

## Histomorphometric analysis of gills in Nile tilapia (*Oreochromis niloticus*) exposed to different concentrations of ammonia

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### Abstract

The rapid development of aquaculture resulted in modern methods such as intensive aquaculture or water circulation systems producing agricultural wastewater with high concentrations of nitrogen pollutants. However, these pollutants and urban, industrial, and agricultural wastewater are harmful to aquatic animals and increase damage in gills and energy loss. The present study aimed to evaluate the histomorphometry of the gills of Nile tilapia exposed to different ammonia concentrations. Juvenile *Oreochromis niloticus* were prepared and kept in the 100l aquariums for adaptation. After completing these steps, the test was conducted based on the increase in susceptibility of Nile tilapia on ammonia. Then, an acute toxicity test was performed in the experimental and control groups for 96 hours according to the standard instructions. For sub-lethal toxicity studies, 120 Nile tilapia were categorized into four groups, including three groups based on different percentages of (10, 20, and 30% LC50 96h) and a control group. The groups were exposed to ammonia for 14 days at a stable physicochemical conditions. Finally, histological analysis was carried out on fish gills. Then standard method of paraffin sections followed and tissue sections, 4-6µ thick were cut and stained with hematoxylin and eosin methods. The data obtained demonstrate that high concentrations of ammonia caused various gills tissue damage as hyperemia, filament swelling, increase in the number of chloride cells, necrosis and cell death, hyperplasia, hypertrophy and changes in behavior such as decreased appetite and mobility. The obtained data showed that an increase in ammonia level could cause irreversible damage to gill structure, and other tissues. Therefore, the habitats of these aquatic animals must be continuously monitored for ammonia levels.

**Key words:** Ammonia, Nile tilapia, Histomorphometric, Lethal concentration

### Introduction

Aquatic animals play an important role in human food supply due to their easy reproduction (Dastan et al. 2017), consumption of relatively little energy, and potentials to produce more foods rich in vitamins, omega three fat, and phosphorus (Morovvati et al. 2017; Shalaby et al. 2021). Therefore, commercially, fish and seafood are among the most valuable protein

sources to feed a growing world population. Tilapia is a genus of cichlid fishes endemic to freshwater habitats (Elbialy et al. 2021). Tilapia, native to Africa, has become the most popular farmed fish globally and one of the most traded seafood commodities (Luquet 2017). In addition to being delicious and cheap, this farmed fish is an ideal choice for human nutrition because it

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does not require the utmost care and eats fast-growing, inexpensive vegetables (Mei et al. 2020). It is the fourth most commonly consumed type of seafood in the United States due to its special characteristics (Espinosa et al. 2019). The tilapia belongs to the percidae family and has a rectangular body with small scales and a long dorsal fin with 23-31 thorns and radii. Most tilapias eat phytoplankton, but some prefer plants and use organic plants in areas where other breeding species feed on plankton. Tilapia species with short and coarse gill spines eat larger food particles. Tilapia's diet can contain large amounts of vegetable protein, lowering its production costs than other cultivars (Abdelghany 2020). Various diseases caused by toxins and pathogenic microorganisms may damage fish in the agricultural environment (Mohamed et al. 2021; Basir and peyghan. 2019). Ammonia is the most important toxic compound for farmed fish and combines nitrogen and hydrogen. This substance is mainly collected by moving air and water down in breeding ponds (Naderi et al. 2014; Sriyasak et al. 2015). Ammonia poisoning is a major cause of fish mortality on farms. The digestion of protein by fish leads to the production of ammonia, which is very toxic. Fish need to throw this waste into the water to remove it from their bodies (Yousefi et al. 2020; Jebur et al. 2019). Ammonia damages fish skin, gills, and other tissues (El-Shafai et al. 2004; Zeitoun et al. 2016; El-Greisy et al. 2016) and causes a variety of symptoms such as respiratory problems (rapid breathing of gills, shortness of breath, swallowing air from the water surface), irregular swimming, and sudden darting movement, skin bruising (bleeding) and excessive mucus production (dark spots on the skin), lethargy, loss of appetite, fish lying in bed, red streaks on the fins and body and red or purple gills (Ali et al. 2020; Mohamed et al. 2020). If the injury with ammonia poisoning continues, streaks or bloodstains appear on the body and fins. Internal damage also occurs in the brain,

