%) in nitrite exposed fish, and the amino acids threonine, arginine, valine and leucine were the most abundant (57%) in nitrate exposed fish (Fig. 3). Among the non-essential FAA, aspartic acid, glutamic acid, serine and tyrosine were the highest (34%) in ammonia exposed; alanine (40.7%) in NO_2^- exposed and glycine (56%) in NO_3^- exposed fish. Student's 'T' test conducted between the total essential FAA contents of control & ammonia, and control & nitrate indicated that the differences were significant ($P<0.05$) while the difference in total FAA content between control and nitrite exposed was not significant ($P>0.05$). The differences in the non-essential amino acid contents of the control and all the exposed were statistically insignificant ($P>0.05$) and hence the data not presented.

4. Conclusion

Nitrogenous compounds that reach the fish culture ponds affect the protein constituents of different tissues and induce their breakdown into fragments. Ammonia exposure causes severe depletion of protein content and alters the amino acid profiles more significantly than nitrite and nitrate species. FAA levels were higher in the toxicant exposed tissues suggesting significant protein turn over in the tissues. A careful survey of the literature has not indicated any attempt in the past to study the protein banding pattern and its trace back to the structural and/or physiological alterations in the protein(s) that would adversely affect fish growth, fecundity and nutritive quality. It is strongly desirable that detailed molecular studies on protein fragmentation and the associated disturbances in the metabolism, in terms of under or over expression of certain proteins or the production of additional stress proteins, due to exposure to nitrogenous compounds are undertaken in the perspectives of qualitative and quantitative fish production, human health and economic status of the fish cultivators.

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