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Environmental Pollution xx (2007) 1–9

ENVIRONMENTAL POLLUTION

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Effects of nickel chloride and oxygen depletion on behaviour and vitality of zebrafish (*Danio rerio*, Hamilton, 1822) (Pisces, Cypriniformes) embryos and larvae

Cornelia Kienle [a,c,*], H.-R. Köhler [a], Juliane Filser [b], Almut Gerhardt [c]

[a] *Department of Animal Physiological Ecology, University of Tübingen, Konrad-Adenauer-Strasse 20, D-72072 Tübingen, Germany*
[b] *Department of General and Theoretical Ecology, University of Bremen, Leobener Strasse, D-28359 Bremen, Germany*
[c] *LimCo International, An der Aa 5, D-49477 Ibbenbüren, Germany*

Received 27 December 2006; received in revised form 16 June 2007; accepted 24 June 2007

Increasing concentrations of nickel chloride and decreasing concentrations of oxygen lead to reduced vitality and locomotory activity in Danio rerio embryos and larvae.

Abstract

We examined acute (2 h exposure of 5-day-old larvae) and subchronic (exposure from fertilization up to an age of 11 days) effects of $NiCl_2 \cdot 6H_2O$ on embryos and larvae of zebrafish (*Danio rerio*), both alone and in combination with oxygen depletion. The following endpoints were recorded: acute exposure: locomotory activity and survival; subchronic exposure: hatching rate, deformations, locomotory activity (at 5, 8 and 11 days) and mortality. In acute exposures nickel chloride (7.5–15 mg Ni/L) caused decreasing locomotory activity. Oxygen depletion ($\leq 2.45 \pm 0.16$ mg O_2/L) also resulted in significantly reduced locomotory activity. In the subchronic test, exposure to $\geq$10 mg Ni/L resulted in delayed hatching at an age of 96 h, in decreased locomotory activity at an age of 5 days, and increased mortality at an age of 11 days ($LC_{20} = 9.5$ mg Ni/L). The observed LOEC for locomotory activity (7.5 mg Ni/L) is in the range of environmentally relevant concentrations. Since locomotory activity was already affected by acute exposure, this parameter is recommended to supplement commonly recorded endpoints of toxicity.

Keywords: Behaviour; $NiCl_2$; O_2; Multispecies Freshwater Biomonitor®

1. Introduction

Zebrafish (*Danio rerio*, Hamilton, 1822) which originally live in stream habitats rich in macrophytes in Southeast Asia (Börries, 2006) have received special attention in research during the last years, especially as model vertebrates in developmental biology and genetics (e.g. Kimmel, 1989; Nüsslein-Volhard, 1994). The embryo test with *D. rerio* (DarT) was proposed as an alternative method for the acute fish test with adult fish (Nagel, 2002) and numerous studies with *D. rerio* embryos and larvae have been conducted so far (Bachmann, 2002; Nagel, 2002; Strmac, 1999; Versonnen et al., 2004). Some studies investigated the effects of pollutants on the behaviour of zebrafish using adolescent or adult fish (Baganz et al., 1998; Levin et al., 2003; Steinberg et al., 1995; Vogl et al., 1999) while others described baseline data on the behaviour of larval zebrafish (Bagatto et al., 2001; Budick and O'Malley, 2000; Orger et al., 2000), but only a single study so far considered the effect of a chemical stressor

* Corresponding author. Department of Animal Physiological Ecology, University of Tübingen, Konrad-Adenauer-Strasse 20, D-72072 Tübingen, Germany. Tel.: +49 7071 7573557; fax: +49 7071 7573560.

E-mail addresses: cornelia.kienle@uni-tuebingen.de (C. Kienle), heinz-r.koehler@uni-tuebingen.de (H.-R. Köhler), filser@uni-bremen.de (J. Filser), almutg@web.de (A. Gerhardt).

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doi:10.1016/j.envpol.2007.06.069

(amino acid chemostimulants) on zebrafish larval behaviour (Lindsay and Vogt, 2004). To further reduce the use of adult fish in ecotoxicological tests, however, it might be reasonable to establish behavioural tests with fish larvae.

Behavioural ecotoxicology deals with the effects of pollutants on the behaviour of organisms, and their link to adjacent levels of biological organisation (e.g. biochemical, physiological or general metabolic processes within the animal as well as population maintenance) (Dell'Omo, 2002). Behaviour integrates the animals' responses to internal (physiological) and external (environmental, social) factors and relates one organism to another (Evans, 1994).

Behavioural tests represent a sensitive method to detect effects of contaminants (Dell'Omo, 2002) compared to conventional endpoints as mortality (e.g. Levin et al., 2003). Moreover, alterations in behaviour are measurable already after a short time (e.g. avoidance, attraction). Lindsay and Vogt (2004) were able to detect effects of amino acid chemostimulants on the behaviour of 4-day-old *D. rerio* larvae within only few minutes of exposure.

To measure behavioural alterations automated online biomonitors can be used. They use living organisms as sensors for alterations in water quality and work in real time (Gruber et al., 1994; Osbild et al., 1995). In our study, the Multispecies Freshwater Biomonitor® (MFB) (LimCo International, Germany) has been used to record the locomotory activity of *D. rerio* larvae.