organs, and central nervous system. Eventually, bleeding (hemorrhage) occurs on the inner and outer surfaces of the fish, leading to death (Kim et al. 2019) (Kim et al. 2020). To date, very few studies have been conducted on the effects of different chemical elements on the tissues of tilapia species in Iran, which has resulted in conflicting results. The purpose of this study was to examine the histomorphometry of the gills of Nile tilapia (*Oreochromis niloticus*) exposed to different concentrations of ammonia to reduce the damage to this species and to prevent wastage of capital and workforce in breeding this species.

### Materials and Methods

A total of 120 juveniles Nile tilapia (mean weight  $35 \pm 1$  g) were prepared and then transferred to the laboratory in a 500-liter tank for two weeks to adapt to environmental conditions. During acclimation, proper daily aeration was performed, and for 2% of the fish body weight by the plate, fish were fed with a commercial dry food (Bio mar, France). During the experimental period, the average temperature of the water was  $27 \pm 1$  °C, dissolved oxygen was  $6.2 \pm 1$  mg/L, and total hardness was  $269 \pm 3$  mg/L. Range finding test was performed on Nile tilapia to find lethal ammonia levels (Suliman et al. 2021). An acute toxicity test was carried out on tilapia fish for 96 hours according to standard instructions. Ammonia (Merck, Germany) was prepared as an ammonium chloride solution. Feeding of juveniles was stopped 24 hours before the acute toxicity test. All effective physicochemical parameters of water, including pH, dissolved oxygen, and temperature, were recorded daily. After determination of the lethal range, the final test for acute ammonia toxicity was performed. For this purpose, four treatments with control group specimens have been examined. The fish in each treatment were placed, and the solution was aerated 24 h before the test

organisms were placed in the 15-liter aquarium. Dead fish were collected from the aquarium environment shortly after observation, and loss numbers were calculated and recorded 24, 48, 72, and 96 hours later. The results of acute toxicity test data were analyzed using probit analysis with a 95% confidence level. For sublethal toxicity studies, fish were categorized into four experimental groups, three of which were selected based on different percentages of LC50 96h (10, 20, and 30%) and placed with a control group in three replication. The fish were exposed to ammonia for 14 days in 100-liter aquarium under controlled physicochemical conditions (Mazandarani et al. 1395; Naji et al. 1388). Feed quantity was 2 percent of body weight per day. The remaining food was removed from the aquarium by exchanging water. Water containing the same amount of ammonia was drawn from the aquarium and was added to the aquarium to keep the ammonia concentration constant.

