Ukrainian Journal of Ecology

ORIGINAL ARTICLE

Etiology and histopathological alterations in some body organs of juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) at nitrite poisoning

N. Grynevych[1], A. Sliusarenko[2], T. Dyman[1], S. Sliusarenko[1], B. Gutyj[2], M. Kukhtyn[3], V. Hunchak[2], V. Kushnir[4]

[1]Bila Tserkva National Agrarian University,
Pl. Soborna 8/1, Bila Tserkva, 09117, Kyiv region, Ukraine
[2]Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies Lviv,
Pekarska Str., 50, Lviv, 79010, Ukraine
[3]Ternopil Ivan Pului National Technical University,
Ruska Str., 56, Ternopil, 46001, Ukraine
[4]State Scientific-Research Control Institute of Veterinary Medicinal Products and Feed Additives,
Donetska Str., 11, Lviv, 79019, Ukraine
Corresponding author E-mail: gnatbc@ukr.net
Submitted: 03.01.2018. Accepted: 18.02.2018

Nitrite is toxic to fishes and is often encountered in recirculation aquaculture systems. The present study was carried out to study gills, liver and kidney histopathology in the juvenile freshwater fish Oncorhynchus mykiss which exposed to nitrite (1.2 ± 0.03 mg L^{-1}) for 96 hours. Histopathological alterations in gills, liver and kidneys observed microscopically showed damage in the tissues while gills, liver and kidneys of control groups exhibited a normal architecture. The most characteristic features observed in nitrite exposed fish were hemorrhages in the oral cavity, in gills and on fish body surface; focal hemorrhages under the liver capsule and in the stratum of parenchyma; atrophy and destructive changes of hepatocytes; granular degeneration of liver and kidney; liver hyaline-drop dystrophy. The study confirmed that histopathological biomarkers of toxicity in fish organs are a useful indicator of environmental pollution.
Key words: nitrite poisoning; histopathology; gills; liver; kidney; rainbow trout

Introduction

The increasing need for growing fish under controlled conditions coupled with limited capacity of water resources has led to the building of aquaculture facilities where up to 95 % of water is being recirculated. Recirculating aquaculture systems (RAS) for growing fish have been used for more than four decades. Attempts to advance these systems to commercial scale food fish production have increased significantly in the last two decades. The interest in RAS is due to their perceived advantages such as greatly reduced land and water requirements in places where water resources are limited; reduced production costs by retaining energy if the culture species require the maintenance of a specific water temperature, and the feasibility of locating production near prime markets. Currently, freshwater recirculating systems are used to raise high value species or species that can be effectively marketed (Badiola et al, 2012).

At the same time the RAS have disadvantages. The most important is the deterioration of the water quality if the water treatment process within the system is not controlled properly. This can cause negative effects on fish growth, increase the risk of infectious disease, increase fish stress, and other problems associated with water quality that result in the deterioration of fish health and consequently loss of production. The water quality in RAS depends on different factors most importantly the source, the level of recirculation, the species being cultured and the waste water treatment process within the system (Timmons et al., 2002; Svobodova et al., 2005; Grynevych and Dyman, 2016).

The fish create and expel various nitrogenous waste products through gill diffusion, gill cation exchange, and urine and faeces excretion; in addition, some nitrogenous wastes are accumulated from the organic debris of dead and dying organisms,

uneaten feed, and from nitrogen gas in the atmosphere (Timmons et al., 2002). Nitrogenous compounds (ammonia, nitrite, and nitrate) are considered as major contaminants in aquaculture wastewater.

Nitrite and nitrate are produced when ammonia is oxidized by nitrifying bacteria concentrated within a biological filter, but they are also found throughout water columns and on surfaces within the recirculating system.

Ammonia in water occurs in two forms: ionized ammonium (NH_4^+-N) and unionized (free) ammonia (NH_3). The latter, NH_3, is highly toxic to fish in small concentrations and should be kept at levels below 0.05 mg L^{-1}. The total amount of NH_3 and NH_4^+-N remain in proportion to one another for a given temperature and pH, and a decrease in one form will be compensated by conversion of the other. The amount of unionized ammonia in the water is directly proportional to the temperature and pH. As the temperature of pH increases, the amount of NH_3 relative to NH_4^+-N also increases (Williams et al., 1986).

Ammonia concentration levels are not a problem in a simple flow-through system but it is a problem when using recycling and reuse systems with biofilters to remove ammonia within the system. However, the fish farmers must take care of the biofilters' functionality to maintain the acceptable ammonia concentration levels in the culture water depending of the culture species requirements (Kroupova et al., 2005; Grynevych, 2016).