Next to biotic factors, abiotic factors determine the constitution and the efficiency of an organism's physiological and behavioural performances in an ecosystem. Abiotic stressors like oxygen depletion can occur during summer in the hypolimnion of eutrophic lakes and in streams dominated by organic matter degradation (Schwoerbel, 1992). Fish from mountain streams usually react most sensitive to oxygen deficiency: whereas salmonids need at least 6 mg O_2/L and show stress in respiration at 40–50% O_2 saturation, the more insensitive carps are capable of living at oxygen contents down to 1 mg/L, resp. 13% saturation (at 26 °C) (Schönborn, 2000).

Nickel(II) chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) is a water-soluble nickel compound, not biologically degradable, very toxic for aquatic organisms and may cause long-term harmful effects (Merck, 2004). Nickel (Ni) is a ubiquitous, naturally occurring trace metal (0.0086% of the earth crust; Duke, 1980), with increased concentrations in waterbodies, e.g. in the area of nickel-processing industries (WHO, 1991). Unpolluted Canadian rivers and lakes exhibit background concentrations of 0.1–10 μg Ni/L but natural waters near industrial sites have been shown to contain between 50 and 2000 μg Ni/L, with a maximum of 183 000 μg Ni/L near a nickel refinery in Sudbury, Ontario (Chau and Kulikovsky-Cordeiro, 1995; Kasprzak, 1987).

The aim of the present study was to examine the effects of nickel chloride on locomotory behaviour, survival and vitality of early life stages of zebrafish (*D. rerio*) in hard water. The innovative approach in our study was based on (1) the evaluation of behaviour as sensitive test parameter for short- and long-term tests, (2) the potential of replacing adult fish by young larvae considering ethical reasons as well as sensitivity aspects and (3) increased ecological realism by adding oxygen depletion as an interfering natural stressor.

The following hypotheses were tested for juvenile zebrafish.

1. Exposure to $NiCl_2$ results in a higher locomotory activity (avoidance reaction).
2. Sensitivity to Ni is exposure time-dependent.
3. Additional environmental stress (oxygen depletion) increases $NiCl_2$ toxicity.

2. Materials and methods

2.1. Test animals and acquisition of eggs

Adult zebrafish (*D. rerio*, strain WIK, MPI for Developmental Biology, Tübingen) of both sexes were kept in the laboratory in 150–230 L aquaria with aerated and filtered water (50/50% mixture of tap and distilled water with a conductivity of approx. 400 μS/cm), with a minimum of 1 L water per fish on the average. Culture conditions were 26 ± 1 °C at a 12 h:12 h light:dark cycle without dimming. The adult fish were fed ad libitum twice per day with dry flake food (Nutrafin Max, Hagen, Germany) and frozen crustaceans or midge larvae (MM Aquaristik, Germany), respectively.

The eggs used in the tests were collected using spawn traps which had been placed on the bottom of each aquarium the evening before spawning was required. In the morning (1 h after triggering the spawning via switching on the light) the spawn traps were removed from the aquaria, the eggs were sieved and cleaned under flowing tap water and transferred to Petri dishes. Embryos and larvae were kept in glass Petri dishes in reconstituted water (OECD-Guideline 203; ISO-Standard 6341-1982), which had been aerated for 12 h before use with an aquarium pump. The Petri dishes with the embryos and larvae were kept in a climate chamber at 26 ± 1 °C and a 12 h:12 h light:dark cycle up to an age of 5 days. Two to four hours after fertilization the fertilized eggs were separated from unfertilized eggs and distributed over several Petri dishes with test water. An appropriate amount of water (~1/3 of the volume) was exchanged daily. After 24 h the eggs were put into new Petri dishes with fresh reconstituted water. Every day the condition of the larvae was checked under a stereomicroscope, and malformed or inactive embryos and larvae were removed.

2.2. Test substance

Nickel(II) chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) (Roth, Germany) was dissolved in reconstituted water in order to prepare a stock solution of 1000 mg Ni/L at pH 7.5. From this stock solution the test solutions were prepared directly before use. Eight different nominal concentrations (0.25, 1, 2.5, 5, 7.5, 10, 12.5 and 15 mg Ni/L) and two negative controls with pure reconstituted water were examined for the acute test. The subchronic test comprised five nominal concentrations (0.5, 1, 5, 10 and 15 mg Ni/L) and one negative control.

2.3. The Multispecies Freshwater Biomonitor® (MFB)

The Multispecies Freshwater Biomonitor (LimCo International, Germany) is an online biomonitor which continuously and quantitatively records the behaviour pattern of animals (Gerhardt et al., 1994). The MFB consists of flow-through sensor chambers, a measuring unit and a personal computer with specific software for data evaluation (Gerhardt, 2001). The measuring principle in the sensor chamber is based on quadrupole impedance conversion (Gerhardt et al., 1994). The behavioural signal of the animal is analysed by a Fast Fourier Transformation, resulting in a histogram of different signal

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frequencies, hence being able to distinguish different types of behaviours, such as locomotion and ventilation (Gerhardt et al., 1994). The chambers, sealed with a lid (mesh size: 0.25 mm) at both ends, used for the fish larvae were 4 cm in length with a diameter of 1 cm, allowing for free movement of the fish (size of fish larvae: ~3.8 mm in length, ~0.5–1 mm in diameter). Previous tests with chambers of different lengths revealed that the above-mentioned size was suitable for short-term exposure of 2 h.

2.4. Acute behavioural tests with nickel chloride

Five-day-old larvae have been chosen based on the results of pilot studies (data not shown) which showed that larvae first display constant swimming activity with an intermediate overall activity and low variation in locomotory activity at this age and thus seemed to be most suitable to allow for the detection of increased as well as decreased activity due to environmental stress.

