Nitrite Toxicity to *Danio rerio*: Effects of Chloride Concentrations during Acclimatization and in Toxicity Tests

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Abstract

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The aquarium fish *Danio rerio* is the most frequently used species of fish for toxicity tests. The aim of the present study was to determine NO_2^- toxicity to *D. rerio* and to evaluate effects of chloride concentrations during acclimatization and in toxicity tests. The semistatic method according to OECD 203 was used in the tests. The 96hLC50 NO_2^- value for *D. rerio* was 242.41 ± 13.67 mg·l^{-1} at 19 mg Cl^-·l^{-1} in diluting water. There was a significant increase in 96hLC50 NO_2^- to 318.02 ± 50.65 mg·l^{-1} and 387.40 ± 43.99 mg·l^{-1} following an increase in Cl^- concentration in diluting water to 50 mg·l^{-1} ($p < 0.05$) and 100 mg·l^{-1} ($p < 0.01$), respectively. A logarithmical relationship between 96hLC50 NO_2^- values and chloride concentrations in diluting water ($R^2 = 0.733$) was demonstrated. A significant increase in 96hLC50 NO_2^- values was found in toxicity tests in *D. rerio* acclimatized to increased Cl^- concentrations for 96 hours before the test. In these cases 96hLC50 NO_2^- was 378.81 ± 27.20 mg·l^{-1} (50 mg·l^{-1} Cl^-; $p < 0.05$) and 484.04 ± 46.34 mg·l^{-1} (100 mg·l^{-1} Cl^-; $p < 0.01$). *D. rerio* belongs to fish species less sensitive to nitrites. Results of the study confirmed the protective effect of chlorides, particularly in fish adapted to higher chloride concentrations prior to the test.

Danio rerio, 96hLC50, NO_2, competition between NO_2 and Cl^-

Increased nitrite concentrations in water is one of frequent problems encountered in both aquariums and on fish farms. Products of fish metabolism and decaying remains of food and faeces are mainly sources of nitrites. The basic product of protein metabolism of freshwater fish is ammonia (NH_3), which is released from the respiratory tract to water. It is converted by nitrification to toxic nitrites and, finally, to nitrates, which are almost non-toxic to fish. In aquariums, fish are most frequently poisoned by nitrites that accumulate in the tank as a result of inadequate efficiency of biological filters (Adamsson et al. 1988; Etscheidt 2003).

Nitrite toxicity to fish varies considerably and depends on a large number of external and internal factors (species and age of fish, water quality) whose importance is continuously verified and re-assessed. Nitrite toxicity to fish has been studied at various levels, different authors have reached sometimes even contradictory conclusions, but no final explanation of the combined effect of individual internal and external factors has been put forward (Lewis and Morris 1986; Svobodová et al. 2005a). The generally deleterious effect of nitrites results from the fact that they may pass from the aquatic environment through the gills to the fish where they react with haemoglobin to produce methaemoglobin. In this way, its oxygen transport capacity is hampered and the fish may die of hypoxia or anoxia (Woo and Chiu 1997; Knudsen and Jensen 1997).

Breeding aquarium fish, and mainly their export, is at present a significant source of income for the economy of the Czech Republic. For that reason, ways to minimize the risk of fish mortalities or damage due to excessive nitrite concentrations in water-recirculating

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systems are being investigated: one of possible ways how to enhance fish tolerance to nitrites is to increase chloride concentrations in water (Crawford and Allen 1977; Mazik et al. 1991; Atwood et al. 2001; Jensen 2003; Lin and Chen 2003; Dvořák 2004; Svobodová et al. 2005a). The effect of increased chloride levels in the environment may result in competitive inhibition of nitrite uptake through the gills and other integumental tissues and/or it could result in elevated plasma and body fluid chloride levels. The level of chloride in the plasma is significant because of the possible competition between nitrite and chloride for entry into the red blood cell. Dietary and environmental chloride levels would then protect against Fe^{2+} in haemoglobin oxidation by nitrite (Perrone and Meade 1977). Nitrite toxicity to fish decreases with increasing chloride concentrations (Crawford and Allen 1977; Russo and Thurston 1977; Svobodová et al. 2005b).

