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Ammonia Induced Biochemical Changes on the Muscle Tissues of the Fish *Cyprinus carpio* FT-IR Study

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ABSTRACT

Ammonia is a naturally occurring highly toxic environmental pollutant. Lethal effects of ammonia (22 ppm) on the biochemical changes of fresh water teleost, *Cyprinus carpio* was studied in FT-IR spectroscopy. The fish exposed to lethal concentrations of ammonia for 96 h showed altered membrane lipids, altered protein profiles and increased glycogen were recorded. Likewise the decrease in α-helix and an increase in random coil structure were also observed when the fish was exposed to lethal concentrations of ammonia. The present study suggests that FT-IR spectroscopy on the muscle tissues on above said fish can be used as a rapid and inexpensive tool in toxicological research and are good biomarkers for field assessment in areas that are subject to a multiplicity of environmental variations.

Key words: FT-IR, ammonium chloride, *Cyprinus carpio*, muscle tissue

INTRODUCTION

Dissolved ammonia is one of the most important pollutant derived from the main source of farming and agricultural activities (Lau *et al*., 2004; Molins-Legua *et al*., 2006). Exposure to high levels of ammonia can cause irritation and serious burns on the skin and in the mouth, throat, lungs and eyes. Ammonium is released into the environment from extensive use of fertilizer, human and animal excreta and waste-water treatment plants. Ammonium, as NH_4^+, is acutely toxic to some fish at concentrations of 200 μg N L^{-1} as pointed out by Gibb (2000). At very high levels, ammonia can even cause death (Deb and Verma, 2010). Fish, as an indicator organism, play an increasingly important role in monitoring of water pollution, because they respond with great sensitivity to changes in the aquatic environment (Aas *et al*., 2001; Palaniappan and Vijayasundaram, 2008). Nutritional value of fish depends on their biochemical composition which is affected by water pollution because fish are constantly exposed to chemicals in polluted and contaminated waters. Palaniappan and Renju (2009) reported that zinc exposure caused significant changes in the biochemical contents of the *L. rohita* muscle tissues. In addition, it also caused an alteration in the protein secondary structures by decreasing the α-helix and increasing the β-sheet contents of muscle tissues. Jagadeesan *et al*. (2005) noticed that the total protein content were found to be decreased in the liver tissues of mice, *Mus musculus* after treatment with median-lethal dose of mercuric chloride.

Fourier Transform Infrared (FT-IR) spectroscopy is a non-disturbing technique which provides quantitative biochemical information about biological samples. It is a valuable technique due to its

high sensitivity in detecting changes in the molecular constituent of tissues, such as lipids, proteins and nucleic acids. The shift in the peak positions, bandwidths and intensities of the bands, all give valuable structural and functional information which may have diagnostic value. With FT-IR spectroscopy, it is possible to monitor changes in the structure and properties of biomolecules such as DNA, RNA, proteins, carbohydrates, lipids in biological tissues and cell simultaneously (Ci *et al*., 1999). The aim of the present study was to investigate the toxic effects of ammonia on the biochemical contents of the muscle tissue of freshwater fish, *Cyprinus carpio* by using FT-IR Spectroscopy.

MATERIALS AND METHODS

Specimens of *Cyprinus carpio* were procured from a local fish farm at Vadalore, Cuddalore District, Tamil Nadu, India and it was acclimatized to the laboratory conditions for 20 days. Water was exchanged daily. Fish were fed *ad libitum* witha mixture of rice bran and groundnut oil cake twice a day. Fish ranging from 6-7 cm in length and weighing 8-10 g were selected for experimental purpose. The water quality was determined according to the method of APHA, AWWA and WPCF (1976) and are as follows: Dissolved oxygen was, 6.2±0.02 mg L^{-1}, pH, -7.2±0.2; Water temperature, 25.0±2.0°C; Salinity, 0.2±0.07 ppt; Total hardness, 13±2.0 mg L^{-1}; Calcium, 5.0±0.1 mg L^{-1}; Magnesium, 8.0±2.0 mg L^{-1} and Total alkalinity, 20.0±0.6 mg L^{-1}. Preliminary studies were carried out to find out the median lethal concentration (LC_{50}) of ammonia for 96 h by probit analysis method of Finney (1978).