The chambers were placed into polyethylene vessels ($208 \times 208 \times 64\ mm^3$, 2 L) filled with 2 L of the respective nickel solution, which were arranged in duplicate in a surrounding black basin (to eliminate disturbance from movement along the vessels) with temperature adjusted water (to $26 \pm 1\ ^\circ C$). Only healthy larvae were used and transferred carefully into the chambers; the remaining air bubbles in the chambers were removed with a Pasteur pipette. Subsequently, the chambers were placed horizontally on the bottom of the test vessel (Fig. 1a). After an acclimation time of 10 min the measurement was started. The behaviour of 11–12 larvae per treatment was recorded continuously for 2 h in intervals of 10 min and for a duration of 4 min each. Several abiotic parameters (temperature, pH, conductivity, oxygen concentration and saturation) were determined at the beginning and the end of each measurement period. The test vessels were illuminated from above during the measurements (58 W neon light, distance to chambers: 145 cm). No food was provided during the experiments.

2.5. Subchronic test with nickel chloride

The test was conducted according to the VMD Guidance Note ''Ecotoxicity testing of medicines intended for use in fish farming'' (Veterinary Medicines Directorate, 1996). The organisms were exposed to Ni from the time of fertilization (≤1 h) up to an age of 11 days in plastic Petri dishes with 30 fertilized eggs each and three replicates per nickel concentration. Plastic Petri dishes were used to avoid possible Ni–glass interactions. After 96 h of embryonic development, the hatching rate and mortality were recorded. Furthermore, mortality and unusual swimming behaviour at the surface were recorded daily up to the 11th day after fertilization. For behavioural measurements in the MFB, four larvae from each replicate were randomly removed for analysis at regular intervals (5, 8 and 11 days after fertilization). The behaviour measurements of the animals were performed in the same Ni concentration as used for the subchronic exposure. No food was provided during the experiments.

2.6. Test with different oxygen levels

The experiments were performed in a completely air-tight construction (Fig. 1b), oxygen was removed by pumping gaseous nitrogen in the test solutions, the surrounding waterbath and the overlying atmosphere via an aeration stone for an appropriate time (~5 to 30 min), depending on the oxygen concentration which was aimed to be reached. To keep the oxygen level constant, the test vessels were arranged in a surrounding glass aquarium ($60 \times 30 \times 30$ cm) with appropriate holes for the cables of the measuring chambers and for the aeration tube. As soon as the appropriate oxygen level was reached, the larvae were placed into the chambers as described in Section 2.4. Subsequently the top was covered by a glass plate and the waterbath and the surrounding atmosphere aerated once again to reach the appropriate oxygen concentration. The air-tightness of the construction was guaranteed through

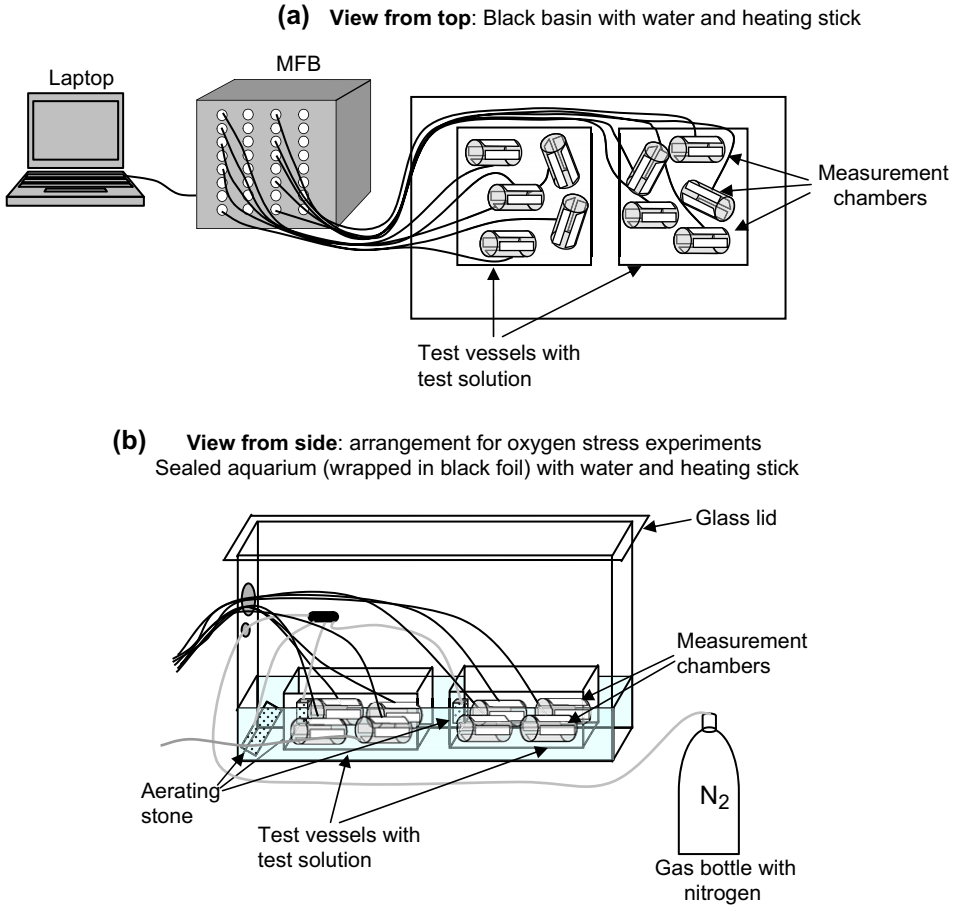

Fig. 1. Experimental setup for behavioural measurements with and without oxygen stress (explanation in the text).

