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Mohammed Abd Kadhim and
Veterinary Directorate, Ministry of Iraqi Agriculture, Baghdad, Iraq

Jamal K Al-Faragi
Department of Pathology and Poultry Diseases, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

Correspondence
Mohammed Abd Kadhim and
Veterinary Directorate, Ministry of Iraqi Agriculture, Baghdad, Iraq

Effect of adding sodium chloride in water on mortality rate, behavior and gill histopathology in *Cyprinus carpio* exposed to acute toxicity of unionized ammonia

Mohammed Abd Kadhim and Jamal K Al-Faragi

Abstract
A toxicity test was conducted for 24 hours periods to evaluate the impact of three lethal concentration of un ionized ammonia (UIA) at three levels of water salinity on mortality rate behavior and gill histopathology of *Cyprinus carpio*. A total of 240 of *C. carpio* (mean weight of 50±5 g) reared in 24 glass aquaria divided to three groups (A,B and C) according to water salinity levels: (A:tap water, B:2 and C:4 g/l NaCl) and subdivided to three groups each group exposed to different lethal concentration of UIA (1.0, 1.5 and 2.0 mg/l) with control groups (with no adding of ammonia). The mortality rate significantly decrease ($p<0.05$) and ($p<0.01$) at levels B and C respectively compared with level A. Fish exposed to 1.0, 1.5 and 2.0 mg/l (UIA) appeared the following toxicity signs: lost equilibrium in water, moved very rapidly, increase ventilation, convulsions, spiral swimming, gasping, increase in mucous secretion in the gills and on the skin and hemorrhage in the gill, these sings were less in extent in fish groups reared in water at levels B and C g/l of NaCl respectively. Histopathological examination of gills showed different degrees of pathological changes which included various degrees of degeneration cells. These changes became sever with increasing concentration of ammonia. These changes were lesser extent in *C. carpio* groups reared in water containing 4g/l NaCl. The current study indicated that there is a relationship between the tolerance of ammonia toxicity and the water salinity level.

Keywords: Water salinity level, Un Ionized Ammonia (UIA), *Cyprinus carpio*, toxicity signs, mortality rate, gill histopathology

1. Introduction
The increasing pollution of aquatic environment with thousands of anthropogenic and natural chemicals is becoming the critical environmental threat facing human and environmental health [1]. Aquatic ecosystems are highly susceptible due to their affinity to accumulate comparatively high levels of chemicals entering from a variety of point and nonpoint to its water bodies such as rivers, streams, marine ecosystem and groundwater [2]. Human activities produce increasing amounts of nitrogenous materials in a significant proportion being in the form of ammonia or compounds that can yield ammonia [3]. Ammonia is the main nitrogenous compounds that represents 60% to 80% of nitrogenous waste of fish, also can be introduced into aquatic systems by direct and indirect means including agricultural sources, effluents and industrial activities. Socioeconomic effects may lead to severe consequences for aquatic populations [4]. Generally, total ammonia containing of non-toxic form (ionized ammonia) called as ammonium (NH_4^+) and toxic form un-ionized ammonia (UIA) referred as ammonia (NH_3). UIA is generally linked with ammonia toxicity because it can easily diffuse across gill tissues into the plasma because of lipid solubility and lack of charges [5]. Fish are a kill when exposed to high levels of ammonia [6]. In case of high ammonia level should focus on protecting fish by addition of common salt (NaCl) in aqua culture water [7]. The ammonia toxicity is severely dependent on factors including dissolved oxygen, pH, salinity, and temperature so these factors play an important role in the survival rate [8]. This study was aimed to evaluate the effects of acute toxicity of unionized ammonia on behavior, mortality rate and gill histopathology of *C. carpio* reared at different levels of salinity.