Preliminary studies were carried out to find out the median lethal concentration (LC_{50}) for 96 h of ammonia (Ammonium chloride was obtained from Merck (Merck Company, Darmstadt, Germany, Glaxo India Limited, Mumbai, India (No. 17584)) and used without further purification. All chemicals used were of analytical grade). For this, appropriate amount of ammonia was dissolved in water freshly every time to prepare a stock solution of 1000 ppm for each toxicant. The median lethal concentration (LC_{50}) of the above toxicant to fish for 96 h of ammonia (22 ppm) were determined. Acute toxicity studies were conducted for 96 h with four replicates. The tanks were filled with 20 L of water and 22 ppm L^{-1} of ammonia was added. Twenty fishes were introduced in to each tub. A common control was also maintained. Toxicants were renewed daily in the experimental tanks. No mortality was observed throughout the experimental period. After this period, the fish were sacrificed, the muscle tissues were dried in a lyophilizer (VIRTIS 6KBEL85) for overnight to remove the water content in the samples. The samples were then ground (with liquid nitrogen) in an agate mortar and pestle in order to obtain muscle powder. The muscle powder was mixed with completely dried potassium bromide at a ratio of 1:100 and then the mixture was subjected to a pressure of 5 t for 5 min in an evacuated die to produce KBr pellet for use in FT-IR spectrometer (Akkas *et al*., 2007). The measurement of FT-IR spectroscopy was performed on a Nicolet-Avatar 300 FT-IR spectrometer equipped with a DTGS detector, installed at Centralized Instrumentation and Services Laboratory, Annamalai University. The spectra covered the wave number ranging from 4000-400 cm^{-1}. Absorption intensity of the peaks was calculated with base-line method using ORIGIN 8.0 Software.

RESULTS AND DISCUSSION

The present study was carried out to analyze the toxic effects of ammonia in the muscle tissues of freshwater fish *C. carpio* by using FT-IR spectroscopy. Figure 1 shows the FT-IR spectra of control and ammonia intoxicated muscle tissues of *Cyprinus carpio* in the 4000-400 cm^{-1} range. The