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Table 1
Combinations of nickel and oxygen concentrations

O_2 (mg/L)							
Nickel (mg/L)	0.81	2.45 ± 0.16	3.23 ± 0.25	4.19 ± 0.28	4.75 ± 0.60	5.33 ± 0.40	7.94 ± 0.24
0	+	+	+	+	+	+	+
0.25				+			+
0.5				+			
1		+		+			+
2.5				+			+
5			+	+			+
7.5		+		+		+	+
10			+	+		+	+
12.5				+			+
15		+	+	+		+	+

sealing with tape. As confirmed by repeated oxygen measurements, this construction kept the oxygen level in the water nearly constant over a period of 2 h. Six different oxygen concentrations between 0.81 and 7.94 mg O_2/L were tested (for detailed data see Section 3). Each oxygen concentration was combined with different concentrations of Ni (Table 1). The behaviour of 9–12 replicate *Danio* specimens was recorded for each treatment.

2.7. Data analysis

For each larva, means of locomotory activities (percentage time spent on locomotion) were calculated separately for the first and the second hours, to take into account possible early warning reactions and the decrease of activity over time. For statistical evaluation the data on "percentage time spent on locomotion" were arcsine transformed from proportional values. Nonparametric methods were chosen because the data were only partially normally distributed (one-sample Kolmogorov–Smirnov test, SPSS 10.0.1, USA). Linear regression analysis (JMP 4.0, SAS systems, USA) was performed in order to detect treatment differences in abiotic parameters. The data of all tests were analysed for significance using Friedman's ANOVA (Statistica 5.0, StatSoft, USA), followed by a Wilcoxon two group test (JMP 4.0, SAS systems, USA) to examine differences between control and exposure treatments. The response surface for mixture data of $NiCl_2$ and oxygen depletion was calculated with Statistica 5.0 (StatSoft, USA) and mixture responses were calculated with the MixTox Model (Jonker et al., 2005). The LC_{20} after 11 days was estimated with Table Curve™ 2D 5.1 (SYSTAT Software Inc., USA).

3. Results

3.1. Abiotic parameters

In the experiments with Ni alone, the abiotic parameters matched optimal conditions for the larvae, such as 25.3 ± 0.8 °C, 7.94 ± 0.24 mg O_2/L (99.6 ± 2.6%), pH: 7.99 ± 0.14 and conductivity: 640 ± 17 µS/cm (mean ± SD of the control treatments, $n = 6$).

The oxygen concentrations in the tests with oxygen depletion were: 0.81 mg O_2/L (~10%, single value), 2.45 ± 0.16 mg O_2/L (31.1 ± 2.4%), 3.23 ± 0.25 mg O_2/L (41.7 ± 3.5%), 4.19 ± 0.28 mg O_2/L (53.6 ± 3.9%), 4.75 ± 0.60 mg O_2/L (60.5 ± 7.0%) and 5.33 ± 0.40 mg O_2/L (68.3 ± 4.7%). The pH increased significantly with decreasing oxygen concentration to 8.39 ± 0.33 ($p < 0.018$, $pH = 8.409 - 0.005\ [O_2]$, $r^2 = 0.414$, $n = 13$). With increasing nickel concentrations, electric conductivity increased significantly to 719 ± 19 µS/cm ($p < 0.001$, conductivity = 648.658 + 4.721 [Ni], $r^2 = 0.815$, $n = 13$), but in a tolerable range for the embryos and larvae.

3.2. Locomotory activity of D. rerio larvae

D. rerio larvae showed nearly constant locomotory movements in the control treatments (Fig. 2). Occasionally, short pauses in locomotion were recorded. The movement pattern was characterized of alternating high peaks (high amplitude,

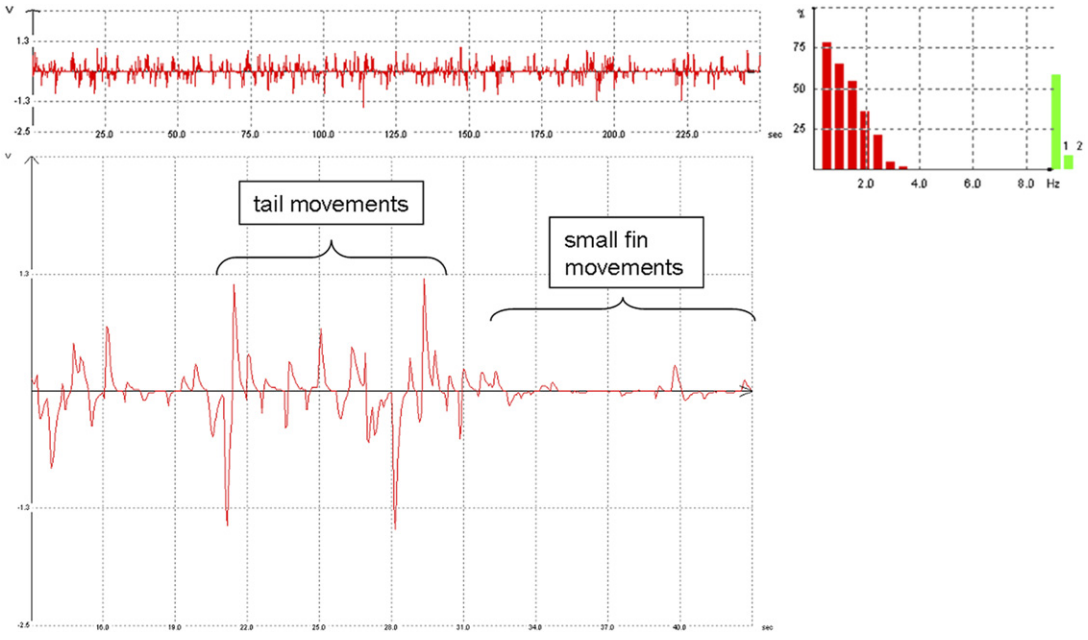

Fig. 2. Example of the spontaneous locomotory movement pattern (left: original signal: amplitude (V) vs. time (s), right: FFT-histogram (activity in % of the time (250 s)) vs. frequency (Hz)) of a 5-day-old *Danio rerio* larva under control conditions.