Nitrite (NO_2^--N) is the intermediate product in the process of nitrification of ammonia to nitrate and it is toxic for the fish because it affects the blood hemoglobin's ability to carry oxygen oxidized the iron in the hemoglobin molecule from the ferrous state to ferric state. The resulting product is called methemoglobin, which has a characteristic brown color, hence the common name "brown colour disease". In addition to ammonia, nitrite (NO_2^--N) poisoning of fish also is an imminent danger in RAS. Nitrite levels should be kept below 0.5 mg L^{-1}. (Nazaruk et al., 2015; Martyshuk et al., 2016; Khariv et al., 2016; Gutyj et al., 2017; Huberuk et al., 2017).

Nitrate (NO_3^--N) is the end product of the nitrification process. As Timmons et al. (2002) note, NO_2^--N is considered as the minimum toxic nitrogen product, with 96-h lethal concentration values more than 1000 mg NO_3L^{-1} for some aquaculture species. In recirculating systems, NO_2^--N levels are controlled by daily water exchanges, but in some systems with low water flow rates this parameter has become increasingly important and concentration levels should be lower than 10 mg L^{-1} NO_2^--N (Williams et al., 1986; Timmons et al., 2002).

Nitrite in the ambient water can be actively taken up across the gill epithelium and accumulate at high concentrations in the body fluids. The toxic effect of nitrite as well as its influence on physiological function in fish is well documented. Nitrite affects the regulation of ions in fish tissue, respiration, cardiovascular function, endocrine function, and excretory mechanisms. However, the chief outcome of nitrite poisoning is the oxidation of hemoglobin to methemoglobin in erythrocytes. Consequently, blood oxygen transport is compromised, since methemoglobin does not bind oxygen (Kroupova et al. 2005; Dolomatov et al., 2011; Staryk et al., 2012; Pinto et al., 2016).

Acute and chronic toxicities of nitrite have been extensively studied in many freshwater species including rainbow trout (*Oncorhynchus mykiss*). Due to fast growth rate, high stocking-density capacities and high consumer acceptability rainbow trout are commercially farmed in many countries throughout the world. Because it is a prominent culture species, the rainbow trout has been used in many fundamental experimental researches.

Histopathological investigations have long been recognized to be reliable biomarkers of stress in fish. Histopathological alterations have been widely used as biomarkers in the evaluation of the health of fish exposed to contaminants, both in the laboratory and field studies. Histopathology, the study of lesions or abnormalities on cellular and tissue levels is useful tool for assessing the degree of pollution, particularly for sublethal and chronic effects (Au, 2004; Chezhian et al., 2012). More than one tissue may be studied for assessment of the biological effects of a toxicant on localized portions of certain organs and also for assessment of subsequent derangements (degradations) in tissues or cells in other locations and this allows for diagnoses of the observed changes (Adeyemo, 2008).

One of the great advantages of using histopathological biomarkers in environmental monitoring is that this category of biomarkers allows examining specific target organs, including gills, kidney and liver, that are responsible for vital functions, such as respiration, excretion and the accumulation and biotransformation of xenobiotics in the fish (Gernhofer et al., 2001). Accumulation of the chemical pollutants is known to adversely affect the liver, kidney, muscles and other tissues of fish. The changes found in these organs are easier to identify than functional ones, and serve as warning signs of damage to animal health. Since gills and gastrointestinal tract in fishes considered the main passage for entrance of pollutants to the internal body organs like liver and kidney through the blood. Gills are the first target of waterborne pollutants due to the constant contact with the external environment (Saoud et al., 2014). The organ most associated with the detoxification and biotransformation process is the liver, and due to its function, position and blood supply it is also one of the organs most affected by contaminants in the water. The monitorization of histological alterations in fish liver is a highly sensitive and accurate way to estimate the effects of xenobiotic compounds in field and experimental studies (Van der Oost et al., 2003; Hadi and Alwan, 2012). The teleostean kidney is one of the first organs to be affected by contaminants in the water (Thophon et al., 2003). Hence, this study was undertaken to examine the pathological effects of nitrite on the gills, kidney and liver of rainbow trout under acute conditions.