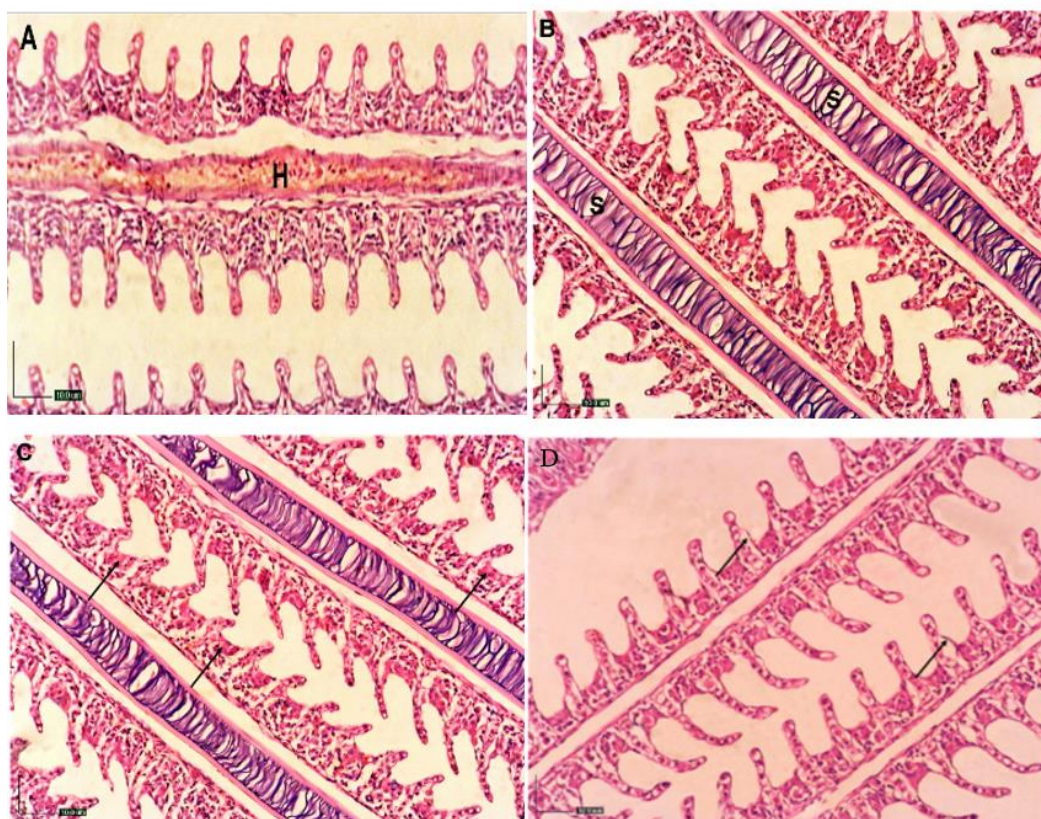
#### *Histological Examination*

At the end of the experiment, the fish were randomly caught and anesthetized with 0.5 g/l clove powder. Specimens prepared in 10% formalin buffer (Basir and Peyghan. 2016) were fixed and transferred in 70% alcohol for 24 hours. They were further processed through a series of graded ethanol's (70, 80, 90, 100% and 100%) and xylene. It should be noted that all these steps were performed by automated tissue processors (Leica ASP300 S, Germany) under the defined program (Suliman et al. 2021). The tissues were molded and then paraffinized by using Tissue-Tek, molded under the melting temperature of 56-58 ° C. Specimens were sectioned to 4 to 6-micron thick by using a microtome, and then they were placed on a slide and put in an oven at 60 ° C for 30 m for deparaffinization of

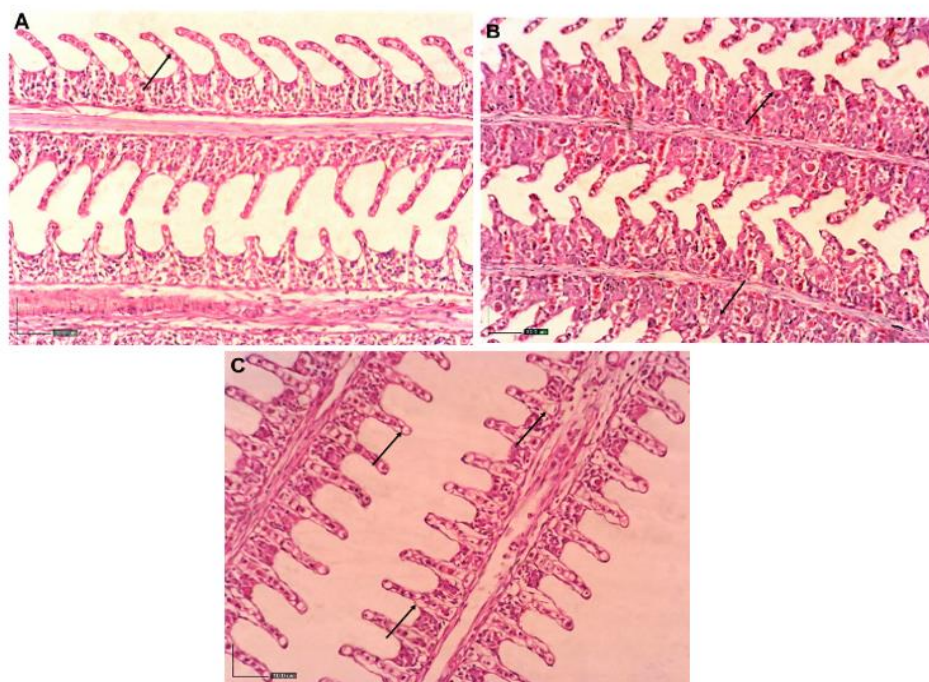
paraffin-embedded sections (Pourkhadje et al. 2014). To stain the specimens, they were deparaffinized in xylene, rehydrated, and stained with hematoxylin and eosin. The slides, 10 slides from the gills, were examined by a light microscope attached to a Dinolite lens and a computer system equipped with DinoCapture software (Morovvati et al. 2012).