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corresponding to tail movements) and weaker movements with lower amplitude (corresponding to small fin movements).

The comparison of the data recorded for the respective first hour of measurement with those recorded for the second hour revealed the effect of Ni and O_2 depletion to be more pronounced during the second hour of movement recording. Therefore, in the following, we exclusively refer to data recorded during the second hour of the measurement.

3.3. Acute test with nickel chloride

The locomotory activity decreased significantly with increasing nickel concentration ($p < 0.001$, activity $= 0.702 - 0.0168$ [Ni], $r^2 = 0.188$, $n = 117$). The LOEC with a significant difference vs. the control was 7.5 mg/L ($p < 0.001$, Friedman's ANOVA; $p < 0.005$, Wilcoxon test) (Fig. 3).

3.4. Subchronic test with nickel chloride

In the subchronic test with $NiCl_2$, larvae exhibited different symptoms of Ni toxicity with increasing exposure time.

A significant delay of hatching was observed at concentration levels of 10 mg/L and above at an age of 96 h ($p < 0.019$, Friedman's ANOVA; $p < 0.046$, Wilcoxon test) (Fig. 4). In treatments with 15 mg Ni/L on the average 23.3% of the larvae had not hatched as compared to 1.1% in the control treatment. No increased mortality could be observed at this age.

Locomotory activity decreased with exposure time. After 11 days of exposure, the activity of the larvae was generally much lower than that in the first days. The most obvious differences in the activity of the larvae between the treatments were recorded at the age of 5 days ($p < 0.001$, Friedman's ANOVA) (Fig. 5), e.g. decreased activity vs. the control was found at 10 mg Ni/L ($p < 0.028$) and at 15 mg Ni/L ($p < 0.042$, first hour; $p < 0.067$, second hour, Wilcoxon test).

Significant differences in the number of larvae which stayed constantly at the water surface (in the following 'surface swimming') were observed ($p < 0.0103$, Friedman's ANOVA) in treatments with 10 mg Ni/L at an age of 8 days and more ($p < 0.034$, Wilcoxon test) and with 15 mg Ni/L at an age of 7 days and more ($p < 0.037$, Wilcoxon test) (Fig. 6). These larvae went back to the surface after having been pushed under water with a pipette tip and were not able to remain in the water column or at the bottom of the Petri dish. This effect prevented behavioural data recording for 10–15 mg Ni/L-exposed larvae at day 11 in our setup with chambers, filled completely with water, without any air space.

Mortality increased significantly at an age of 11 days after fertilization at concentrations of 10–15 mg Ni/L ($p < 0.016$, Friedman's ANOVA; $p < 0.046$ and $p < 0.043$, Wilcoxon test) up to $39.4 \pm 5.3\%$. The LC_{20} at 11 days was 9.52 mg Ni/L.

3.5. Acute test with O_2-deficiency

A lower and less frequent locomotory activity of larvae in oxygen-deficient water (2.45 mg O_2/L) compared to control larvae in water with 7.94 mg O_2/L was recorded ($p < 0.004$,

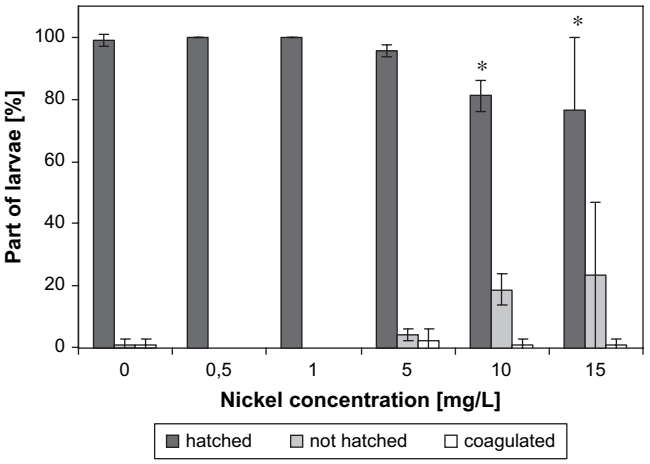

Fig. 4. Hatching rate and mortality of 96-h-old *D. rerio* larvae. Percentage of hatched or coagulated (dead) larvae vs. the nickel concentration (mg/L) (means ± SD, 30 larvae per replicate for each concentration, three replicates each). Significant differences to the control: $*p < 0.05$ (Wilcoxon test).

