Nitrite Toxicity to *Danio rerio*: Effects of Subchronic Exposure on Fish Growth

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Abstract

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The aim of this study was to investigate the long-term effects of subchronic exposure to sublethal levels of nitrite, ranging from 15 to 130 mg·l^{-1} NO_2^-, on growth in aquarium fish *Danio rerio*. The juvenile growth test according to OECD 215 was used in the experiments. Fish weight was measured at the beginning of the experiment and then using the same method, fish weight was observed 28 days after fish stocking. Compared to the control, growth suppression was detected from the concentration of 73 mg·l^{-1} NO_2^- ($P < 0.05$) and a significant inhibition of fish body growth was shown from 130 mg·l^{-1} NO_2^- ($P < 0.01$). An exponential relationship between nitrite concentrations and specific growth rate ($R^2 = 0.896$) was detected.

Cyprinus rerio, zebrafish, NO_2^-, growth test

In recent years, the harmful effects of nitrite on fish have attracted much attention. Elevated concentrations of nitrite can be found in water receiving nitrogenous effluents, in various hypoxic environments and in effluents from industries producing metal, dyes, and celluloid (Pitter 1999). Increased nitrite concentrations in water are also one of the most frequent problems encountered both in aquariums and on fish farms (Adamsson et al. 1998; Etscheidt 2003; Dvořák 2004; Svobodová et al. 2005). Nitrite (NO_2^-) naturally occurs in fresh water and it is an intermediate product in bacterial nitrification and denitrification processes in the nitrogen cycle. In oxygenated waters nitrite is transformed by nitrification to nitrate and in anoxic conditions the elementary nitrogen is the product of biological denitrification (Pitter 1999). Problems with high nitrite toxicity in freshwater animals stem from the fact that NO_2^- has an affinity for the branchial Cl^- uptake mechanism, presumably the Cl^-/HCO_3^- exchanger in chloride cells in gills. Thus, whenever nitrite is present in the ambient water, a part of the Cl^- uptake will be shifted to NO_2^- uptake (Jensen 2003). Nitrite problems typically are more likely to occur in closed intensive culture systems due to insufficient, inefficient or malfunctioning filtration systems removing waste ammonia from water by means of nitrification (Kroupová et al. 2005). Particularly in aquacultural facilities with water re-use systems, high levels of nitrite have been found to cause severe physiological disturbances and have resulted in mass fish mortalities (Svobodová et al. 2005). The accumulation of organic matter leads to the release of microbial metabolites, such as ammonia, nitrite and hydrogen sulphide into the water column, and may exert chronic stress on fish during the culture (Das et al. 2004). The resultant stress may ultimately lead to exhaustion, disease, and mortality in fish (Francis-Floyd 1990).

Literature dealing with the long-term toxicity of sublethal nitrite concentrations corresponding to 10% of 96hLC50 suggests that such concentration should not be detrimental to freshwater fish. Neither growth suppression nor tissue damage was observed (Wedemeyer and Yasutake 1978; Colt et al. 1981). Acclimatisation of fish to elevated nitrite concentrations was studied by Tucker and Schwedler (1983), Doblander and Lackner (1997) and Máchová et al. (2004).

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Acute toxicity of nitrite has been investigated and described in a number of fish species (Bowser et al. 1983; Crawford and Allen 1977; Russo and Thurston 1977; McConnell 1985; Lewis and Morris 1986; Kroupová et al. 2005; Pištěková et al. 2005; Kroupová et al. 2006; Voslářová et al. 2006), but the effects of chronic nitrite exposure on their survival and growth have not been well documented yet. However, detailed knowledge of the impact of water quality factors on fundamental production characteristics such as growth performance, food conversion efficiency, and animal welfare is needed in order to effectively exploit the benefits of aquacultural systems (Siikavuopio and Saether 2006). In particular, fish farms using recirculation systems therefore require detailed knowledge on the levels of nitrite that can be tolerated (Kamstra et al. 1996).

Thus, the aim of this present study was to investigate the effects of subchronic exposure to nitrite at concentrations ranging from 15 to 130 $mg \cdot l^{-1}$ NO_2^- on growth in aquarium fish *Danio rerio* - the species of fish most frequently used in the world for toxicity tests (OECD and ISO methodological guidelines).