Zebrafish (*Danio rerio*) are at present the species most frequently used for toxicity tests (OECD and ISO methodological guidelines). For that reason, we also used zebrafish in our study. The aim of the present study was to determine the acute nitrite toxicity level for *D. rerio*, and to examine acute nitrite toxicity at various chloride concentrations and acute nitrite toxicity to that species of fish after a brief acclimatization to elevated chloride concentrations.

Materials and Methods

Tests of acute toxicity were performed on aquarium fish *Danio rerio* (*Cyprinus rerio* (Hamilton 1822) according to „Catalog of Fishes 2004“*)* aged 2 - 3 months (25 - 35 mm body length). The procedure complied with OECD No. 203 Acute Toxicity Test on Fish - Semistatic Method guidelines. The fish were acclimatized for 96 hours to different chloride concentrations before 96-hour acute toxicity tests were performed. A total of 5 series of experiments with NO_2^- concentrations ranging from 67 to 633 mg·l^{-1} and different chloride concentrations were made:

1. fish acclimatized for 96 h with 19 mg·l^{-1} Cl^-, Cl^- test concentration 19 mg·l^{-1}
2. fish acclimatized for 96 h with 19 mg·l^{-1} Cl^-, Cl^- test concentration 50 mg·l^{-1}
3. fish acclimatized for 96 h with 19 mg·l^{-1} Cl^-, Cl^- test concentration 100 mg·l^{-1}
4. fish acclimatized for 96 h with 50 mg·l^{-1} Cl^-, Cl^- test concentration 50 mg·l^{-1}
5. fish acclimatized for 96 h with 100 mg·l^{-1} Cl^-, Cl^- test concentration 100 mg·l^{-1}

In each test series, 5 tests of acute toxicity were made, with 10 fish used for each concentration and for the control group.

Basic physical and chemical indices of diluting water used in the acute toxicity test were as follows: $ANC_{4.5}$ (acid neutralisation capacity) 1.15 mmol·l^{-1}; CODMn (chemical oxygen demand) 1.9 mg·l^{-1}; total ammonia 31.4 mg·l^{-1}; NO_3^- 19.1 mg·l^{-1}; PO_4^{3-} 0.01 mg.l^{-1}; sum of Ca + Mg 14 mg·l^{-1}. Water temperatures in the test ranged 23 ± 1 °C, oxygen saturation of water was above 60% (ranging from 85 to 96%), pH ranged from 8.04 to 8.66.

During the tests, the fish condition was checked at 24-hour intervals and the number of dead fish was recorded for different concentrations. No fish from control groups died during any of the tests. The results obtained (number of fish dying in individual test concentrations) were processed by the probit analysis (EKO-TOX 5.1 software) to determine the 96hLC50 NO_2^- value. For statistical processing of results (basic statistics, correlations between LC50 NO_2^- and Cl^- concentrations in diluting water, significance of differences between 96hLC50 values from individual test series), Unistat 5.1. software was used.

Results

The acute NO_2^- toxicity to *D. rerio* as expressed by the 96hLC50 value was 242.41 ± 13.67 mg·l^{-1}. This result was obtained with chloride concentration naturally occurring in the diluting water used, i.e. 19 mg·l^{-1}. The 96hLC50 NO_2^- values for *D. rerio* obtained in acute toxicity tests with different chloride concentrations in diluting water are given in Fig. 1. Significant differences in 96hLC50 NO_2^- values were found between tests with chloride concentrations of 19 and 50 mg·l^{-1} ($p < 0.05$), 19 and 100 mg·l^{-1} ($p < 0.01$) and 50 and 100 mg·l^{-1} ($p < 0.05$). Statistical evaluation of 96hLC50 NO_2^- values obtained in acute toxicity tests when diluting water with increasing chloride concentrations was used (19 - 100 mg·l^{-1}) demonstrated a logarithmic relationship between 96hLC50 NO_2^- and chloride concentrations in diluting water (Fig. 2). The resulting regression equation is y = -15.26 + 86.69*Ln(x) ($R^2 = 0.733$), where *x* is chloride concentrations in diluting water (in mg·l^{-1} Cl) and *y* is the value of 96hLC50 (in mg·l^{-1} NO_2^-).