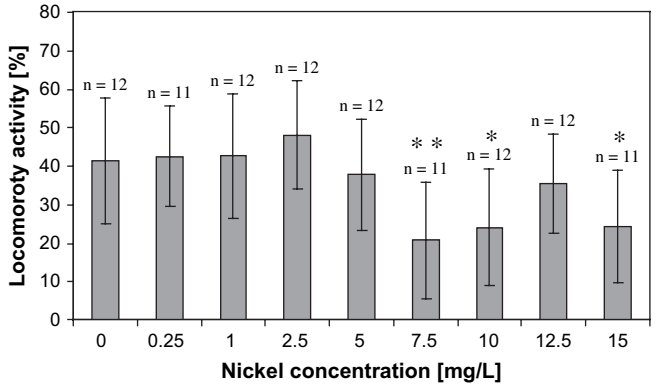

Fig. 3. Acute exposure: concentration–response relationship of locomotory activity (%) (0.5–2 Hz frequency band) of 5-day-old *D. rerio* larvae against the Ni concentration (mg/L) (mean ± SD). Significant differences to the control (0 mg Ni/L): $**p < 0.01$; $*p < 0.05$ (Wilcoxon test).

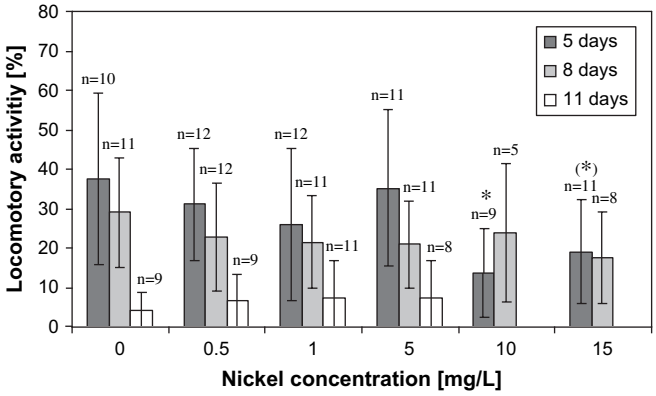

Fig. 5. Subchronic exposure: concentration–response relationship of locomotory activity (%) (0.5–2 Hz frequency band) of 5-day-old *D. rerio* larvae against the concentration (mg/L) (mean ± SD). Significant differences vs. control: $*p < 0.05$; $(*)p < 0.067$ (Wilcoxon test).

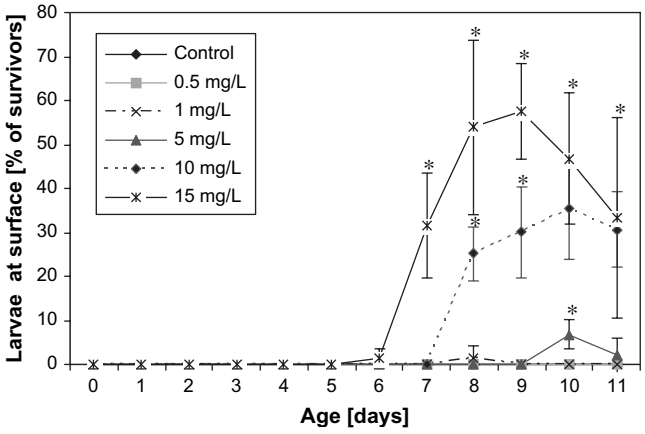

Fig. 6. Proportion of *D. rerio* larvae (% of survivors) which stayed constantly at the water surface at different nickel concentrations (mean ± SD); number of larvae for each replicate 30 (days 0–5); 26 (days 6–8); 22 (days 9–11), three replicates each. Significant differences to control treatment: $*p < 0.05$ (Wilcoxon test).

Wilcoxon test). The locomotory activity between treatments with 2.45 and both 4.19 and 7.94 mg O_2/L differed significantly ($p < 0.001$, Wilcoxon test). After 2 h of measurement, mortality occurred in treatments with 0.81 (~100%), 2.45 (42%) and 3.23 mg O_2/L (25%). Accordingly no sublethal effect threshold for oxygen depletion on the behaviour of zebrafish larvae could be detected.

In combination of oxygen deficiency and nickel treatment, locomotory activity decreased with increasing nickel concentrations in tests with high oxygen saturation levels (4.19 mg O_2/L and higher) (Fig. 7). At lower oxygen concentrations (<4.19 mg O_2/L), nickel had rather a stimulating than an inhibitory effect on locomotory activity. Mixture toxicity

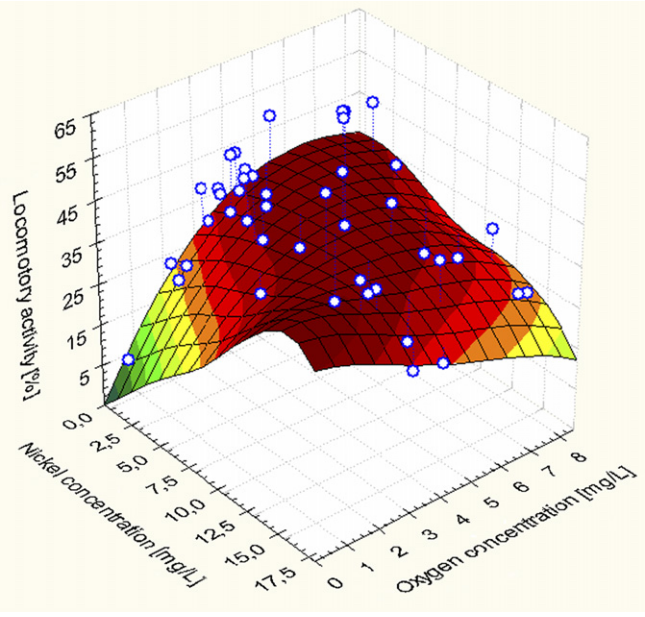

Fig. 7. Locomotory activity of *D. rerio* larvae 5 days after fertilization in acute tests with $NiCl_2$ at different oxygen concentrations ($n = 9–12$).

modelling (MixTox model; Jonker et al., 2005) indicated a significant antagonistic action of O_2 deficiency and Ni treatment ($p < 0.003$).