Materials and Methods

Tests of prolonged exposure to nitrite on the growth of juvenile fish were performed on aquarium fish *Danio rerio* (*Cyprinus rerio*, Hamilton 1822) according to the "Catalogue of Fishes 2004"). The procedure complied with the OECD No. 215 Fish, Juvenile Growth Test guidelines and CSN ISO 10229 (75 7760). The experiment was carried out in triplicate repetitions, with 20 fish used for each concentration (15, 40, 73, 107, 130 $mg \cdot l^{-1}$ NO_2^-) and for the control group. Fish were caught with a fine net and placed into a glass dish with diluting water and weighed (always 5 fish). The tank-fish weight was expressed as the difference between the weight of the dish with water and fish and the weight of the dish with water. The average weight of fish used in the experiment was 0.477 ± 0.069 g. After being weighed, fish at the age of 20 days were placed in test chambers and exposed to a range of sub-lethal concentrations of nitrite (dosed as $NaNO_2$) dissolved in water. The test was performed semi-statically. The duration of each test was 28 days. Fish were fed 8% of body weight by dried *Artemia salina* without nutshells per day; the food ration was based on initial fish weights and was recalculated after 14 days. At the end of the test, the fish were weighed again. Food was withheld from the fish for 24 h prior to weighing.

Basic physical and chemical indices of diluting water used in the toxicity tests were as follows: $ANC_{4.5}$ (acid neutralisation capacity) 3.56 - 3.75 $mmol \cdot l^{-1}$; COD_{Mn} (chemical oxygen demand) 1.44 - 1.91 $mg \cdot l^{-1}$; total ammonia < 0.05 $mg \cdot l^{-1}$; NO_3^- 20.8 - 24.35 $mg \cdot l^{-1}$; NO_2^- < 0.002 $mg \cdot l^{-1}$; Cl^- 18.5 - 19.1 $mg \cdot l^{-1}$; sum of Ca ± Mg 14 $mg \cdot l^{-1}$. Diluting water was changed every 48 h. Before changing the water, the maximum following physical and chemical indices were recorded in the control tank: $ANC_{4.5}$ (acid neutralisation capacity) 4.6 $mmol \cdot l^{-1}$; COD_{Mn} (chemical oxygen demand) 2.08 $mg \cdot l^{-1}$; total ammonia < 0.05 $mg \cdot l^{-1}$; NO_3^- 24.8 $mg \cdot l^{-1}$; NO_2^- 0.036 $mg \cdot l^{-1}$; Cl^- 20.1 $mg \cdot l^{-1}$. During the tests, the living conditions were checked at 24-hour intervals and the number of dead fish was recorded in each concentration. Water temperature in the tests ranged 23 ± 1 °C, oxygen saturation of water was above 60% (ranging from 76 to 93%), pH ranged from 8.17 to 8.48.

Tank-average specific growth rates were calculated using this formula according to the OECD No. 215:

$$r = \frac{\log_e W_2 - \log_e W_1}{t_2 - t_1} \cdot 100$$

$\overline{\log_e W_1}$ – average of the logarithms of the values W_1 for the fish in the tank at the start of the study period

$\overline{\log_e W_2}$ – average of the logarithms of the values W_2 for the fish in the tank at the end of the study period

t_1, t_2 - time (days) at start and end of the study period

The results were analysed using the statistical package Unistat 5.1. Data was subjected to one-way ANOVA and subsequently to Dunnett's test in order to assess the statistical significance of differences in tank-average fish specific growth (r) between test groups with different concentrations and that of the control group. The estimation of the LOEC = lowest observed effect concentration (NOEC = no effect observed concentration) was based on ANOVA followed by Dunnett's test for identification of the lowest concentration for which this difference is (is not) significant at a 0.05 probability level.

Results

In a series of three growth tests, no mortality occurred in any of the test groups of fish throughout the 28-day experimental period. Fig. 1 shows an overview of the results of body weight measurements before and after the series of three trials (means ± standard deviations). The initial body weights were similar between groups, but at the end of the trial, body weights were lower in the used test concentrations of NO_2^- as compared to the control group.

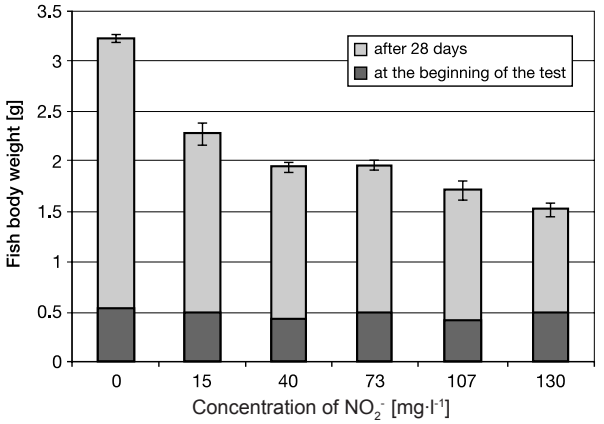

Fig. 1. Comparison of body weight of control (0 mg·l^{-1}) and test fish (nitrite concentrations from 15 to 130 mg·l^{-1})