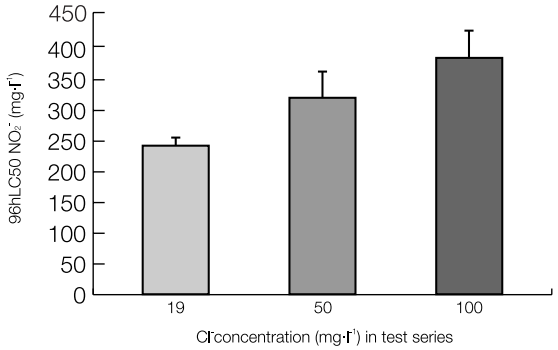

Fig. 1. Effects of higher chloride concentrations in diluting water to 96hLC50 NO_2^- in *Danio rerio* (test series 1, 2 and 3)

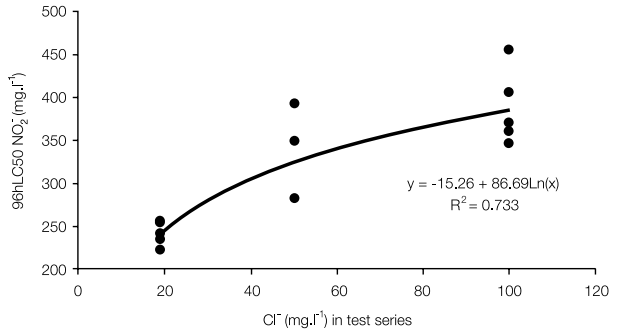

Fig. 2. Relationship between 96hLC50 NO_2^- values in *Danio rerio* and chloride concentrations in diluting water (test series 1, 2 and 3)

An increase in 96hLC50 NO_2^- was found in fish that were acclimatized in diluting water with higher Cl^- concentrations (50 and 100 mg·l^{-1}) for 96 hours before the test start. Differences between 96hLC50 NO_2^- in fish acclimatized to increased chloride concentrations and fish acclimatized to diluting water containing chlorides at 19 mg·l^{-1} concentration was statistically significant at Cl^- concentrations of 50 mg·l^{-1} ($p < 0.05$) and 100 mg·l^{-1} ($p < 0.01$) (Fig. 3). The relation of 96hLC50 NO_2^- to chloride concentrations in diluting water following acclimatization to higher chloride concentrations (Fig. 4) is expressed by the regression equation y = -186.46 + 145.24*Ln(x) ($R^2 = 0.922$), where *x* is chloride concentration in diluting water (in mg·l^{-1} Cl^-) and *y* is the value of 96hLC50 (in mg·l^{-1} NO_2^-).

Discussion

Considerable differences in fish species sensitivity to nitrite exposure have been reported by, e.g., Lewis and Morris (1986). They noticed that data from acute toxicity tests vary within a very wide range of results not just between different families of fish, but within families as well. At chloride concentration of 20 mg·l^{-1}, they found 96hLC50 NO_2^- of 21.7 mg·l^{-1} for the rainbow trout, values over 207 mg·l^{-1} for the common carp, 217 mg·l^{-1} for *Pimephales promelas* and 355 mg·l^{-1} for *Lepomis macrochirus*. The 96hLC50 NO_2^- value for *D. rerio* found in the present study (242.41 ± 13.67 mg·l^{-1}) at 19 mg·l^{-1} concentration of

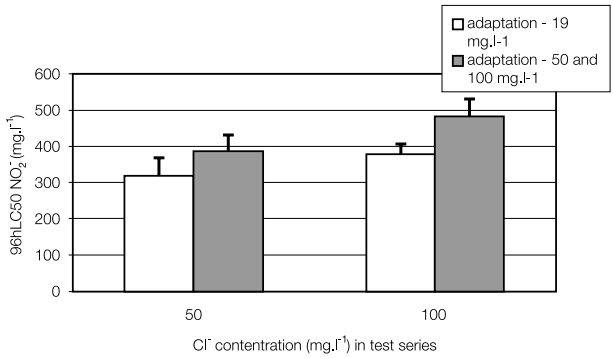

Fig. 3. Comparison between different 96hLC50 NO_2^- values obtained in acute toxicity tests at different Cl^- concentrations on *Danio rerio* fish briefly acclimatized prior to tests to various chloride concentrations (comparison between results of series 2 + 4 and 3 + 5)