Material and Methods

Fish acquisition and experimental conditions

Freshwater fish Rainbow trout (*Oncorhynchus mykiss*) juveniles were obtained from the full-system trout farm East-Ukrainian Center of Valuable Fish Species Breeding «Mzha». On arrival in the laboratory, the fish were placed in glass aquarium (26'x18'x18.5') cm with aerated tap water and were fed with commercially pellets. Water replaced every 24h after feeding in

order to maintain a healthy environment for the fish. This ensures sufficient oxygen supply for the fish and the environment is devoid of any accumulated metabolic waste. The feeding was withheld for 24 h before the commencement of the experiment and to keep the specimens in the same metabolic state. Fish were acclimatized for 2 weeks under a natural photoperiod and an average temperature of 12°C. The average water quality conditions were as follows: pH, 7.2; temperature 8.7-10.1 °C; dissolved oxygen concentration, 7.4 mg L^{-1} O_2; total hardness, 28.4 mg L^{-1} $CaCO_3$; alkalinity, 32.6 mg L^{-1} $CaCO_3$; 0.004 mg L^{-1} NH_3; 0.007 mg L^{-1} NO_2^--N. Variation coefficients were lower than 10% for all parameters.

A total of 20 juvenile fish of both sexes were used. The average weight of the fish was 48.4 ± 1.3 g.

Experimental setup

Separate circular plastic tub of 50 L of water capacity was taken and concentration of nitrite 1.2 ± 0.03 mg L^{-1} were added. This level was maintained through the addition of sodium nitrite ($NaNO_2$, Merk, 99.5 % purity) and were chosen based on previous LC50–96 h experiments with rainbow trout (William et al., 1986; Croupova, 2008). 10 healthy fishes were introduced into this tub. A control tub (no toxicant) with 50 L of water and 10 fishes were also maintained. During the 96 hrs. of acute toxicity experiment water was renewed at the end of 24 h and freshly prepared solution was added to maintain the concentration of sodium nitrite at a constant level.

Fish were observed every 12 h and removed when immobile and respiratory movements ceased. Mortality was determined after 96 h exposure.

Microscopy examinations

Three fish with nitrite poisoning were taken for histological examination. Following necropsy gills, liver and kidney were fixed in 10% buffered formalin for 48 hours. Tissue were trimmed to the thickness of 0.5 cm in size and the block were subsequently dehydrated in a series of alcohol, clean with xylene and embedded in paraffin wax. The tissues were sectioned at about 4–5 µm on microtome and mounted on glass slides, dewaxed and stained with hematoxylin and eosin (HE) (Bancroft et al., 1996). Finally, the prepared sections were examined and photographically enlarged using light microscopy.

Results and discussion

Etiology

No fish died during the acclimation period before acute nitrite exposure and no control fish died during toxicity test. Behavioral responses of fish to most toxicants are the most sensitive indicators of potential toxic effects. The pattern of acute nitrate poisoning of juvenile rainbow trout was uniformly presented. Fish began moving towards the influx of water. The excitation and acute sensitivity to mechanical and light factors were increasing. Subsequently, the development of strong tonic-clonic seizure (jerking motion) and fin trembling were present. Fish exposed to nitrite had a rapid respiration rate, loss of equilibrium, erratic swimming, flashing and crowding at the water surface. After long periods of motionlessness, it was seen that the fish lied down on the tub bottom and suddenly started to move. Fish mouth was open. Fins and gill covers were raised. The trout died instantly. The stiffness of death was well-defined. The entire body surface and gills were covered with mucus and have well-defined pinpoint focal hemorrhage. One of the main clinical signs of nitrate poisoning was the presence of hemorrhages in the oral cavity.

Histology of gills

Fish gills are multifunctional organs needed for respiration, osmoregulation, acid-base balance and nitrogenous excretion. The large surface area and direct continuous contact with the surrounding water make the gills the first target to waterborne chemicals. Pollutants enter the organism through the gills and exert their primary toxic effects on the bronchial epithelium. Thus, morphological changes in fish gills are among the most commonly recognized responses to environmental stressors and are indicative of physical and chemical stress in marine as well as freshwater habitats. Histopathological changes in gills such as epithelial lifting, hypertrophy, hyperplasia, epithelial necrosis, edema, and fusion of secondary lamellae are the major effects reported in fish exposed to various types of pollutants and toxic substances (Fernandes et al., 2007; Chezhian et al., 2012; Saoud et al., 2014).

Gill preparations of fish reared in nitrite free water had normal gill architecture and typical structural organization of gill filaments. The structure of the lamellae observed was comparable to gills described by Kroupova et al. (2008) for healthy fish. The gill arch was composed of healthy looking long gill filaments (primary lamellae) bearing distinct secondary lamellae on both sides and well separated with evident interlamellar space. Secondary lamellae were evenly arranged, running parallel to each other. Gills also had evident interfilamentous space and interlamellar space.