2. Materials and Methods

2.1 Fish Test

Approximately, 240 healthy of *C. carpio* with an average weight of (50±5g) were brought from a commercial fish farm. Fish were acclimatized for two weeks in laboratory conditions prior the experiment. Fish were reared in chlorine free tap water, the chemo-physical parameters of the water were measured during the experimental period as follows: [Temperature (ºC) 24 ± 1, Dissolved O_2 (mg/L) 6.10± 0.5, pH 7.60 ± 0.2]. During the acclimatization period commercial chemical kits were used to help monitor water quality to ensure that the levels of ammonia and nitrite were maintained below harmful levels. The photoperiod was maintained 12 light/ 12 dark with daily feeding (once per day) of commercial feed.

2.2 Experimental design

Post acclimatization period three toxic concentrations of UIA (1, 1.5 and 2) mg/l NH_3 alongside with control (with no adding of ammonia) were used in duplicate for each treatment group (to determine the short term (24 h) ammonia tolerance level of *C. carpio*). These concentrations used in the experiment were based on the toxicity test results of [9]. Ammonium chloride (NH4Cl MW=5) was mixed with dechlorinated tap water using a magnetic stirrer (IKA) to obtain the required ammonia concentration according to method described by [10]. The ammonia concentrations in mg/l were determined by a colorimetric direct nesslerization method [11], to form a yellow color, the color intensity of the solution determine from the TAN concentration (Table1) according to temperature and pH calculated of the water by using a multiplication factor (pk_a) according to method described by [12].

In this study each concentration of ammonia was conducted at three levels of salinity as follows: A: de-chlorinated tap water, B: 2 g/l NaCl and C: 4 g/l NaCl) obtained by add NaCl for tap water, fish were not fed during experimentation and were monitored to detect signs of toxicity and to remove dead fish directly. The experiment was conducted in 24 glass aquaria and after 24 hours periods, At the end of the experiment, three fish from each treatment were dissected for histopathological examination.

Table 1: Ratio of TAN and UIA (ppm) with standard error in experimental water.

Test number	temperature	pH	Total Ammonia-N	UIA
T1	24±0.4	7.6± 0.2	36 ± 0.55	1 ± 0.15
T2	24±0.4	7.6± 0.2	50 ± 0.80	1.5 ± 0.18
T3	24±0.4	7.6± 0.2	66 ± 0.25	2 ± 0.22

2.3 Histopathological study

Gill tissues were fixed in 10% formaldehyde solution for 72 hours. Then, the tissues were processed routinely and prepared into paraffin blocks. The blocks of the gill tissues were then cut at 5µm thickness and were stained with Haematoxylin and Eosin (H&E) stain. Standard histological methods were followed according to [13] for histopathological examination.

2.4 Statistical analysis

The Statistical Analysis System-SAS (2012) program [14] was used to study the effect of difference in study parameters. The data obtained in this study were analyzed using One Way analysis of variance (ANOVA) followed by Duncan multiple range tests to compare between means.

3. Results

3.1 Mortality Rate

The percentage of mortality in experimental groups are illustrated in Table 2 which represent the evaluation of adding of NaCl in three levels (A; tap water, B; 2 g/l and C; 4 g/l of NaCl). The mortality rate of *C. carpio* showed significant decrease (P<0.05) at level B and at level C (P<0.01) compared to level A.

Table 2: Effect of different concentrations of NaCl on the mortality rate of *C. carpio* exposed to different concentrations of NH_3 for 24 hours period.

Conc. of NaCl (g/l)	NH_3Conc.(mg/l)				LSD value
	0	1	1.5	2	
	Mortality rate (%)				
A: tap water	0	5	25	50	6.368 **
B: 2 g/l	0	0	22	40	5.437 **
C: 4 g/l	0	0	15	25	5.021 **
LSD value	0.00 NS	2.50 *	3.56 **	6.013 **	---

*(P<0.05), **(P<0.01).

