Acute nitrite exposure of fish using common carp

Acute nitrite exposure of fish using common carp as a model species

The acute exposure of carp (*Cyprinus carpio* L.) to enhanced nitrite concentration at a low molar ratio of Cl^-/NO_2^- (0. 21) for 48 hours caused high mortality and increased blood plasma nitrite to concentrations 7 times that of the ambient water. Jensen et al. (1987) observed that exposure of carp to nitrite for 48 hours resulted in plasma concentrations 5. 4 times that of their ambient water. The present study showed that nitrite also accumulated to a lesser extent in the liver and muscle of carp. However, only limited data are available for comparison of nitrite accumulation in tissues. Margiocco et al. (1983), who exposed rainbow trout (*Oncorhynchus mykiss* Walbaum) to a lower nitrite concentration (molar ratio Cl^-/NO_2^- 1. 75) for 48 hours, found nitrite levels in the muscle similar to that of the ambient water, but in liver, nitrite levels 9 times the environmental concentrations. In a study of nitrite accumulation in Siberian sturgeon (*Acipenser baerii* Brandt) exposed to nitrite (Cl^-/NO_2^- 0. 23) for 18 hours, Gisbert et al. (2004) observed highest nitrite concentrations in skeletal musculature, followed by the blood plasma and liver.

The present study showed that the concentration of nitrite at a molar ratio of Cl^-/NO_2^- (2. 57) did not result in the degree of health impairment that was seen with the lower ratio (0. 21), and no mortality was observed. Also nitrite levels measured in the blood plasma and tissues did not differ significantly from control values. This observation verified the protective effect of enhanced chloride concentration on nitrite toxicity and documented that enhanced chloride concentrations protect fish from nitrite uptake and

accumulation in the body. This supports the theory that nitrite has an affinity for the branchial chloride uptake mechanism in the fish gills (Williams and Eddy, 1986). However, a safe molar (or weight) ratio of chloride to nitrite in aquaculture has yet to be established. European Inland Fisheries Advisory Commission (EIFAC, 1984) recommends, for salmonids and coarse fish species, values of Cl^-/NO_2^- molar ratio at 6. 7 and 3. 2, respectively. The present study showed that even the lower molar ratio Cl^-/NO_2^- of 2. 57 did not cause health impairment in carp under laboratory conditions. In contrast, observations at an aquaculture facility with a water re-use system showed that cases of death in catfish (*Silurus glanis* L.) and tench (*Tinca tinca* L.) occurred at levels of 13. 0 and 7. 4, respectively, despite these levels being considered safe according to EIFAC recommendations. Apparently other factors influencing nitrite toxicity to fish need to be taken into account, and the EIFAC levels proposed as optimum values may require re-evaluation.

In addition to chloride, water temperature was also found to markedly influence nitrite accumulation in fish. Nitrite accumulated to significantly higher levels at higher temperature in both the blood plasma and in selected tissues of carp. The nitrite concentrations in the plasma at 14 °C and 20 °C represented levels 3. 5 times and 7 times that of the surrounding medium, respectively. Information on the influence of temperature on nitrite accumulation in fish has been limited. In crayfish (*Astacus astacus* L.) a lowering of temperature significantly retarded nitrite accumulation (Jeberg and Jensen 1994). Since fish and crayfish are poikilothermic, their physiological processes are highly affected by environmental temperature. This means that the metabolic rate, heart rate, enzyme activities, and blood

flow increase as body temperature rises and decrease at lower body temperature. For example, Metz et al. (2003) observed temperature-enhanced branchial enzymatic pump activity in common carp. In the present experiment, increased temperature may also have affected activity of enzymes involved in ion uptake through the gills, consequently causing more rapid nitrite accumulation in the blood plasma and tissues of carp. Chloride cell proliferation may by another factor contributing to greater nitrite accumulation at higher temperatures (Schmidt et al. 1998).

From the blood plasma, nitrite diffuses into red blood cells, where it oxidises haemoglobin to methaemoglobin which is unable to bind oxygen; thus reducing the oxygen-carrying capacity of the blood (Bodansky, 1951; Cameron, 1971). Methaemoglobin formation can be considered a key physiological disturbance; however, the toxicity of nitrite results from a combination of effects rather than from a single one.

In the present experiments acute nitrite poisoning was also generally associated with a decrease in haematocrit value, erythrocyte count, haemoglobin concentration, and eventually with a decrease in mean corpuscular volume. Several theories may explain these pathological changes.