#### **Results**

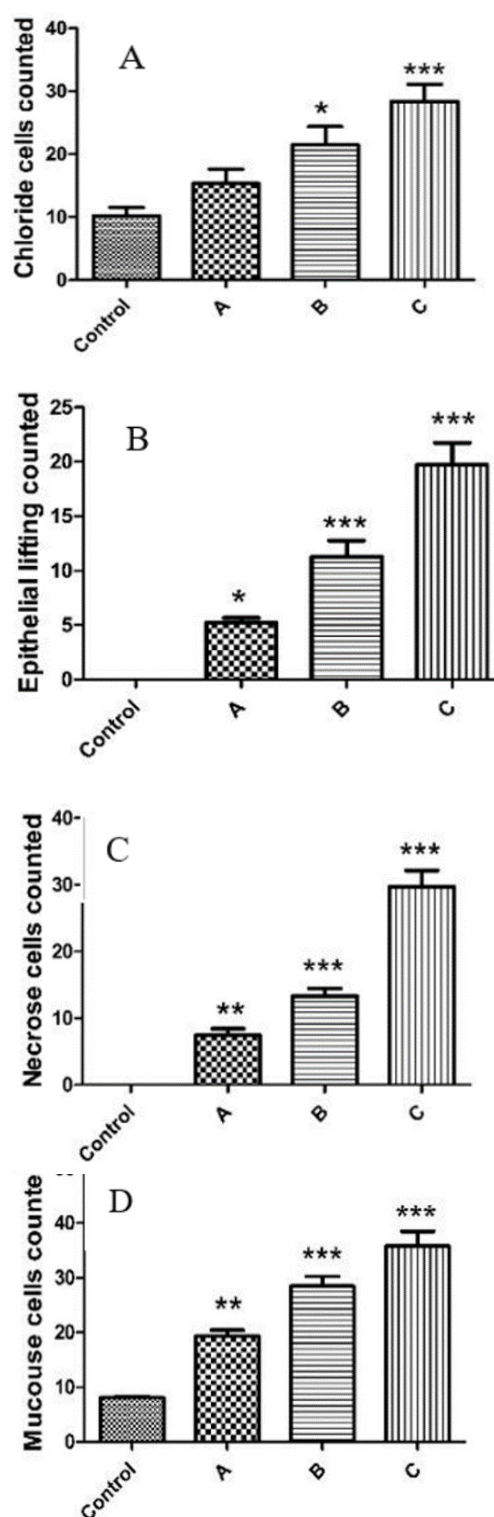
Results showed no mortality rate during the adaptation period in different treatments under total ammonia sublethal level (0.9, 1.8, and 2.7 mg/L) and control treatment for two weeks. In microscopic studies of Nile tilapia fish exposed to different concentrations of ammonia, especially in high concentrations and in the last days of the experiment, some symptoms of ammonia poisoning were observed, including decreased appetite, decreased mobility, nervous conditions, and swallowing air from the water surface, acceleration in respiration and opening and closing of the gill operculum. The gills were severely hyperemic, and the accumulation of mucus was noticeable in all parts. Bleeding on the outer surface, and accumulation of mucus on the surface of the fish's body were also quite obvious. The results of microscopic studies of fish gills exposed to different concentrations of ammonia compared to control fish showed changes and lesions in gill tissue. Tissue changes such as hyperemia and hemorrhage, hyperplasia and adhesion of lamellae, hypertrophy, filament swelling, detachment of lamella epithelium, and increase in chloride cell count and cell necrosis were also observed. Overall, the severity of these complications increased significantly from low- to high-concentration treatment ( $p < 0.05$ ) (Figures 1 and 2).