Fig. 2 demonstrates the results of specific growth rate r_2 (means ± SD) of test groups in comparison with the control group as follows from the chart. A decrease in fish growth caused by nitrite concentration was shown from concentrations of 73 mg·l^{-1} NO_2^-. This decrease was significant ($P < 0.05$) in the concentrations of 60 and 107 mg·l^{-1} NO_2^- and highly significant ($P < 0.01$) in the concentration of 130 mg·l^{-1} NO_2^-. It was identified LOEC = 73 mg·l^{-1} NO_2^- and NOEC = 40 mg·l^{-1} NO_2^-.

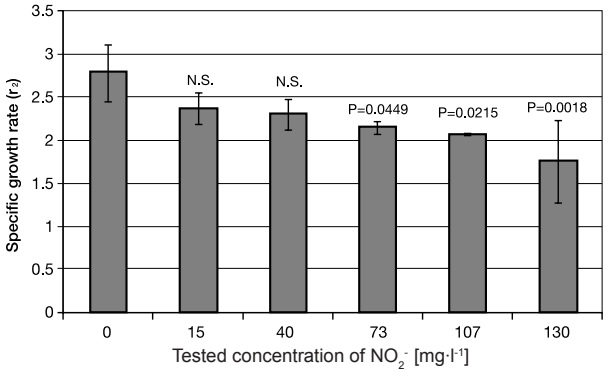

Fig 2. Comparison of specific growth rate r_2 and tested nitrite concentration (NO_2^- concentrations from 15 to 130 mg·l^{-1})

Fig. 3 shows the specific growth rates of groups in relation to different concentrations of nitrite. The increased NO_2^- concentration resulted in lower fish growth. The relationship of nitrite concentrations to growth is expressed by a regression equation, which demonstrates an exponential relationship between these values. The resulting regression equation for larvae of *D. rerio* is $y = 2.6263e^{-0.0028x}$ ($R^2 = 0.896$), where *x* represents nitrite concentrations in diluting water (in mg·l^{-1} NO_2^-) and *y* represents the specific growth rate (r_2).

During the series of three experiments all conditions were applied for the tests to be valid (no fish mortality in the control groups, final weight after 28 days > 150%, dissolved oxygen concentrations were at least 60% and water temperature did not differ by more than ± 1 °C).

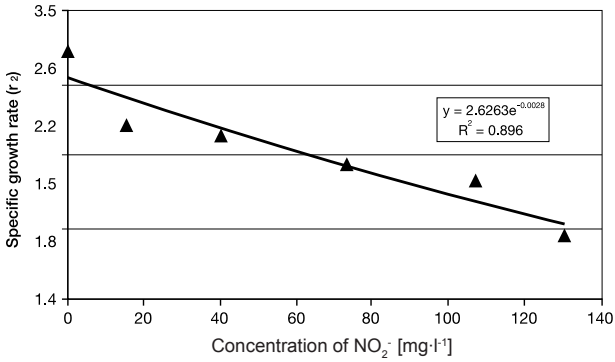

Fig 3. Relationship between nitrite concentrations in diluting water and specific growth rate r_2 in *Danio rerio* larvae

Discussion

Few studies have dealt with the long-term effect of nitrite on the growth of fish. Long-term toxicity of nitrites has been quantified in terms of mortality, growth suppression and tissue damage (Wedemeyer and Yasutake 1978; Colt et al. 1981; Lewis and Morris 1986; Kamstra et al. 1996; Siikavuopio and Saether 2006). In our trial we focused only on the influence of nitrite on fish growth. In previous experiments 96hLC50 values of NO_2^- were determined in juvenile zebrafish (Voslářová et al. 2006). The acute NO_2^- toxicity to *D. rerio* aged 20–25 days as expressed by the 96hLC50 was 386.00 ± 29.75 mg·l^{-1}. The scale of nitrite concentrations from 15 to 130 mg·l^{-1} NO_2^- was used in following growth tests. These values ranged between 4% and 34% of the 96hLC50 value of NO_2^-. Our results show the effect of growth suppression but there was only a significant effect in higher nitrite concentrations used in the trials (107 and 130 mg·l^{-1} NO_2^-). It represents 19% and 34% of 96hLC50 NO_2^- for zebrafish. Similarly, Siikavuopio and Saether (2006) found that the growth of juvenile cod *Gadus morhua* was significantly reduced when exposed to all treatment levels, with reduced growth in the high concentration group in the first period of experimentation (day 1–31) and in all experimental concentrations during later periods.