4. Discussion

In the present study fish larvae were examined for the first time in the MFB which proved to be well suitable for such young larvae. Small fin and tail movements could be distinguished, which resembled the signals of swimming movements and movements with the small fins of the three-spined stickleback, also recorded in the MFB (Craig and Laming, 2004). The electrical field of the MFB did neither disturb behaviour of adult three-spined stickleback (Craig and Laming, 2004) nor crustaceans (Kirkpatrick et al., 2005).

In our study acute behavioural investigations were most sensitive with decreasing effects on the locomotory activity at 7.5 mg Ni/L and above whereas significant decreasing effects on hatching rate, locomotory activity and mortality in the subchronic test occurred first at 10 mg Ni/L and above (see Table 2). According to these results the first hypothesis (Exposure to $NiCl_2$ results in a higher locomotory activity (avoidance reaction)) has to be rejected for exposure to Ni alone, but could be accepted for combined exposure to Ni and reduced oxygen levels. The second hypothesis (Sensitivity to Ni is exposure time-dependent) could be accepted. According to Triebskorn et al. (1997) behavioural answers should be integrated as short-time and long-time indicators of contaminations with high ecological relevance.

The observed effect concentrations for Ni are in the range of measures near industrial sites (50–2000 μg Ni/L in natural waters near industrial sites and 183 000 μg/L near a nickel refinery; Chau and Kulikovsky-Cordeiro, 1995; Kasprzak, 1987). In nature decreased locomotory activity in fish might lead to increased downstream drift and/or predation risk, hence representing an ecologically relevant parameter for the species' health and survival. A similar decreasing effect of metals on the activity of rainbow trout and brook trout (*Salvelinus fontinalis*) exposed to aluminium as well as walleyes (*Stizostedion vitreum vitreum*) exposed to mercury was reported in Atchison et al. (1987).

The delay of hatching at ≥10 mg Ni/L might be caused by an interaction of nickel with the hatching enzyme chorionase, a metal-protease (Hagenmaier, 1974). This effect is supported by other data, e.g. studies for zebrafish (45 μg/L (Geometric Mean of NOEC and LOEC, GM NOEC–LOEC), 6 mg Ni/L (LOEC) at an age of 96 h) (Dave and Xiu, 1991; Grabner, 2005) and studies for carp (*Cyprinus carpio*) (6 mg Ni/L) (Blaylock and Frank, 1979).

The additionally observed 'surface swimming' at ≥10 mg Ni/L could possibly be explained by a delay of hatching in these concentrations. Here the yolk sac seemed to be resorbed to a minor degree than in the control larvae of the same age. In histological sections small lipid droplets, presumably non-resorbed degradation products of the yolk, were visible below the swim bladder (R. Triebskorn, Tübingen, personal communication) in the respective nickel treatments, which might have provided

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Table 2
Comparison of effect concentrations in different studies concerning nickel toxicity to fish

Species	Age	Acute studies				
		Parameter	Nickel	pH	Water hardness (mg/L) (as $CaCO_3$)	Source
Cyprinus carpio (carp)	E + L	Hatching rate	6 mg/L	7.4	128	Blaylock and Frank (1979)
Oncorhynchus mykiss (rainbow trout)	A	Attraction	6 μg/L	7–7.5	28.4	Giattina et al. (1982)
O. mykiss	A	Avoidance reaction	>19 μg/L	7–7.5	28.4	Giattina et al. (1982)
Danio rerio (zebrafish)	E + L	Hatching rate	45 μg/L	7.5–7.7	100	Dave and Xiu (1991)
D. rerio	E + L	Hatching rate	10 mg/L	8.0	231.4	Present study
D. rerio	L	Diminished locomotory activity	7.5 mg/L	8	231.4	Present study
Subchronic and chronic studies						
O. mykiss	E + L	Growth	35 μg/L	7.0	53	Nebeker et al. (1985)
O. mykiss	E + L	Embryo survival, swim-up, hatching, fingerling survival, growth	>466 μg/L	7.9	89	Brix et al. (2004)
D. rerio	E + L	Mortality (after 14 days)	90 μg/L	7.5–7.7	100	Dave and Xiu (1991)
D. rerio	E + L	Mortality (after 11 days)	10 mg/L	8.0	231.4	Present study

A, adult; L, larvae; E, embryos. Displayed are the lowest effect thresholds.

buoyancy and therefore were responsible for the swimming behaviour at the surface.

Increased mortality of *D. rerio* due to exposure with Ni has been observed in another study as well. Dave and Xiu (1991) observed increased mortality at 360 μg Ni/L (GM NOEC–LOEC) and above when exposing *D. rerio* larvae (from 2 to 4 h after fertilization up to an age of 16 days without feeding) to nickel sulfate hexahydrate ($NiSO_4 \cdot 6H_2O$) (at a water hardness of 100 mg/L (as $CaCO_3$), pH 7.5–7.7). The fact that the mortality inducing concentrations in this study are clearly below those of the present study can be explained by the prolonged exposure time (11 vs. 16 days). So the starvation stress could have been considerably higher. Additionally it is also possible that the *D. rerio* strain used by Dave and Xiu (1991) was more sensitive.