Jensen et al. (1987, 1990, 1992) discovered that nitrite stimulated potassium chloride co-transporter in carp erythrocytes, which led to an osmotically mediated efflux of water out of the cells and induced red blood cell shrinkage. This was accompanied by increased plasma potassium concentration. Increase in extracellular potassium, a major intracellular

cation, can be considered also as an indication of haemolysis. Jensen et al. (1987; 1990) assumes that red blood cell shrinkage may be followed by loss of haemoglobin solubility, resulting in haemoglobin crystals and structural damage to erythrocytes, consequently leading to haemolysis. However, haemolysis can be also caused by oxidative injury to erythrocytes. Recent investigations on mammals reveal that nitrite plays an important role in the NO cycle and is one of the sources of nitric oxide (Gladwin et al., 2000). Among other agents, deoxyhaemoglobin reduces nitrite to nitric oxide and works as nitrite reductase (Gladwin et al., 2004). Nitric oxide can be a source of free radicals. If the concentration of free radicals exceeds the capacity of detoxication mechanisms, oxidative injury of erythrocytes will occur, leading to denaturing of important proteins, including haemoglobin, and oxidation of membrane components in erythrocytes (Romero et al., 2006). It is also possible that the decrease in erythrocyte count can be caused by accelerated aging of erythrocytes as a consequence of enhanced metabolic demands by the activity of detoxification enzymes, including methaemoglobin reductase.

In carp exposed to nitrite, a significant number of erythrocytes were characterized by a conspicuously clear cytoplasm, visible on blood smears. This phenomenon is usually attributed to low haemoglobin levels caused by decrease in haemoglobin synthesis (Klaassen, 2001).

The data indicated some changes in nitrogen metabolism in carp exposed to nitrite at low molar ratios of Cl^-/NO_2^-. Elevated concentrations of plasma ammonia, urea-N, and uric acid were detected.

Information on the interaction of nitrite with nitrogen metabolism in fish is limited, but Jensen (2003) hypothesised that nitrite exposure could lead to depletion of adenylates in muscle and other tissues, due to hypoxia and anaerobic anaerobic glycolysis. Conversion of AMP to NH_3 (and IMP), via AMP deaminase, would then elevate the production of ammonia, which could react with glutamate, forming glutamine, subsequently increasing urea production.

Elevated blood urea levels in teleosts may serve as a clinical indication of respiratory and excretory compromise resulting from respiratory epithelial cell hypertropy and hyperplasia (Nelson et al., 1999). This is in accordance with findings of the present study, which revealed that nitrite presence in ambient water caused several histopathological changes, including hyperplasia of the respiratory epithelium with fusion of secondary lamellae, as well as hyperplasia and an elevated eosinophile granular (chloride) cell count.

Elevated plasma levels of uric acid can be explained as a protection of fish against oxyhaemoglobin oxidation in the blood. Smith and Nunn (1984) reported that uric acid inhibited the oxidation by nitrite of bovine oxyhaemoglobin. Furthermore, Doblander and Lackner (1996) found that oxidation (i. e., detoxification) of nitrite to nitrate by iron-containing proteins was enhanced in trout hepatocytes by the addition of uric acid. Uric acid is known to be an antioxidant protecting haem proteins from oxidation.

In carp exposed to nitrite, plasma osmolality was constant. However, certain alterations in the plasma electrolyte status were evident. Nitrite poisoning

caused a marked increase in plasma potassium concentrations and slight decrease in plasma Na^+ concentration compared to controls. Jensen et al. (1987) originally observed that nitrite induced extracellular hyperkalaemia in carp. The rise in plasma K^+ is explained as the nitrite-stimulated release of K^+ from skeletal muscle and red blood cells (Jensen 1990; Knudsen and Jensen 1997), although haemolysis may also contribute to increasing potassium levels, as hypothesized above. Contrary to the results of several authors (Jensen et al *.* 1987; Knudsen and Jensen 1997), plasma Cl^- levels were not affected by nitrite poisoning. Nitrite is a competitive inhibitor of chloride uptake and *vice versa* (Williams and Eddy 1986); thus, chloride influx is reduced by the presence of nitrite in ambient water. On the other hand, in fish exposed to nitrite, there is a concomitant loss of K^+ and Cl^- from skeletal musculature (Knudsen and Jensen 1997), while chloride levels remained unchanged, possibly due to the interaction of the above mentioned effects.

Common carp showed high ability to recover from nitrite poisoning under nitrite-free conditions. In the surviving fish, the recovery of erythrocyte counts and haemoglobin concentrations was observed after 24 hours in nitrite-free water. Methaemoglobin levels and nitrite concentrations in the blood plasma, liver, and muscle decreased slightly, but not significantly. Those parameters reached control values after 144 hours under nitrite-free conditions. Haematocrit also reached normal values by the end of the recovery period. Normal plasma urea and uric acid concentrations were observed after 24 hours post-nitrite exposure. Ammonia concentrations returned to normal levels by the end of the recovery period. Potassium levels

decreased during the recovery period and had dropped significantly below control values by the end of the experiment. Knudsen and Jensen (1997), who observed a similar K^+ deficit, hypothesised that skeletal musculature might recover its normal K^+ levels at the expense of the extracellular K^+, thus reducing plasma potassium levels. In surviving fish; most of the nitrite-induced effects were reversible.

Sub-chronic nitrite exposure of fish using rainbow trout as a model species

In general, the results of the sub-chronic toxicity test revealed the high sensitivity of rainbow trout to nitrite and showed that there were considerable differences between effects of acute and sub-chronic nitrite exposure. Only limited and inconsistent data are available concerning the effects of long-term exposure of fish to low nitrite concentrations, hence results reported here are difficult to compare with other published data.

