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Nitrite-Induced Methemoglobin Formation and Recovery in Rainbow Trout (*Oncorhynchus mykiss*) at High Chloride Concentrations

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Nitrite is the intermediate compound of the nitrification process, the biological oxidation of ammonia to nitrate. Under aerobic conditions, ammonia is readily oxidized to nitrite by *Nitrosomonas* bacteria and, nitrite, in turn, is oxidized to nitrate by *Nitrobacter* bacteria (Russo and Thurston 1991). The process can be modelled (Tarazona and Muñoz 1989; Drtil et al. 1993) and in well-oxygenated systems the rate is limited by the conversion of ammonia to nitrite; thus, nitrite concentrations in most aquatic systems are usually low. Typical exceptions to this rule are aquaculture systems (Russo and Thurston 1991); groundwater in some geographic places; the hypolimnion of eutrophic water bodies (Lewis and Morris 1986), or surface waters receiving high loads of nitrogen-rich organic matter including sewage treatment plant effluents (Russo et al. 1981; Tarazona and Muñoz 1989).

Nitrite toxicity has been profusely studied and recently reviewed (Lewis and Morris 1986; Russo and Thurston 1991). Reported acute LC_{50} values range from 0.19 to 0.88 mg NO_2-N L^{-1} for salmonid fish and 2.3 to 190 mg NO_2-N L^{-1} for non-salmonid fish (Russo and Thurston 1991) but with large variations within the same species depending on other factors. Nitrite toxicity is affected by several water quality conditions, including pH, chloride, sulfate, nitrate, phosphate and calcium (Perrone and Meade 1977; Wedemeyer and Yasutake 1978; Russo et al. 1981; Huey et al. 1984). Special attention has been given to chloride anions. According to Russo and Thurston (1977) chloride concentrations show a linear relationship with the 96-hr LC_{50} values. The

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decrease of nitrite toxicity by chloride ions has been explained by a competitive interaction between both ions for the uptake sites of branchial chloride cells (Williams and Eddy 1986).

A major cause of nitrite toxicity is the oxidation of blood hemoglobin iron to its ferric state, forming methemoglobin, a derivative incapable of binding with oxygen (Steward and Stolman 1961; Russo and Thurston 1991), resulting in hypoxia and death.

Methemoglobin has a potential use as a biomarker of nitrite exposure. In aquaculture and natural systems, the use of biomarkers requires the previous knowledge of time-evolution curves. Most studies on methemoglobin formation and recovery have been done under low chloride conditions (Huey et al. 1984; Williams and Eddy 1988a). This paper shows the time evolution curves of methemoglobin in rainbow trout exposed to nitrite at a high chloride concentration.

MATERIALS AND METHODS

Rainbow trout (*Oncorhynchus mykiss*) 18.9 ± 0.4 cm length were purchased from Uña fish farm (Cuenca, Spain) and acclimated to laboratory conditions for more than two weeks. Fish were fed a commercial trout food (DIBAQ AE-7) once a day.

Fish were randomly separated in groups of three animals and exposed in 30 L glass aquaria; one of these groups was used as control. One aquarium of a flow-through system was used for each concentration. Decalcified groundwater was used as dilution water at a constant flow rate of 20.45 ± 0.07 L h^{-1}; its water quality characteristics appear in Table I. Reagent grade sodium nitrite (Merck, Germany) was added by a metering pump at a constant flow rate of 0.851 ± 0.004 mL min^{-1}. Three nitrite concentrations ranging between 0.5 and 3 mg L^{-1} were achieved using appropriate stock solutions, the control group received nitrite-free dilution water. Nitrite concentrations were analyzed daily using the spectrophotometric method (Rodier 1981).

Fish were exposed for 8 days and observed during an additional post-exposure period of 5 days. At 72, 192, 240 and 312 hr fish were anesthetized (2-phenoxy ethanol, 0.3%) and bled from the caudal vein using insulin heparinized syringes with 21G x 1.½", 0.8 x 40 Nº2 Luer needles, for the measurement of methemoglobin and hematocrit. Fish were identified by their head-tail length.

The percentage of methemoglobin was determined by the

method described by Steward and Stolman (1961), after 10 min in an ultrasonic bath, using U.V. detection at 635 nm. Hematocrits were measured with an Orto Lector.