Gill preparations of fish exposed to nitrite nitrogen had dilations of central axes and infiltration of red blood cells with hyperplasia of spiked and shortened secondary lamellae.

Blunt ended secondary lamellae with aneurysms, and hypertrophy and hyperplasia of epithelial cells were observed in some histological preparations. The branchial and filament arteries of gill filament were blood-filled, and the convex surface of branchial arches had focal hemorrhage (Fig. 1).
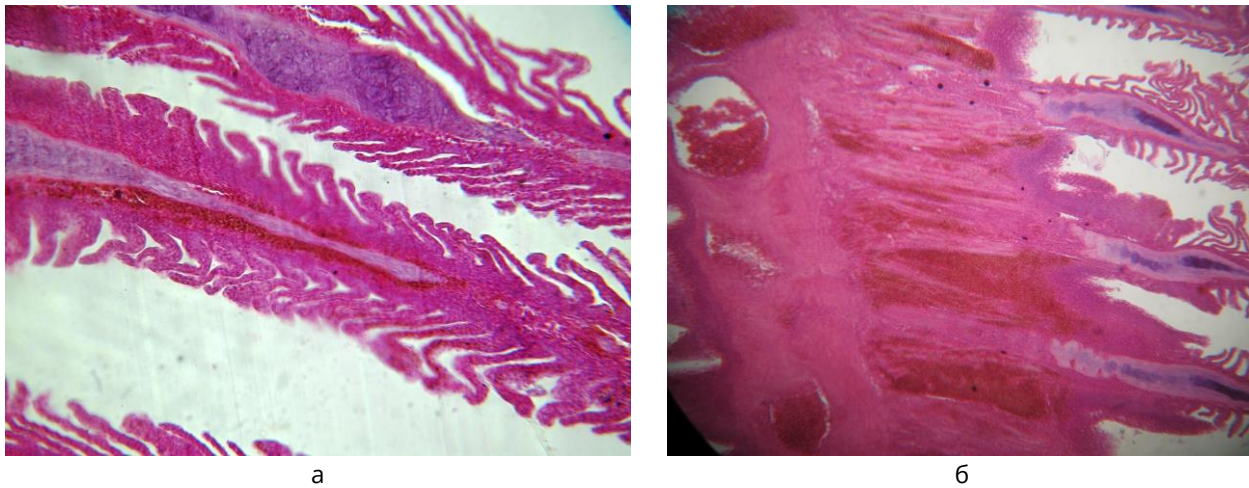
Figure 1. Light micrographs of gills of juvenile rainbow trout at nitrite poisoning: a – blood-filled filament arteries; b – focal hemorrhage on branchial arches. [Formalin fixed-H&E stained preparation, X 400].

Histology of liver

The liver is important in many aspects of nutrition, including lipid and carbohydrates storage and changes in liver structure may be useful as biomarker that indicate prior exposure to environmental stressors. Stressors associated alterations of hepatocytes may be found in the nucleus or cytoplasm or both. The liver plays a major role in complex enzymatic processes of thyroid hormones conversion. So, liver dysfunction and disease affects thyroid hormone metabolism (Hadi and Alwan, 2012).

The liver of control fish exhibits a normal architecture and there were no pathological abnormalities. The hepatocytes present a homogenous cytoplasm and a large central or subcentral spherical nucleus.