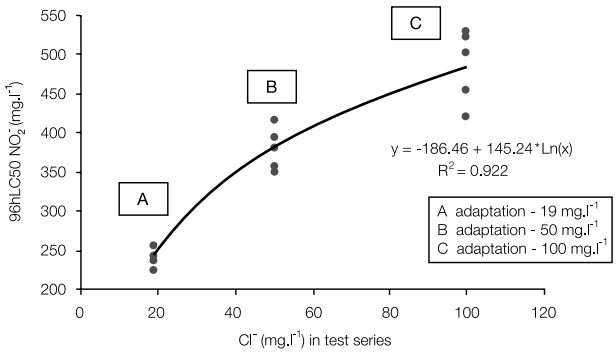

Fig. 4. Relationship between 96hLC50 NO_2^- values and chloride concentrations in diluting water for *Danio rerio* fish acclimatized to Cl^- concentrations 19, 50, 100 mg·l^{-1} before the 96hLC50 test (test series 1, 4 and 5)

Cl^- is comparable with the above results. In a study of the aquarium fish *Poecilia reticulata*, Kroupová et al. (2004) on the other hand found 96hLC50 NO_2^- lower by about one order of magnitude (25 mg·l^{-1}) as compared with results for *D. rerio*. Besides different species sensitivity, an important role in this case was probably played by a low chloride concentration in diluting water (10 mg·l^{-1}).

A significant effect of chloride concentrations in diluting water on 96hLC50 NO_2^- was demonstrated in acute toxicity tests on *Danio rerio*. A logarithmic relationship between 96hLC50 NO_2^- values and chloride concentrations in diluting water (R^2 = 0.733) was demonstrated. A linear relationship has been described in other fish species (Crawford and Allen 1977; Russo and Thurston 1977; McConnell 1985; Lewis and Morris 1986; Kroupová et al. 2004).

Although increases in 96hLC50 NO_2^- as a result of increased chloride concentrations were statistically significant, the differences in values found for *D. rerio* were not as great as those found in other fish species. Crawford and Allen (1977) found that mortality of small chinook salmon in seawater occurred at nitrite concentrations 50 to 100 times higher than in fresh water. Kroupová et al. (2004) reported an increase of 96hLC50 NO_2^- in *Poecilia reticulata* from 25 mg·l^{-1} (Cl^- 10 mg·l^{-1}) to 159 mg·l^{-1} (Cl^- 55 mg·l^{-1}), i.e. more than six fold

increase over the original value. In *D. rerio*, chloride concentration increase from Cl^- 19.0 $mg \cdot l^{-1}$ to 50 $mg \cdot l^{-1}$ in the environment resulted in only a 31% increase in 96hLC50 NO_2^- (from 242.41 ± 13.67 $mg \cdot l^{-1}$ to 318.02 ± 50.65 $mg \cdot l^{-1}$). It may be deduced that *D. rerio* is less sensitive to ambient nitrites than other species of fish. A greater protective effect of higher chloride concentrations might be therefore expected for this fish. For instance, Lewis and Morris (1986) concluded that the strength of the chloride effect is the greatest for the least sensitive species and the smallest for the most sensitive species. No such protective effect was confirmed in *D. rerio*.

It can be concluded from the computed regression equation of relationship between 96hLC50 NO_2^- and chloride concentrations in diluting water that 1 $mg \cdot l^{-1}$ increase in chloride concentration in diluting water results in the case of *D. rerio* in an increase of 8.27 $mg \cdot l^{-1}$ in 96hLC50 NO_2^-. Kroupová et al. (2004) reported an increase of 1.41 $mg \cdot l^{-1}$ in 96hLC50 NO_2^- in *Poecilia reticulata*. In their experiments on rainbow trout, Russo and Thurston (1977) found an increase of 0.95 $mg \cdot l^{-1}$ in 96hLC50 NO_2^-. In his study with *Pimephales promelas*, McConnell (1985) showed that an increase in chloride concentration in water by 1 $mg \cdot l^{-1}$ increased the 96hLC50 value by 6.57 $mg \cdot l^{-1}$ NO_2^-. The comparison of sensitivity of the above species of fish according to their 96hLC50 NO_2^- values, leads to a conclusion that the most sensitive species is the rainbow trout (Russo and Thurston 1977), while *Poecilia reticulata* is less sensitive (Kroupová et al. 2004) and the least sensitive is *Pimephales promelas* (McConnell 1985). It can be concluded further that an increase in chloride concentration increases nitrite tolerance in the less sensitive species to a larger extent than in the more sensitive ones. The results obtained for *D. rerio* corroborate the above hypothesis because their 96hLC50 of 242.41 ± 13.67 $mg \cdot l^{-1}$ grew by 8.27 $mg \cdot l^{-1}$ when chloride concentration in diluting water increased by 1 $mg \cdot l^{-1}$.