In acute exposures oxygen concentrations of 2.45 ± 0.16 mg O_2/L and below both alone and in combination with low nickel concentrations resulted in significantly decreased activity compared to the control. Oxygen stress resulted in increased mortality in treatments with 3.23 ± 0.25 mg O_2/L and lower (both alone and in combination with Ni). At high nickel and low oxygen concentrations, the combined stressors possibly elicited an avoidance/escape response, reflected by higher locomotory activity compared to the single stressors. Decreased oxygen concentrations were associated with an increase in pH of up to 0.4 units. This should not strongly influence the toxicity of Ni as well as the zebrafish larvae. In studies of Hoang et al. (2004) an increase in pH from 7.97 to 8.54 increased the 96 h LC_{50} for fathead minnows (*Pimephales promelas*) only slightly, from 1.75 to 1.80 mg Ni/L, at a water hardness of 100 mg/L (as $CaCO_3$).

Naturally, zebrafish live in streams with high plant density at the riparian zone (Börries, 2006), so in these waterbodies low oxygen concentrations may locally occur. Various studies have shown that embryos and larvae of zebrafish can cope with low oxygen concentrations in certain age stages (Braunbeck et al., 2005; Padilla and Roth, 2001). These studies indicate that an age of 5 days seems suitable for the investigation of effects of oxygen depletion on *D. rerio* larvae. In earlier stages the tolerance to low oxygen levels is still very high (Braunbeck et al., 2005; Padilla and Roth, 2001). The oxygen consumption of 5-day-old larvae is higher than that at the age of 7 and 8 days old (Grillitsch et al., 2005).

No data were available on combined effects of pollutants and oxygen depletion. As a significant antagonistic action of O_2 deficiency and Ni treatment was detected in our study, the third hypothesis (Additional environmental stress (oxygen depletion) increases $NiCl_2$ toxicity) could be accepted.

One reason for the relatively low toxicity of nickel to *D. rerio* larvae in the present study could be the low bioavailability of nickel at the relatively high water hardness (13 °dH; 231.4 mg/L as $CaCO_3$) and the high pH of the used reconstituted water (~8.0). According to Ji and Cooper (1996) at a $NiCl_2$ concentration of 10^{-1} and 10^{-2} M and a pH of 8.0–8.4 almost 100% of Ni is available as $Ni(OH)_4^{2-}$. The shift in pH in our study as well as the tested concentration range of 0.5–15 mg Ni/L (corresponding to 8.52×10^{-3}–2.56×10^{-1} M $NiCl_2$) therefore should not have affected this availability. As for many other metals, toxicity of nickel on aquatic organisms decreases with increasing water hardness (Hoang et al., 2004; Pyle et al., 2002). Differences in bioavailability and therefore in toxicity for fish were emphasised in several studies (Hoang et al., 2004; Pyle et al., 2002). For larval fathead minnows the LC_{50} increased from 0.40 mg Ni/L when exposed in soft water (water hardness 20 mg/L as $CaCO_3$) to 1.57 mg Ni/L in hard water (water hardness 52 mg/L as $CaCO_3$) (Pyle et al., 2002). As mechanism for the toxicity of nickel to fish, several papers mentioned a respiratory rather than an ionoregulatory mechanism (Brix et al., 2004; Pane et al., 2003). Ionoregulatory toxicants like cadmium and copper disturb the Na or Ca balance at the gill which leads to several physiological dysfunctions that eventually cause mortality (Brix et al., 2004). Respiratory toxicity on the contrary resulted in the accumulation of metals at the gills

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and related with this diminished oxygen consumption (Pane et al., 2003).

Adult rainbow trout reacted most sensitive to exposure to nickel by an avoidance reaction (Giattina et al., 1982). Reasons for the strong differences to the LOEC in the present study (7.5 mg/L) may be differences in water parameters, test and exposure systems, or a higher sensitivity of adult rainbow trout. Exposure of carp (*C. carpio*) to 6 mg Ni/L resulted in more dramatic effects on hatching rate (51.7% compared to 92.3% in the control) (Blaylock and Frank, 1979) than at the highest nickel concentration (15 mg/L) in the present study (77% compared to 98.9% in the control at the age of 96 h), probably due to the higher water hardness in this study (see Table 2). In general, adult rainbow trout seem to react more sensitive (attraction) to exposure with nickel than embryos and larvae of zebrafish, rainbow trout and carp. It has to be kept in mind that the results are only limited comparable because of the different test systems.

5. Conclusions

Nickel and low oxygen concentrations lead to diminished locomotory activity of 5-day-old zebrafish larvae in acute and subchronic exposures. In subchronic Ni exposures hatching rate and locomotory activity of the larvae were found to be equally sensitive but occurred at different age stages. Combined exposures to high Ni and low oxygen concentrations seemed to elicit an escape response of the *D. rerio* larvae.

Ecotoxicological studies based on behavioural parameters, which have yet been mainly conducted with adult fish, are also appropriate to fish larvae, since (1) behaviour was shown to be more sensitive in respect to exposure time and concentration than conventional parameters like mortality, and (2) a reduced use of adult fish is required for ethical reasons.

Acknowledgments

The study was supported by the EU Integrated project NoMiracle (Novel Methods for Integrated Risk assessment of Cumulative Stressors in Europe; http://nomiracle.jrc.it) contract no. 003956 to Dr. Almut Gerhardt (LimCo International) under the EU-theme ‘‘Global Change and Ecosystems’’ topic ‘‘Development of Risk Assessment Methodologies’’, coordinated by Dr. Hans Løkke at NERI, DK-8600 Silkeborg, Denmark.

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