Visual examination of the liver of juvenile rainbow trout at nitrite poisoning shows that it was slightly enlarged. It had a dull, gray-brown color, and soft consistency. Focal hemorrhages under the capsule and in the stratum of parenchyma were observed during the histological examination of the liver cross-sections (Fig. 2a). The central veins and capillaries were blood-filled (Fig. 2b).
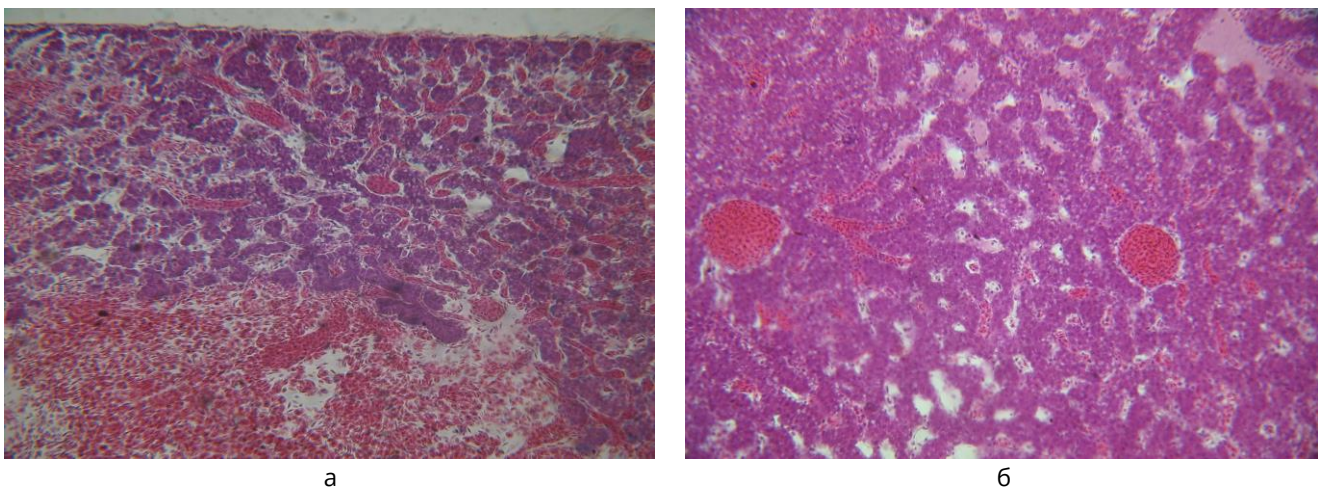

Figure 2. Light micrographs of liver of juvenile rainbow trout at nitrite poisoning: a – hemorrhages in liver; b – blood-filled vessels. [Formalin fixed-H&E stained preparation, X 400].

Hepatocyte atrophy was observed in areas of hemorrhage. Cells had the destructive changes. In addition, in the liver cells cytoplasm, the presence of protein granules of different sizes was revealed (Fig. 3a). Such hepatocytes were slightly enlarged. In some cells, the cytoplasm was turbid and homogeneous.

On the periphery of the hemorrhage areas, liver cells necrosis was observed (Fig. 3b).
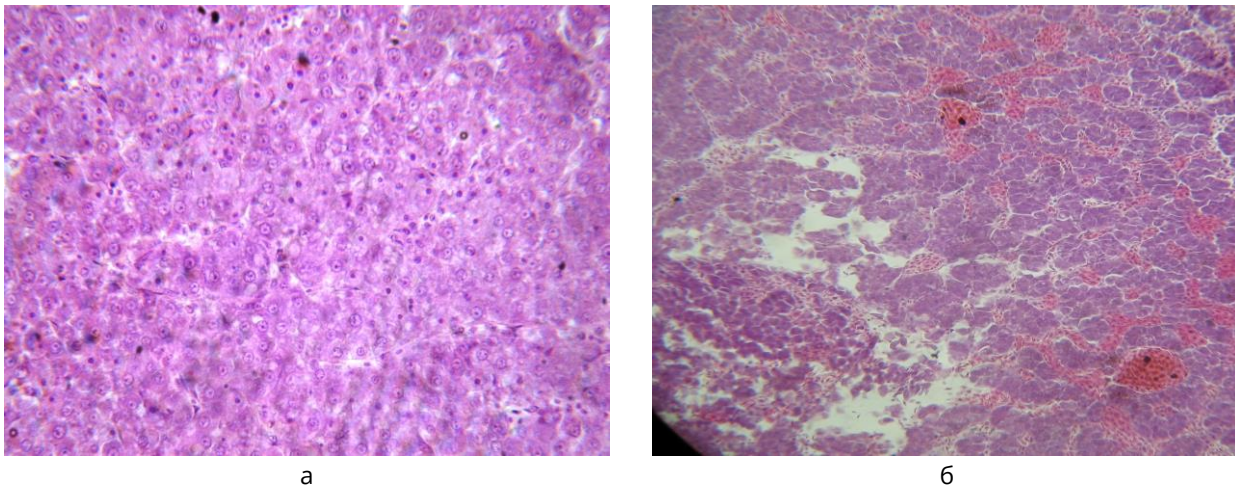
Figure 3. Photomicrographs of the liver of rainbow trout at nitrite poisoning:
a – protein granular degeneration; b – areas of liver necrosis. [Formalin fixed-H&E stained preparation, X 400].

Histology of kidney

Kidney a vital organ of body and proper kidney functioning is important to maintain the homeostasis. Kidney is not only involved in removal of wastes from blood but it is also responsible for selective reabsorption, which helps in maintaining volume and pH of blood and body fluids, erythropoiesis and help in regulating blood pressure by producing the enzyme rennin. Kidney is one of those organs, which are severely affected by different toxic chemicals. Necrosis of hematopoietic tissue, vacuolation of tubule cells, dilation of glomerular capillaries and degeneration of epithelial cell lining are some of the pathological changes observed in fish kidney of various toxins by different researchers (Iqbal et al., 2004; Hadi and Alwan, 2012) but there is a limited amount of studies about the effect of nitrate on kidney histology.