3.2 Clinical signs and behavior of fish

After exposure to different concentrations of UIA, C. *carpio* showed various changes in behaviors including: increased respiration rate, a slight restlessness, lost their equilibrium compared to control group. In later stages, *C. carpio* showed gasping for air, rapid movement and irregular respiration and there were small hemorrhages, in the anterior part of the ocular cavity especially at high levels of ammonia. Gills were severely congested and contain a large amount of mucus secretion and may have slight or severe gill bleeding, fish lie on their side and spasmodically opens wide their mouth and gills opercula lastly reacts aggressively to outside motivations and loses their balance then leap out of the aquaria and their muscles spasm, then the body surfaces become pales and die (Figure 1). The degree of clinical signs was different among groups increased with the increase of ammonia concentration and slightly decreased in fish reared with addition of NaCl in water (Table 2).

Table 3: Semi quantitative scoring of clinical signs of *C. carpio* (-) no alterations, representing normal behavior; (+) the alterations appear in one fish; (++) the alterations appear in 2 to 5 fish; (+++) – the alterations appear in more than 5 fish.

S. No	Clinical Signs	Control			T11mg/l NH_3			T2 1.5 mg/l NH_3			T3 2 mg/l NH_3		
		water without add NaCl	water with add 2g/l NaCl	water with add 4g/lNaCl	water without add NaCl	water with add 2g/l NaCl	water with add 4g/l NaCl	water without add NaCl	Water with add 2g/l NaCl	water with add 4g/l NaCl	water without add NaCl	water with add 2g/l NaCl	water with add 4g/l NaCl
1	A slight restlessness, and increased respiration	-	-	-	+++	++	++	+++	+++	+++	+++	+++	+++
2	Congregate close to the water surface. In later stages, gasp for air	-	-	-	+	+	-	++	++	+	+++	+++	++
3	The gills are heavily congested and contain a considerable amount of mucus	-	-	-	++	++	++	+++	+++	++	+++	+++	+++
4	The skin is light in color, and covered with a thick or excessive layer of mucus	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++
5	rapid movements	-	-	-	+	+	+	+++	+++	+++	+++	+++	+++
6	May have slight to severe bleeding of the gills	-	-	-	+	+	+	+++	+++	++	+++	+++	+++
7	Small hemorrhages occur, mainly at the base of the pectoral fins and in the anterior part of the ocular cavity	-	-	-	+	+	+	+++	+++	++	+++	+++	++
8	Fish react violently to outside stimuli; they lose their balance leap out of the water, and their muscles twitch in spasms	-	-	-	+	+	+	+++	+++	++	+++	+++	+++
9	Affected fish lie on their side and spasmodically open wide their mouths and gill opercula	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++
10	The organs inside the body cavity are congested	-	-	-	+	-	-	+++	+++	+++	+++	+++	+++