In addition to the previously described positive effect of higher ambient chloride concentrations on nitrite toxicity to fish, our experiments also showed that this effect was greater in fish that had been acclimatized to higher chloride concentrations (50 and 100 $mg \cdot l^{-1}$) prior to the toxicity tests. If fish were placed to diluting water with Cl^- concentrations of 50 $mg \cdot l^{-1}$ and 100 $mg \cdot l^{-1}$ 96 hours prior to the beginning of tests, their 96hLC50 NO_2^- values were 378.76 ± 27.20 $mg \cdot l^{-1}$ and 484.14 ± 46.34 $mg \cdot l^{-1}$, respectively. It can be concluded from the computed regression equation of the relation between 96hLC50 NO_2^- and chloride concentrations in diluting water that an increase of 1 $mg \cdot l^{-1}$ in chloride concentration results in an increase of 13.83 $mg \cdot l^{-1}$ in 96hLC50 NO_2^- in the case of *D. rerio* acclimatized to higher chloride concentrations. However, in the absence of acclimatization period, the increase in 96hLC50 NO_2^- was only 8.27 $mg \cdot l^{-1}$. This is probably related to changes in the microscopic structure of the gills occuring due to higher chloride concentrations during the acclimatization period. Svobodová et al. (2005b) noticed an increase in the number of eosinophilic chloride cells on the gills of carp exposed for 96 hours to diluting water containing 100 $mg \cdot l^{-1}$ of chlorides.

Toxicita dusitanů pro danio pruhované (*Danio rerio):* vliv koncentrace chloridů v průběhu adaptace a v testu toxicity

Akvarijní ryba *Danio rerio* je nejpoužívanějším druhem ryb k testům toxicity. Cílem předkládané práce bylo stanovit toxicitu NO_2^- pro *D. rerio* a zhodnotit efekt koncentrace chloridů v průběhu adaptace a při vlastním testu toxicity. Testy byly provedeny semistatickou metodou podle OECD 203. Zjištěná hodnota 96hLC50 NO_2^- pro *D. rerio* byla 242,41 ± 13,67 $mg \cdot l^{-1}$ při koncentraci Cl^- v ředicí vodě 19 $mg \cdot l^{-1}$. K signifikantnímu zvýšení hodnot 96hLC50 NO_2^- došlo po zvýšení koncentrace Cl^- v ředicí vodě v testu toxicity, a to na hodnotu 318 ± 50,65 $mg \cdot l^{-1}$ (50 $mg \cdot l^{-1}$ Cl^-; $p < 0,05$), resp. 387,40 ± 43,99 $mg \cdot l^{-1}$ (100 $mg \cdot l^{-1}$ Cl^-; $p < 0,01$). Byla prokázána logaritmická závislost hodnot 96hLC50 NO_2^- na koncentraci

chloridů v ředicí vodě (R^2 = 0,733). Signifikantní zvýšení hodnot 96hLC50 NO_2^- bylo zjištěno v testech toxicity na *D. rerio* 96 hodin před testem adaptovaných na zvýšené koncentrace Cl^-. V těchto případech byla hodnota 96hLC50 NO_2^- 378,81 ± 27,20 mg·l^{-1} (50 mg·l^{-1} Cl^-; $p < 0,05$) a 484.04 ± 46,34 mg·l^{-1} (100 mg·l^{-1} Cl^-; $p < 0,01$). *D. rerio* patří mezi druhy ryb méně citlivé k dusitanům. V práci byl potvrzen ochranný vliv chloridů, a to především u ryb před testem adaptovaných ke zvýšeným koncentracím chloridů.

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