Results of this histological study showed a typical structural organization of the kidney in the untreated fish. The kidney was composed of numerous renal corpuscles with well-developed glomeruli and a system of tubules. The proximal segment was covered by tall columnar epithelial cells with basal nuclei and brush border located along the cell apices. The distal segment was lined with large, relatively clear columnar epithelial cells with central nuclei and the brush border was reduced or absent. The glomerulus was larger in diameter than the distal segment, containing columnar epithelial cells with basal nuclei and no brush border (Heabee, 2006).

Acute nitrite exposure has led to histopathological alterations in the kidney of exposed juvenile rainbow trout (Fig. 4). Gross changes included irregular diameters of renal tubules, glomerular expansion, renal corpuscle damage, severe degeneration in the tubules cells, in addition to the infiltration of edematous fluid between the tubules, hemorrhage and diffusion the erythrocytes in the interstitial fluid.

Hyaline-like inclusions were noticed in the cytoplasm of convoluted tubules. They were of pink color (Fig. 4a). Epithelial cells were enlarged and poorly demarcated. The epithelial cells cytoplasm of the most convoluted tubules was fine-grained. Their lumens were narrowed and contained protein precipitate (Fig. 4b).
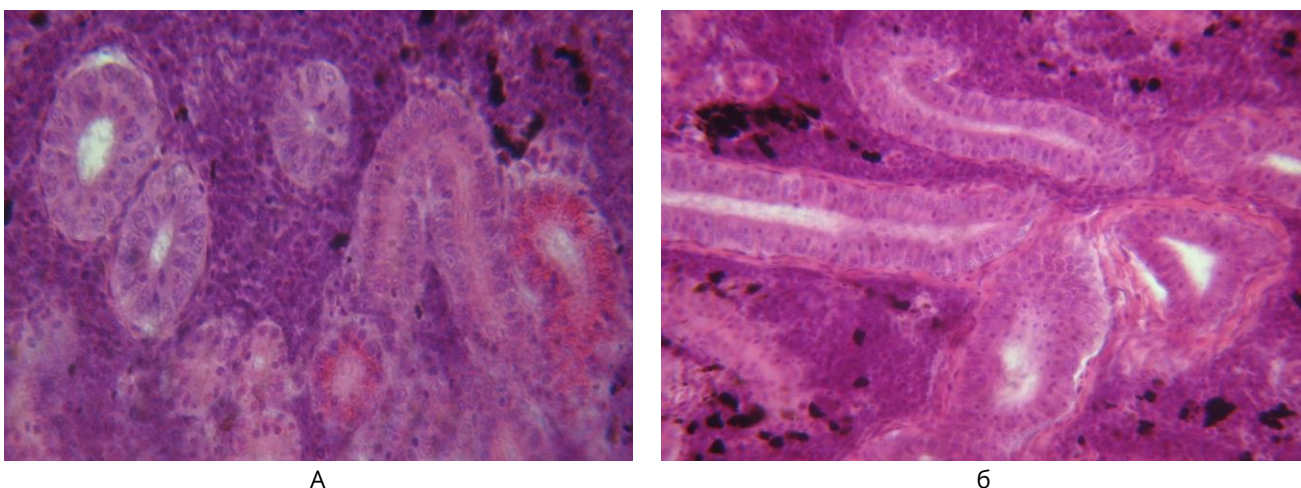

Figure 4. Photomicrographs of the kidney of rainbow trout at nitrite poisoning:
a – hyaline-drop dystrophy of kidney tubule eepithelial cells; b – granular degeneration of convoluted tubules epithelial cells. [Formalin fixed-H&E stained preparation, X 400]

Conclusions

In the present study, excessive nitrite caused severe histopathological alterations in the gills, liver and kidney of experimental juvenile rainbow trout *Oncorhynchus mykiss* and may be the reason for the mortality observed. The most characteristic

features observed in nitrite exposed fish were hemorrhages in the oral cavity, in gills and on fish body surface; focal hemorrhages under the liver capsule and in the stratum of parenchyma; atrophy and destructive changes of hepatocytes; granular degeneration of liver and kidney; liver hyaline-drop dystrophy.

The study confirmed that histopathological biomarkers of toxicity in fish organs are a useful indicator of environmental pollution.