In contrast to our experiment, no growth suppression was observed by Wedemeyer and Yasutake (1978). They found no significant growth suppression of steelhead during 6-month exposures to nitrite concentrations as high as 10% of the 96hLC50. The same results were recorded by Kamstra et al. (1996), who observed sub-lethal effects of nitrite on the growth of European eel, *Anguilla anguilla*. In the range of concentrations studied (0, 3.29, 16.45, 32.9, 65.8 mg·l^{-1} NO_2^-), no significant effect of nitrite on the maximum growth rate was demonstrated. This was explained by the extremely low uptake of chloride in the gills of this species. Williams and Eddy (1986) state that a shared uptake route for nitrite and chloride is also supported by the fact that fish with high branchial Cl^- uptake rates (rainbow trout, perch, pike) are more sensitive to nitrite than species with low uptake rates (eel, carp, tench). Colt et al. (1981) detected that the minimum amount of nitrite capable of causing detectable growth suppression over 31 days was equal to 44% of the minimum nitrite concentration required to induce mortality in channel catfish. Bowser et al. (1983) showed that the minimum nitrite concentration required to cause mortality of channel catfish would equal approximately half of the 96hLC50, which implies that the minimum amount of nitrite capable of causing detectable growth suppression in channel catfish under the conditions studied by Colt et al. (1981) would be approximately one-fifth of the 96h LC50. The maximum growth suppression at such concentrations would

be approximately 10%; the maximum growth suppression actually observed by Colt et al. (1981) at any nonlethal concentration was 21%. In our study, growth suppression was proven from 107 mg·l^{-1} NO_2^- (approximately 25% of 96hLC50) compared to the control; fish growth suppression was 53% at the highest concentration.

Some authors state that fish are able to acclimate to nitrite, but the nature of the mechanism underlying this adaptation is unclear. Tucker and Schwedler (1983) conducted their experiment on channel catfish and the results clearly indicated that these fish acclimated to nitrite. The fish that were not previously exposed to > 0.01 mg·l^{-1} N-NO_2^- (NO_2^-/Cl^- molar ratio < 0.003) developed higher levels of methaemoglobin than the fish with immediate past history of exposure to relatively high NO_2^-/Cl^- molar ratios. These fish were exposed to > 3.0 mg·l^{-1} N-NO_2^- for the preceding 2 weeks (NO_2^-/Cl^- molar ratio approximately 0.26). The ability to adapt to the increased nitrite concentrations was reported by other authors including Doblander and Lackner (1997) and Máchová et al. (2004).

Annex 2 to the Government Decree no. 71/2003 Coll. specifies the indicators and quality ratings of surface waters suitable for life and reproduction. The rate of nitrites therein is determined at the amount of ≤ 0.9 mg·l^{-1} (*Cyprinidae*). The resulting LOEC (73 mg·l^{-1} NO_2^-) and NOEC (40 mg·l^{-1} NO_2^-) values in our experiment show concentrations that highly exceed this norm. However, they are the result of a subchronic growth test lasting only 28 days. Therefore, it would be advisable for further research to focus on the effects of the long-term impact of nitrites on fish.

Toxicita dusitanů pro danio pruhované (*Danio rerio):* vliv subchronické expozice na růst ryb

Cílem předložené práce bylo zjistit vliv dlouhodobého účinku dusitanů v rozmezí od 15 do 130 mg·l^{-1} NO_2^- na růst akvarijních ryb fish *Danio rerio* - druh, který je celosvětově nejčastěji využíván v testech toxicity. Testy byly provedeny semistatickou metodou podle OECD 215. Byly změřeny počáteční hodnoty hmotnosti ryb při nasazení do pokusu a stejným způsobem byly změřeny hmotnosti ryb na konci pokusu (po uplynutí 28 dnů). Porovnáním těchto hodnot byla prokázána inhibice růstu ryb. Při porovnání kontroly s jednotlivými zvolenými koncentracemi dusitanů byla zjištěna významná inhibice růstu od koncentrace 110 mg·l^{-1} NO_2^- ($P < 0,05$) a vysoce významná inhibice růstu od koncentrace 195 mg·l^{-1} NO_2^- ($P < 0,01$). Porovnáním indexu růstu v jednotlivých testovaných koncentracích byla zjištěna exponenciální závislost ($R^2 = 0,896$).

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