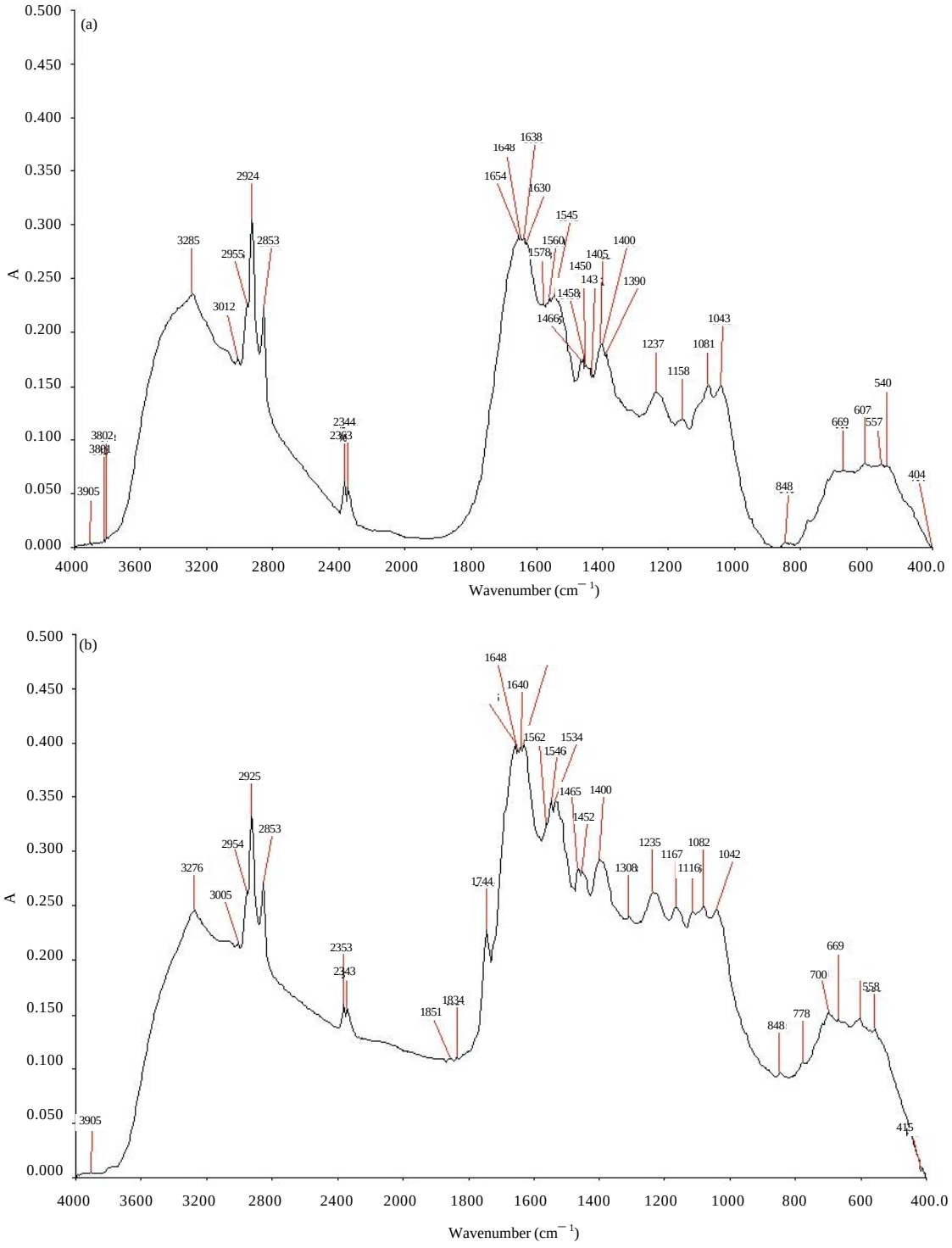

Fig. 1(a-b): Representative FT-IR spectrum of the (a) Control and (b) Ammonia chloride intoxicated *Cyprinus carpio* muscle tissues in the 4000-400 cm^{-1} region

spectrum consists of several bands arising from the functional groups belonging to proteins, lipids, nucleic acids, carbohydrates and amino acids. The main absorption bands and their assignments

Table 1: FT-IR spectra showing vibrational assignment of control, ammonia chloride intoxicated muscle tissue of fish *Cyprinus carpio*

Control	Ammonia	Peak assignment
3285	3276	Amide A: mainly N-H stretching of proteins
2955	2954	CH_3 asymmetric stretch: mainly lipids
2924	2925	CH_2 asymmetric stretch: mainly lipids
1654	1656	Amide I: C = O stretching of proteins
1648	1648	Amide I: C = O stretching of proteins (random coil)
1545	1546	Amide II: N-H bending/C-N stretching of proteins
-	1534	Amide II: N-H bending/C-N stretching of proteins
1466	1465	CH_2 bending: mainly lipids
1458	-	CH_3 asymmetric bending: mainly lipids
1450	1452	Bending vibrations of CH_2 in the lipids and proteins
1400	1400	CH_3 asymmetric bending: mainly lipids,
1390	-	COO- symmetric stretch: fatty acids and amino acids
1237	1235	PO_2 symmetric stretch: mainly nucleic acids
1158	1167	CO-O-C asymmetric stretch: glycogen and nucleic acids
1081	1082	PO_2 symmetric stretch: mainly nucleic acids
669	669	CH_2 bending, carbohydrates, proteins and lipids (sterols of fatty acids)

were defined in the Table 1. The band observed at 3285 and 3276 cm^{-1} corresponds to amide A (mainly N-H stretching of protein with negligible contribution from O-H stretching of intermolecular hydrogen bonding, since unbound water was removed from the system), respectively. The absorption bands observed at 2955 and 2954 cm^{-1} are assigned the asymmetric and symmetric stretching of CH_3 group: The former band mainly monitors lipids while the later mainly monitors the proteins in the biological systems. The band observed at 2924 and 2925 cm^{-1} is assigned to CH_2 asymmetric stretch which mainly monitors lipids with little contribution from proteins, carbohydrates and nucleic acids. The absorption bands centered at 1654 and 1534 cm^{-1} correspond to amide I and amide II vibrations of structural proteins, respectively. The former band is associated with the protein amide C'O stretching vibrations and the latter arises from amide N-H bending vibration coupled to C-N stretching vibration of the polypeptide and protein backbone (Cakmak *et al*., 2006; Akkas *et al*., 2007).

The band observed at 1744 cm^{-1} is indicative of stretching vibration of C-O bonds due to non-ionic carboxyl groups (-COOH, -$COOCH_3$) and may be assigned to hydrogen bonding between carboxylic acids or their esters (Li *et al*., 2007) and ammonia ions. Among the various bands found, the spectral peak at 1,400 cm^{-1} for the asymmetric N-H stretching was chosen for the quantitative determination of ammonium in the pure compound and in the real samples. The band observed at 1450 and 1452 cm^{-1} corresponds to CH_2 bending; mainly lipids with little contribution from proteins. The absorption noticed at 1390 cm^{-1} is assigned as COO^- symmetric stretching, mainly fatty acids and amino acids from proteins. Cakmak *et al*. (2006) had reported that the band observed at 1237 and 1235 cm^{-1} corresponds to PO^{-2} asymmetric stretching of nucleic acids with little contribution from phospholipids and 1081 and 1082 cm^{-1} are mainly due to symmetric stretching of PO^{-2} group in nucleic acids and phospholipids. Dovbeshko *et al*. (2000) reported that the band at 669 cm^{-1} may be due to CH_2 of lipids.