One-way analysis of variance and Student's "t" tests were used to analyze the data. Significant differences were stated at the $p<0.05$ level.

RESULTS AND DISCUSSION

Nitrite concentrations in the exposure aquaria were 0.68 ± 0.05; 1.69 ± 0.05 and 3.09 ± 0.11 mg $N\text{-}NO_2$ L^{-1}. Nitrite concentrations in control aquaria were always below the detection level. Chloride concentrations were 82.36 ± 0.43 mg NaCl L^{-1}; no differences between aquaria were observed.

Table I. Physicochemical characteristics of the dilution water used for the bioassay. Two daily analyses for pH, temperature and dissolved oxygen; n=6 for the other parameters. Organic matter represents permanganate oxygen demand.

Parameter	Mean ± std
Organic matter (mg O_2 L^{-1})	0.50 ± 0.13
Chloride (mg ClNa L^{-1})	82.36 ± 0.43
Hardness (mg CO_3Na L^{-1})	74.44 ± 0.40
Sodium (mg L^{-1})	31.38 ± 9.18
Orthophosphates (mg PO_4^{3-} L^{-1})	0.10 ± 0.11
Total ammonia (mg NH_3 L^{-1})	0.065 ± 0.040
Nitrite (mg NO_2-N L^{-1})	ND
Carbon dioxide (mg L^{-1})	7.78 ± 3.02
Alkalinity (mg CO_3Ca L^{-1})	195.53 ± 21.88
Dissolved oxygen (mg L^{-1})	10.80 ± 0.50
pH	7.47 ± 0.41
Temperature (ºC)	14.0 ± 0.5

Fish were observed several times each day and no mortality was noted. One fish at the lowest nitrite concentration died during the post-exposure period; clinical data showed infection of the bleeding area as the cause of the death.

Hematocrits did not show significant differences through the exposure and recovery periods, nor between groups; showing a negligible effect of the periodic bleeding.

Figure 1 shows the evolution of the methemoglobin percentages in the three exposed groups and in the controls. Only the highest nitrite concentration produced a significant increase in methemoglobin values, with the extreme value, near 50%, after 192 hr of