With acute nitrite exposure (at a low molar ratio of Cl^-/NO_2^-), fish, regardless of species, accumulate the toxicant in the body to levels far exceeding those found in ambient water (e. g. Fontenot et al., 1999; Avilez et al., 2004). In contrast, the plasma nitrite levels did not reach environmental levels in any experimental group at the end of our sub-chronic toxicity test. Slightly elevated nitrite levels compared to controls were found only in the plasma of fish exposed to concentrations of 1. 0 mg l^{-1} NO_2^- and greater. The molar ratio between chloride and nitrite ranged from 1300 to 4. 3 in the experimental groups, implying a possibility that the chloride concentration was sufficiently high to prevent nitrite accumulation.

A difference with sub-chronic exposure when compared to acute exposure was observed in methaemoglobin content, an important indicator of nitrite poisoning. The methaemoglobin levels did not exceed control values in any of the experimental groups; thus there was no correlation between methaemoglobin concentration in the blood and nitrite concentrations in the plasma or in the ambient water. This phenomenon may be attributed to acclimation to elevated nitrite levels in the water as well as to the fish's ability to reconvert methaemoglobin to haemoglobin. Huey and Beitinger (1982) first reported that a methaemoglobin reductase system is present in channel catfish (*Ictalurus punctatus* Rafinesque), which can mediate the reduction of methaemoglobin to haemoglobin. The enzyme is presumed to be present in other fish species, as evidenced by a return to basal methaemoglobin levels when fish are placed into nitrite-free water following nitrite poisoning (Huey et al., 1984; Knudsen and Jensen, 1997; Gisbert et al., 2004). However, whether the enzyme system can be accelerated is controversial. Woo and Chiu (1997) found the system unresponsive in sea bass (*Lates calcarifer* Bloch). However, Avilez et al. (2004) measured increased activity of NADH-methaemoglobin reductase in *Brycon cephalus* after 96 h exposure to several nitrite concentrations, suggesting that the enzyme system may be activated by nitrite concentration or duration of exposure.

High mortality was observed in the group of fish exposed to the highest nitrite concentration (3 mg l^{-1} NO_2^-). However, in surviving fish, haemoglobin concentrations, haematocrit values, and leukocyte counts were comparable to those of controls and significantly lower than in the groups

exposed to lower nitrite concentrations, perhaps due to differences in individual susceptibility to nitrite in rainbow trout. This phenomenon was observed previously by Margiocco et al. (1983), Arillo et al. (1984), and Aggergaard and Jensen (2001). Aggergaard and Jensen (2001) separated rainbow trout, subjected to acute exposure to nitrite, into groups based on level of nitrite accumulation and time of death. They observed that the first group accumulated high levels of nitrite in the plasma and died within 24-48 hours, while the second group showed lower levels and survived. A possible explanation for these individual differences in nitrite accumulation may be variations in number and surface area of branchial chloride cells (Perry et al., 1992) or a variable nitrite detoxification and elimination mechanism (Aggergaard and Jensen, 2001).

High sensitivity of rainbow trout to long term exposure of nitrite even at very low concentrations was observed in the present study. At the lowest concentration (0. 01 mg l^{-1} NO_2^-), an increase in plasma glucose and a decrease in potassium levels were detected in comparison with the control group, and changes in the gill consisting of segmental hyperplasia of the respiratory epithelium of secondary lamellae was observed. The values of NOEC and LOEC were estimated, on the basis of growth rate inhibition data, at 0. 01 mg l^{-1} and 0. 2 mg l^{-1} NO_2^-, respectively.

Conclusions

Results confirmed that elevated nitrite concentrations combined with low chloride levels in water caused marked haematological and biochemical changes in fish. Furthermore, macroscopic and histological changes were

observed in fish gills after acute and sub-chronic nitrite exposure. Not only high levels of nitrite can cause severe health deterioration and mortality; levels in the order of 0. 01 mg l^{-1} NO_2^- cause also considerable physiological changes in trout, substantiating the strict water quality criteria for fresh waters declared in EU Council directive 78/659/EEC (0. 01 mg l^{-1} NO_2^- for salmonid waters). However, the sensitivity of fish to nitrite is highly individual. Some fish die within 24-48 hours of nitrite exposure, while others survive and recover nearly completely in fresh water; thus in surviving fish, most of the nitrite-induced effects are reversible. Less marked changes in all the parameters investigated were observed in fish exposed to nitrite with higher chloride concentrations in ambient water. Enhanced chloride content reduced both nitrite uptake and its toxicity. Mortality and methaemoglobinaemia during nitrite poisoning were not related to water temperature. However, other haematological and biochemical parameters (NO_2^- in the blood plasma, muscle, and liver; plasma Na^+ concentration; haemoglobin concentration; haematocrit; erythrocyte count) were more affected by nitrite exposure at higher temperature.