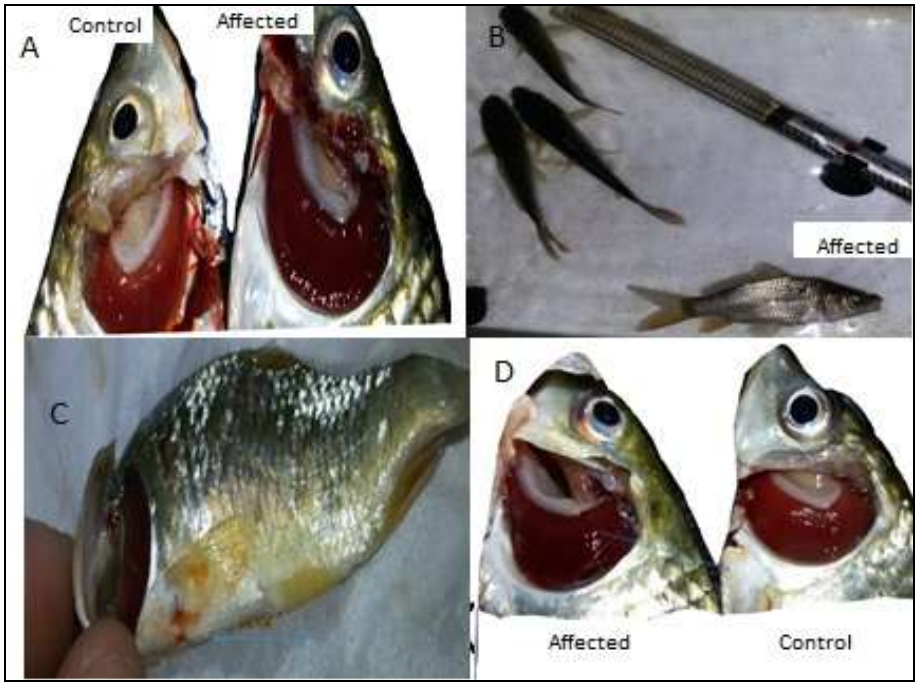

Fig 1: Clinical sings of ammonia toxicity,(A) Sever congested of the gill exposed to high ammonia concentration (B): Fish placed on their side and spasmodically open wide their mouths and gill opercula after exposed to high ammonia concentration,(C): Small hemorrhages occur at the base of the anterior part of the ocular cavity (D): The gill is heavily congested and contain a considerable amount of mucus and small hemorrhages occur in the anterior part of the ocular cavity.

3.3 Histopathological Study

Gill tissues from the control fish exhibited normal structure with no recognizable changes observed in the gills. Examination of gill tissues of *C. carpio* after exposure to three different concentrations from ammonia (1,1.5 and 2 mg/l NH_3) for 24 hours reared in water with and without addition 4 g/l NaCl showed histopathological changes in gills extended with increasing ammonia concentration this changes including: epithelial hyperplasia, secondary lamellar fusion (result from hyperplasia of epithelial cells, epithelial lifting

(separation of epithelial from basement membrane) and edema of lamella (Figures 2 and 3). These lesions became sever with increasing levels of ammonia. These changes were lesser in extent in *C. carpio* groups reared in water containing 4g/l NaCl.

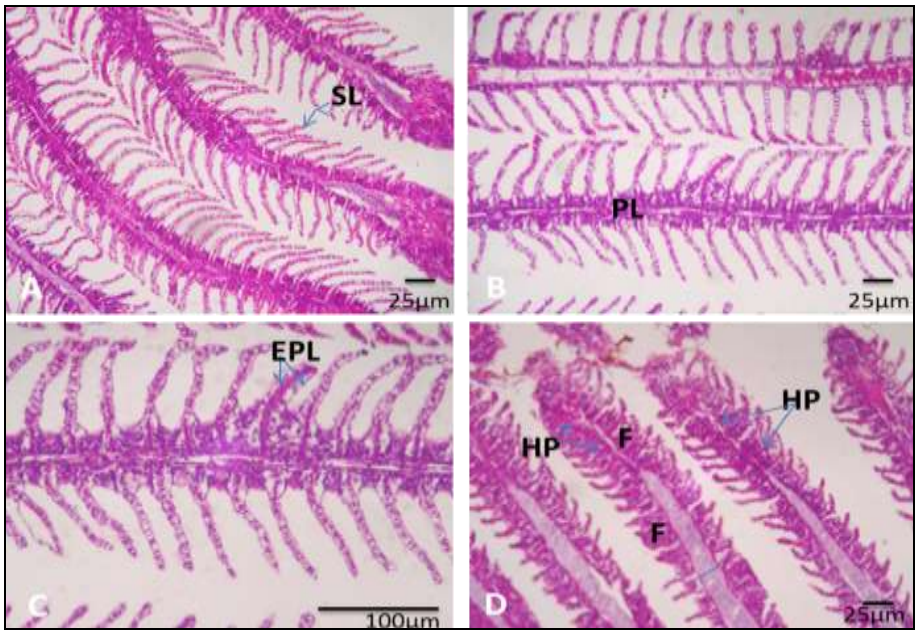

Fig 2: Histological sections through the gill tissue of *C. carpio* following acute exposure to three different concentrations of ammonia for 24 hours with and without addition of NaCl in rearing water. (**A**) The gills of control fish without ammonia and with addition of 4g/l NaCl, showing the structure of the primary lamellar epithelium (PL) and the secondary lamellae (SL). (**B**) The gills of control fish without ammonia with addition of 4g/l NaCl. (**C**) Following exposure to 1 mg/l of ammonia concentration with the addition 4g/l NaCl, the gill showing epithelial lifting (EPL). **(D)** Following exposure to 1 mg/l ammonia without addition NaCl, the gill demonstrating hyperplasia of the secondary lamellae (HP) with fusion (F). H&E.