References

Adeyemo, O.K. (2008). Histological Alterations Observed in the Gills and Ovaries of *Clariasgariepinus* exposed to environmentally relevant lead concentrations. Journal of Environmental Health, 70(9), 48–51.

Au, D.W.T. (2004). The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. Marine Pollution Bulletin, 48, 817–834. doi: 10.1016/j.marpolbul.2004.02.032

Badiola, M., Mendiola, D., Bostock, J. (2012). Recirculating Aquaculture Systems (RAS) analysis: main issues on management and future challenges. Aquacultural Engineering, 51, 26–35. doi: 10.1016/j.aquaeng.2012.07.004

Bancroft, J.D., Stevens, A., Turner, D.R. (1996). Theory and practice of histological techniques**,** 4th Edition, Churchill Livingston, Edinburgh, London, New York. doi: 10.1002/(SICI)1096-9896(199710)183:2<243::AID-PATH770>3.0.CO;2-F

Chezhian, A., Senthamilselvan, D. & Kabilan, N. (2012). Histological changes induced by ammonia and ph on the gills of fresh water fish *Cyprinus carpio var. communis* (Linnaeus). Asian Journal of Animal and Veterinary Advances, 7, 588–596. doi: 10.3923/ajava.2012.588.596

Dolomatov, S.I., Shekk, P.V., Zukow, W., Kryukova, M.I. (2011). Features of nitrogen metabolism in fishes. Reviews in Fish Biology and Fisheries, 21(4), 733–737. doi 10.1007/s11160-011-9212-z

Fernandes, C., Fontainhas-Fernandes, A., Monteiro, S.M., Salgado, M.A. (2007). Histopathological gill changes in wild leaping grey mullet (*Liza saliens*) from the Esmoriz-Paramos coastal lagoon, Portugal. Environmental Toxicology, 22, 443-448. doi: 10.1002/tox.20269

Gernhofer, M., Pawet, M., Schramm, M., Müller, E., Triebskorn, R. (2001). Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams. Journal of Aquatic Ecosystem, Stress and Recovery, 8, 241–260. doi: 10.1023/A:1012958804442

Grynevych, N. (2016). Osoblyvosti vykorystannia biofiltriv z riznymy typamy napovniuvacha v ustanovkakh zamknutoho vodopostachannia v akvakulturi [Features of biofilters with defferent types of filler plants in closed water acuaculture]. Scientific Messenger LNUVMBT named after S.Z. Gzhytskyj, 18, 3(70), 57–61 (in Ukrainian) doi: 10.15421/nvlvet7013.

Grynevych, N.E., Dyman, T.M. (2016). Sezonni zminy gidrohimichnih pokaznykiv vody za vykoristannia ustanovok zamknutogo vodospozhivannia dlia vyroschuvannia raiduzhnoi foreli [Seasonal changes of hydrochemical indices of water in Recirculating acquaculture systems for growing of rainbow trout]. Scientific Messenger of veterinary medicine BNAU, 2(130), 33–39.

Gutyj, B., Nazaruk, N., Levkivska, A., Shcherbatyj, A., Sobolev, A., Vavrysevych, J., Hachak, Y., Bilyk, O., Vishchur, V., Guta, Z. (2017). The influence of nitrate and cadmium load on protein and nitric metabolism in young cattle. Ukrainian Journal of Ecology, 7(2), 9–13. doi: 10.15421/2017_14

Gutyj, B., Leskiv, K., Shcherbatyy, A., Pritsak, V., Fedorovych, V., Fedorovych, O., Rusyn, V., & Kolomiiets, I. (2017). The influence of Metisevit on biochemical and morphological indicators of blood of piglets under nitrate loading. Regulatory Mechanisms in Biosystems, 8(3), 427–432. doi: 10.15421/021766

Hadi, A.A., Alwan, S.F. (2012). Histopathological changes in gills, liver and kidney of fresh water fish, Tilapia zillii, exposed to aluminum. International Journal of Pharmaceutical and Life Sciences (IJPLS), 3(11), 2071–2081.

Huberuk, V., Gutyj, B., Gufriy, D., Binkevych, V., Hariv, I., Binkevych, O., Salata, R. (2017). Impact of antioxidants on enzym activities of glutatione system of bulls bodies antioxidant defense under acute nitrate and nitrite toxicity. Scientific Messenger LNUVMBT named after S.Z. Gzhytskyj, 19(77), 220–224

Iqbal, F., Qureshi, I., Ali, M. (2004). Histopathological changes in the kidney of Common Carp, Cyprinus Carpio, following Nitrate exposure. Journal of Research (Science). Bahauddin Zakariya University, Multan, Pakistan, 15 (4), 411–418.