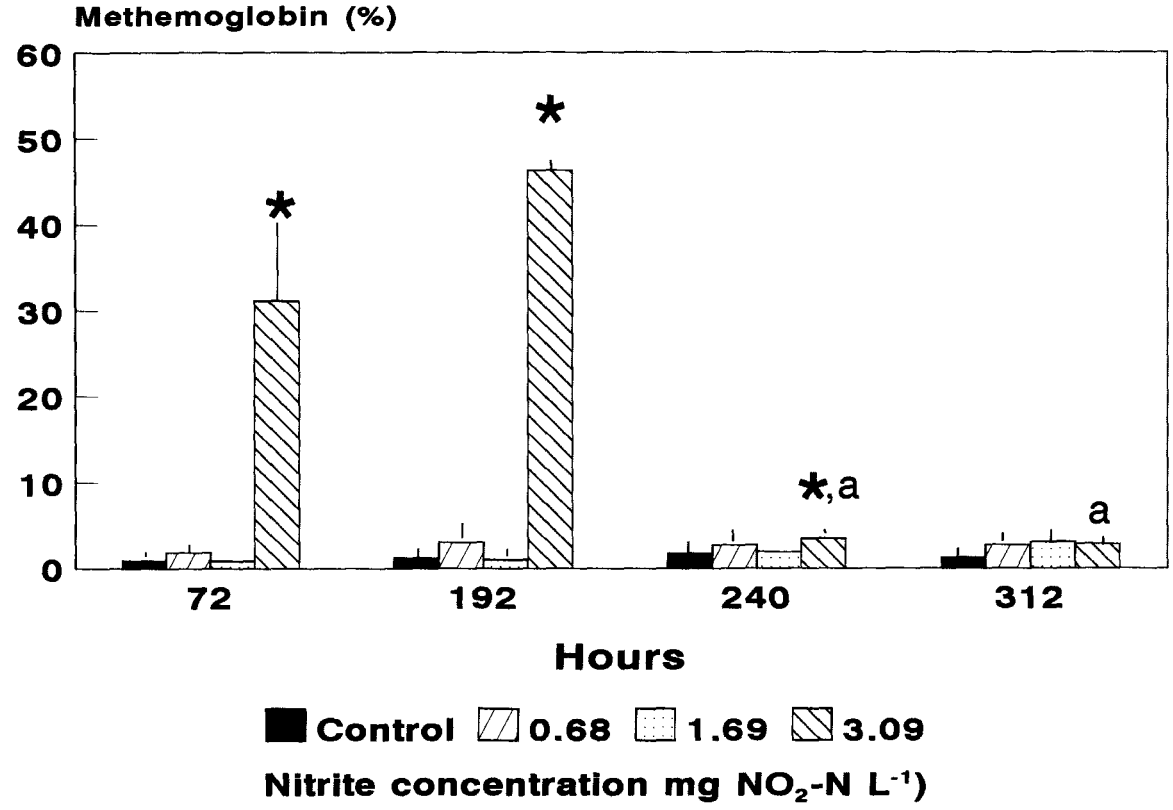

Figure 1. Time evolution of methemoglobin percentages (mean + std) during nitrite exposure (0-192 hr) and recovery (192-312 hr) in rainbow trout (n=3). (*) Significant differences of means versus control mean. (a) Significant differences of means versus the highest mean value of the same concentration.

exposure. The recovery of methemoglobin levels was very rapid; 48 hr after transfer to nitrite-free water, only a slight increase could be observed and after 120 hr levels had returned to pre-exposure values and showed no differences when compared to control fish.

The induction and recovery of methemoglobin levels were in agreement with published data observed for nitrite exposures at low chloride water concentrations (Huey et al. 1984). The methemoglobin formation was linear ($r=0.95$), with a formation rate of 0.24% h^{-1}. This must be considered appropriate for the exposure conditions according to the data provided by Williams and Eddy (1988a; 1988b), which calculated formation rates around 3-6% h^{-1} using two/thirds of the 24-hr LC_{50}. The methemoglobin recovery rate was 0.92% hr^{-1}, which is within the range 0.6-2.15% hr^{-1} reported for the in vitro reduction of methemoglobin (Williams and Eddy 1988b).

The methemoglobin levels observed in the control fish, 0.63 to 1.79%, were similar to those normally reported in the blood of unexposed rainbow trout, which range between 0.9 and 3.6% (Smith and Russo 1975; Lewis and Morris 1986).

According to Russo and Thurston (1977) there is a linear relationship between chloride concentrations and 96-hr LC_{50}s. Using this relationship the highest nitrite concentration employed in this experiment was approximately one/eighth of the estimated 96-hr LC_{50} value. This is in good agreement with the lack of mortality observed after 192 hr of exposure and the high methemoglobin increase, considering that only slight differences between the 96-hr and 192-hr LC_{50} values must be expected (Thurston et al. 1978).

Nitrite concentrations below 2 mg N-NO2 L^{-1} did not lead to increases in methemoglobin levels or clinical alterations. This is in concordance with the low toxicity of nitrite in chloride-rich waters, even at the sublethal level (Russo and Thurston 1991). These data are in agreement with the lack of detrimental effects observed in freshwater fish by nitrite concentrations equal to 10% or less of the 96-hr LC_{50} (Lewis and Morris 1986)

The extent of methemoglobinemia formation is directly correlated to plasma nitrite levels (Eddy et al. 1983; Tomasso 1986). According to Freeman et al. (1983) the return of fish to nitrite-free water causes the decrease of blood plasma nitrite levels through efflux of nitrite to the water via gills. Then, the methemoglobinemia disappears due to the activity of methemoglobin reductase systems, which have been demonstrated in the

blood of many fish species (Freeman et al. 1983; Scott and Harrington 1985). Particularly, a NADH-methemoglobin-reductase system has been observed in rainbow trout (Huey and Beitinger 1982).

The rapid and high increase of methemoglobin levels during sublethal nitrite exposures, and their rapid recovery after a few days, suggest the capability of methemoglobin percentages as a biomarker for the presence of toxic levels of nitrite in natural environments and fish farms. The agreement between the results presented in this paper and those reported for low chloride waters allows the use of this biomarker in any kind of water, even those with significant variations in chloride concentrations.

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