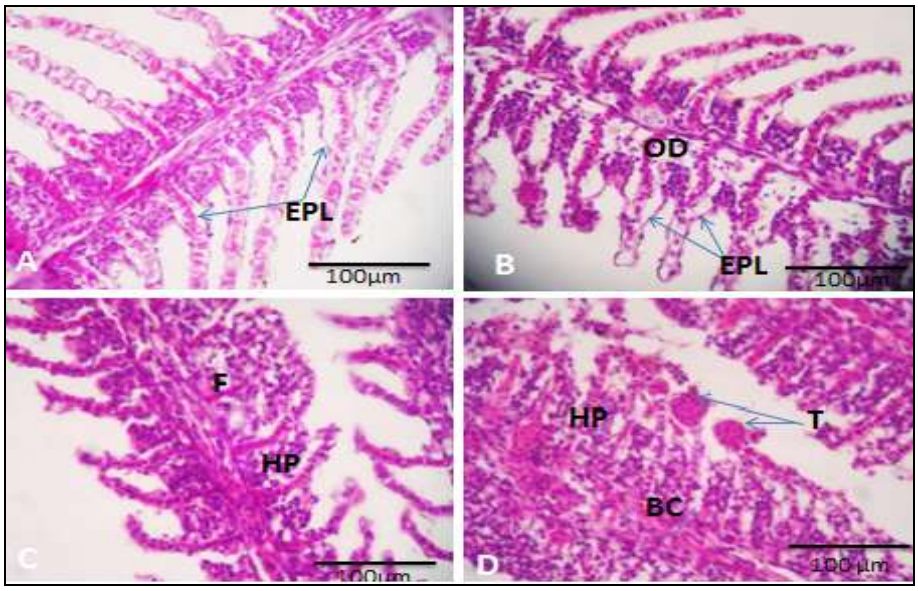

Fig 3: Histological sections through the gill of *C. carpio* following acute exposure to three different ammonia concentrations for 24 hours with and without the addition of 4g/l NaCl in rearing water. (**A**) Following exposure to 1.5 mg/l of ammonia concentration with addition 4g/l NaCl showing epithelial lifting (EPL). (**B**) Following exposure to 1.5 mg/l of ammonia concentration without addition 4g/l NaCl demonstrating edema (OD) with epithelial lifting (EPL). (**C**) Following exposure to 2 mg/l of ammonia concentration with addition 4g/l NaCl showing hyperplasia (HP) and fusion of secondary lamellae (F) (**D**) Following exposure to 2 mg/l of ammonia concentration without addition 4g/l NaCl demonstrating hyperplasia of the secondary lamellae (HP), dilation of the central venin with blood congestion (BC) and telangiectasis (T). H & E stain.

4. Discussion

Fish are comparatively sensitive to changes in water quality and have a relatively long lifespan compared to other aquatic animals. Hence, fish can give indication of the general health of the aquatic environment. The present study showed disturbance in behavior of *C. carpio* after exposure to different levels of toxic ammonia including: increased respiration rate, a slight restlessness, lost their equilibrium gasping for air, rapid movement and irregular respiration compared to control group. These findings are in agreement with [15] who reported the high ventilation frequency, lethargy and loss of appetite in Nile tilapia, *Oreochromis niloticus* after exposure to UIA. Also, [16] documented that the increase in ventilation rate and lost equilibrium in *Oreochromis*

niloticus after exposure to high concentrations of ammonia. The increase of ventilation rate could be attributed that NH_3-induced effect on the O_2 binding capacity of hemoglobin; a decrease in the number of circulating red blood cells; an increased energy consumption for the keeping of the osmotic balance [17]. Increase in mucous secretion in the gills and on the body surface, hemorrhage in the gills and darkening on the skin, observed in the current study has been reported by [18, 19, 20 21]. The hyper-secretion of mucous is considered to be a common defense mechanism that prevents ammonia particles from their further entrance to gills.