**Figure 1: Gills tissue micrograph of Nile tilapia in experimental groups (H&E, x40). A) Hyperemia (H) in ammonia-affected groups 0.9 mg/L for two weeks; B) Filament swelling (S) in ammonia-affected groups 1.8 mg/L over two weeks C); Increase in the number of chloride cells (arrows) in ammonia-affected groups 2.7 mg/L, D); Peeling of the epithelium (arrows) in the groups affected by ammonia 0.9 mg/L in two weeks.**



**Figure 2: Gills tissue micrograph of Nile tilapia gills in experimental groups (H&E, x40). A) necrosis and cell death (arrows) in ammonia-affected groups 0.9 mg/L over two weeks, B); Hyperplasia Cellular (arrows) in ammonia-affected groups 1.8 mg/L for two weeks C); Cellular hypertrophy (arrows) in ammonia-affected groups 2.7 mg/L for two weeks.**



**Figure 3:** According to the figures: Graph A: Increase in the number of chloride cells, Graph B: Increase in the number of epithelial protrusions, Graph C: Increase in the number of necrotic cells, Graph D: Increase in mucosal cells in the gill of the Nile tilapia ammonia-affected groups, 0.9 mg/L (A), 1.8 mg/L (B), and 2.7 mg/L (C) (Different symptoms indicate a significant difference in level of  $p < 0.05$ ).

## Discussion

In recent years, tilapia has become the second most important fish in aquaculture after carp worldwide (Šilovs 2018). By region, asia accounts for over 70% of world tilapia production (Mo et al. 2018). Diseases affecting aquatic animals, especially farmed fish, are among the most significant causes of human and capital loss in the region, increasing the risk of cultivating these organisms (Mercante et al. 2018; Abdi et al. 2011). Ammonia is a toxic compound with many toxic effects and destroys the gill tissue and skin of aquatic animals. It is increased by the excretion of fish in aquaculture water, leading to the extinction of this species (Mo et al. 2018). Ammonia in aqueous media can be detected in both ionized and non-ionized types, while the latter is highly toxic because it penetrates the epithelium of aquatic organisms (Kim et al. 2017). In this study, histomorphometry of the gills of Nile tilapia exposed to different ammonia concentrations was examined for the first time in Iran. The results showed that increased ammonia concentrations lead to tissue damage such as gill damage, paleness, various tissue damage, and changes in behavior such as decreased appetite, decreased mobility, nervous conditions, swallowing air from the water surface, acceleration in respiration, and opening and closing the gill cover eventually resulted in death in aquatic animals. Banihashemi (2013) reported similar features and symptoms in histopathological effects of ammonia gills of *Acipenser persicus*. The results of microscopic studies of fish gills exposed to different concentrations of ammonia compared to control fish showed changes and lesions in gill tissue. Tissue changes such as hyperemia and hemorrhage, hyperplasia and adhesion of lamellae, hypertrophy, filament swelling, detachment of lamella epithelium, and increase in chloride cell count and cell necrosis were

also observed. Similar histopathological changes and complications were reported by other substances in similar cases on other aquatic animals (Elbialy et al. 2021; Jebur and El-Demerdash. 2019; Naji et al. 2009). Overall, the severity of these complications increased significantly from low to high concentration treatment and showed a significant difference compared to specimens in the control group. Banihashemi et al (2013). examined and determined the lethal concentration of ammonia and its effect on the histopathological status of gills, kidneys, and liver of ozone fish. Their results showed that it is possible when the gills are exposed to ammonia, hyperemia, hyperplasia, secondary lamellar adhesion, primary lamellar swelling, hemorrhage, and cell necrosis. Besides, complications such as hyperemia, bile aggregation, cellular necrosis, and cell atrophy were observed in the liver, and all complications such as hyperemia, interstitial tissue degeneration, cellular necrosis, Bowman's dilatation, and hemosiderin were observed in the treatments. In general, the most relevant injuries have been observed in the gills of these fish (Banihashemi et al. 2013;