Kroupova, H., Machova, J., Piackova, V., Blahova, J., Dobsikova, R., Novotny, L., Svobodova, Z. (2008). Effects of subchronic nitrite exposure on rainbow trout (Oncorhynchus mykiss). Ecotoxicology and Environmental Safety, 71, 813-820.

Kroupova, H., Machova, J., Svobodova, Z. (2005). Nitrite influence on fish – a review. Vet Med – Czech, 50, 461–471. doi: 10.17221/5650-VETMED

Khariv, M., Gutyj, B., Butsyak, V., & Khariv, I. (2016). Hematolohichni pokaznyky orhanizmu shchuriv za umov oksydatsiinoho stresu ta za dii liposomal'noho preparatu [Hematological indices of rat organisms under conditions of oxidative stress and liposomal preparation action]. Biological Bulletin of Bogdan Chmelnitskiy Melitopol State Pedagogical University, 6(1), 276–289 (in Ukrainian). doi: 10.15421/201615

Nazaruk, N.V., Gutyj, B.V., Gufrij, D.F. (2015). Vplyv metifenu ta vitamiksu se na aktyvnist' aminotransferaz syrovatky krovi bychkiv za nitratno–kadmijevogo navantazhennja. Naukovyj visnyk L'vivs'kogo nacional'nogo universytetu veterynarnoi' medycyny ta biotehnologij im. G'zhyc'kogo. 17, 1(1), 121**–**126 (in Ukrainian).

Martyshuk, T.V., Gutyj, B.V., Vishchur, O.I. (2016). Level of lipid peroxidation products in the blood of rats under the influence of oxidative stress and under the action of liposomal preparation of «Butaselmevit», Biological Bulletin of Bogdan Chmelnitskiy Melitopol State Pedagogical University, 6 (2), 22–27. doi: 10.15421/201631

Pinto, D., Maltezi, L., Stringhetta, G., Pellegrin, L., Nitz, L., Figueiredo, M., Garcia, L. (2016). Ammonia and nitrite acute toxicity in juvenile piavuçu Leporinus macrocephalus (Actinopterygii, Anostomidae) Pan-American Journal of Aquatic Sciences, 11(4), 292–300.

Saoud, I., Naamani, S., Ghanawi, J., Nasser, N. (2014). Effects of Acute and Chronic Nitrite Exposure on Rabbitfish *Siganus rivulatus* Growth, Hematological Parameters, and Gill Histology. J Aquac Res Development, 5(6), 263. doi:10.4172/2155-9546.1000263

Svobodova, Z., Machova, J., Poleszczuk, G., Hjda, J., Hamaakova, J., Kroupova, H. (2005). Nitrite Poisoning of Fish in Aquaculture Facilities with Water-recirculating Systems. Acta Veterinaria Brno, 74, 129–137. doi: 10.2754/avb200574010129

Staryk, L.I., Gutyj, B.V., Vasiv, R.O., Gufrij, S.D. Murs'ka, D.F. (2012). Vplyv sukupnogo vvedennja pirydoksynu gidrohlorydu z askorbinovoju kyslotoju na biohimichni ta morfologichni pokaznyky krovi bychkiv pry gostromu nitratno–nitrytnomu toksykozi. Naukovyj visnyk L'vivs'kogo nacional'nogo universytetu veterynarnoi' medycyny ta biotehnologij im. G'zhyc'kogo. 14, 2(1), 306–312 (in Ukrainian).

Thophon, S., Kruatrachue, M., Upathan, E. S., Pokethitiyook, P., Sahaphong, S., Jarikhuan, S. (2003). Histopathological alterations of white seabass, *Lates calcarifer* in acute and subchronic cadmium exposure. Environmental Pollution, 121, 307–320. doi: 10.1016/S0269-7491(02)00270-1

Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T. and Vinci, B.J. (2002). Recirculating Aquaculture Systems, 2nd Edition. Cayuga Aqua Ventures, Ithaca, NY 14850, USA. 800 p. NRAC Publication No. 01-002.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environmental Toxicology and Pharmacology, 13, 57–149. doi: 10.1016/S1382-6689(02)00126-6

William, M., Lewis, Jr., Morris, D. (1986). Toxicity of nitrite to fish: a review. Transactions of the American Fisheries Society, 111, 183–195. doi: 10.1577/1548-8659(1986)115<183:TONTF>2.0.CO;2