The present study also showed significantly decrease of mortality rate with increase salinity concentration, these results indicate that solution cation influence the NH_3 toxicity to the fish. This result is in agreement with [22] who reported the effect of salinity on ammonia toxicity in Sea bream, *Sparus aurata* exposed to different ammonia concentrations at three different salinities that the toxic effect of (TAN) decreased with increasing salinity levels. Although, [23] found the LC_{50} for 24h NH_3 was significant increase in lake trout and Atlantic salmon when addition (1 or 10 g/l NaCl) in reared water. Also,[23] found the survival of Atlantic salmon smolts on exposure to constant concentrations of ammonia the 24-h LC_{50} of UIA is 0.15 mg/l NH_3 in fresh water and 0.3 mg/l NH_3 in 30% sea water.

In contrary, [24] reported that an increase in environmental sodium salinity (3g/l) did not produce a visible change in UIA toxicity to golden shiners in the range tested. However, [25] found that the tolerance of blue tilapia to acute un-ionized ammonia exposure was not influenced by salinity and also found that the using of brackish water for blue tilapia culture may not be a single factor to reduce the toxicity of high ammonia, but [26] reported that through the test shrimp, *Penaeus semisulcatus*, an increased tolerance to ammonia when reared in 40 ppt salt, as compared to 15 ppt. Similar results reported by [27] in rainbow trout.

[28] Reported that the salinity stress in freshwater fish affects primarily gills as the major organ involved both in osmoregulation and waste nitrogen excretion, also [29] suggested the increased salinity reduces the osmoregulatory cost of hyperventilation induced by toxicant exposure.

Histopathological studies of gills showed different degrees of pathological lesions which included various levels of degeneration, these changes including: hyperplasia, secondary lamellar fusion, telangiectasia, epithelial lifting, edema of lamella, proliferation of mucous cells, these changes were lesser in extent in *C. carpio* groups reared in water with addition NaCl. Such changes in gill tissue were reported in several effects of stressors and contaminants on various fish species [30]. The absorption of toxic chemicals through the gills is enhanced by increasing the permeability to water and ions through the gill epithelium and by inhibition of ions exchange activity of the chloride cells [31]. The epithelium hyperplasia, epithelial lifting and fusion of the secondary lamellae in gills due to ammonia exposure in most cases represent general defense mechanisms because the lifting lamellar epithelium and edema increased the distance between the external environment and the blood, thus serving as a barrier to the entrance of contaminants [32]. While, [33] stated that hyperplasia may indeed have a protective function but it may also inhibit the respiration and excretory functions of the gills.

Histopathological changes caused by ammonia consist of epithelial lifting are in line with [34, 35, 36] who observed separation of epithelial from the basement membrane and enlargement of lamellar capillaries (telangiectasia) in rainbow trout exposed to ammonia. Also, These findings were supported by [37] who observed lamellar fusion and edema in gill tissue of silver catfish, *Rhamdia quelen* exposed to different NH_3 levels. As well as, [38] observed various histopathological alterations including lamellar fusion and epithelial lifting in gill of slimy sculpin, *Cottus cognatus* exposed to 0.8-1.6 mg NH_3/l.

5. Conclusions

Results from this study indicate that ammonia concentrations hold potential to affect the health of common carp including: behavior, histological and mortality rate. The current study indicated that there is a relationship between the tolerance of ammonia and the salinity level as the toxicity of ammonia increased with decreased the salinity concentration. The present study shows that histopathological manifestations in gills are useful biomarker for ammonia toxicity in *C. carpio.*

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