In the present study, the control amide A band area was observed at 3285 and this value significantly decreased to 3276 cm^{-1} in ammonia treated fish. Similar observation was made by Palaniappan and Renju (2009) who reported the band area at 3303.51 cm^{-1} in zinc exposed fish. They stated that this large shift might imply a variation in the strength of protein and amide

hydrogen bonding due to changes in the plasma chemistries. The decrease in CH_2 and CH_3 asymmetric stretching band was observed in control at 2955 and 2924 cm^{-1} and in ammonia treated fish at 2954 and 2925 cm^{-1}. Toyran *et al.* (2008) reported that the frequencies of the CH_2 stretching bands of the acyl chains depend on the degree of conformational order/ disorder state of lipids. Similar observation was also made by Palaniappan and Vijayasundaram (2008) reported that the liver tissues of fish *Labeo rohita* exposed to arsenic showed a decreased proportion of the CH_2 groups.

The amide I and II peaks were observed at 1654, 1648 and 1545 in control and at 1656, 1648, 1646 and 1634 in the ammonia-intoxicated muscle tissue homogenate of fish. The amide I peak is shifted to 1654 cm^{-1} and an additional shoulder at 1648 cm^{-1} (random coil) also appears thus indicating alteration of the protein conformation from α-helix to random coil (Chu *et al.*, 2001). Palaniappan *et al.* (2008) studied that the increase in the intensities and area values of the amide bands in the ammonia-intoxicated tissues may be due to the altering of protein synthesis capacity and the protein structure. Diplock *et al.* (1991) suggested that the exposure of proteins to free radical-generating systems may induce secondary structural changes, since secondary structure is stabilized by hydrogen bonding of peptide backbone and interference with the functional groups of the peptide bonds may cause secondary structural modifications. The decrease in the band areas and absorbance intensity of both amides I and II bands indicate the destructive effect of arsenic, as opined by Makrides (1983) who stated that free radical damage could cause a reduction in protein synthesis. The author also postulated that the amide I and II region shifts corresponds to the α-helix protein conformational change. Samuel *et al.* (2005) reported that an oxidative action of arsenic is supported by the observation that arsenic treatment decreases the protein content in the brain tissues of rat. Palaniappan and Renju (2009) obseved that these changes reflect the loss of protein levels in the arsenic intoxicated liver tissues. This loss of protein provides verification of increased protein oxidation in the liver tissues with arsenic intoxication. Loss of function of protein may result from a change in critical side chain or from a break in the hydrogen or disulfide bonds which maintain the secondary and tertiary structures.

Palaniappan and Renju (2009) noticed that the zinc exposure causes significant alterations in the protein secondary structure by decreasing the α-helix and increasing the β-sheet contents of muscle tissues. In the present study also, the significant alteration in amide I and II may be due to the altering of protein synthesis capacity and the protein structure or alterations in the protein secondary structure by decreasing the α-helix and increasing the β-sheet contents of muscle tissues. The shift in the position of CH_2stretching bands observed in the present work reveals that ammonia functions by disordering the lipid system through increasing the number of ethylene gauche conformers, these changes might involve an alteration in lipid and protein profiles, leading to a modification in membrane composition. The conformational changes of proteins may be caused by the binding of zinc ions to some amino acids of polypeptide chain which affect the lipid-protein interactions within the plasma membrane (Akahori *et al.*, 1999). Similar observation was made by Filipe *et al.* (1995) who reported that the reason for the increase in the biochemical content in the zinc-intoxicated tissues may be due to the antioxidant properties of zinc inhibiting effect on the spontaneous lipid peroxidation in rat brain. In the present study also, similar reduced CH_2 was observed in ammonia exposed muscle tissues which may be due to ammonia intoxication. Jagadeesan and Mathivanan (1999) evaluated that the depletion of protein profile induces diversification of energy to meet the impending energy demands during the toxic stress. Jana and

Bandyopadhya (1987) and Vincent and Ambrose (1994) reported a similar observation in fish *Cyprinus carpio* and *Catla catla* when exposed to lindane and cadmium, respectively. In the present study, changes in fatty acids, ammonia acids, glycogen and nucleic acids were also observed and this may be due to the ammonia intoxication.

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