Magouz et al. 2021). Shalaby et al (2021). examined the lethal concentration and behavioral changes in acute ammonia and nitrite poisoning in Common carp. They found that adding ammonia and nitrite to the studied concentration increased the mortality rate of fish exponentially. The present study is another example of studies proving necrotic changes in the gills and tissues of fish due to ammonia poisoning, which can lead to death if the duration is increased. In a study investigated the effect of oral menthol extract on the growth of Nile tilapia and showed that ammonia increases cortisol and glucose levels as well as oxidative stress in this aquatic species, which is consistent with the findings of the present study (Yilmaz et al. 2020; Mo et al. 2018). Mercante et al. (2018) also confirmed the increase in tissue problems due to the increase in the exposure time of fish to aquatic ammonia and showed that this exposure could lead to increased complications of gills, cortisol level and oxidative stress leading to death in aquatic animals (Termeh Yusefi et al. 2018), and it was in line with the results of the present study.

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### Conflict of interest

We need to declare no conflict of interest.

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## آنالیز هیستومورفومتری آبشش تیلاپای نیل (*Oreochromis niloticus*) در معرض غلظت‌های مختلف آمونیاک

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### چکیده

توسعه‌ی سریع آبی‌پروری منجر به روش‌های مدرنی مانند آبی‌پروری فشرده یا سیستم‌های گردش آب برای تولید فاضلاب کشاورزی با غلظت بالایی از آلاینده‌های نیتروژن شد. اما این آلاینده‌ها و پساب‌های شهری، صنعتی و کشاورزی برای آبیان مضر بوده و باعث افزایش آسیب در آبشش‌ها و اتلاف انرژی می‌شود. مطالعه‌ی حاضر با هدف بررسی هیستومورفومتری آبشش‌های ماهی تیلاپا نیل در معرض غلظت‌های مختلف آمونیاک انجام شد. ماهیان جوان پس از تهیه و برای سازگاری در آکواریوم‌های صد لیتری نگهداری شدند. پس از انجام این مراحل، آزمایش بر اساس افزایش حساسیت ماهی تیلاپا نیل به آمونیاک انجام شد. سپس تست سمیت حاد طبق دستورالعمل استاندارد به مدت ۹۶ ساعت انجام گرفت. برای مطالعات سمیت زیر کشنده، ۱۲۰ ماهی تیلاپا نیل به چهار گروه، شامل سه گروه بر اساس درصدهای مختلف (۱۰، ۲۰، و ۳۰ درصد LC50 96h) و یک گروه کنترل دسته‌بندی شدند. گروه‌ها به مدت ۱۴ روز در شرایط فیزیکی‌وشیمیایی پایدار در معرض آمونیاک قرار گرفتند. در نهایت، تجزیه و تحلیل بافت‌شناسی بر روی آبشش ماهی انجام شد. سپس روش استاندارد مقاطع پارافینی و برش‌های بافتی به ضخامت ۴-۶ میکرون برش داده شد و با روش‌های (E&H) رنگ‌آمیزی شد. داده‌های به دست آمده نشان می‌دهد که غلظت‌های بالای آمونیاک باعث آسیب‌های مختلف بافت آبشش مانند پرخونی، تورم رشته، افزایش تعداد سلول‌های کلرید، نکروز و مرگ سلولی، هیپرپلازی، هیپرتروفی و تغییرات رفتاری مانند کاهش اشتها و تحرک می‌شود. داده‌های به دست آمده نشان داد که افزایش سطح آمونیاک می‌تواند آسیب‌های جبران‌ناپذیری به ساختارهای آبشش و سایر بافت‌های مربوط به آن وارد کند. بنابراین، زیستگاه این آبیان باید به طور مداوم از نظر سطح آمونیاک کنترل شود.

**کلمات کلیدی:** آمونیاک، تیلاپای نیل، هیستومورفومتری، غلظت کشنده

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