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Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (*Danio rerio*) embryos and larvae ☆

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ABSTRACT

In order to assess the combined toxicity of environmental chemicals with different modes of action in acute (2 h) and subchronic (11 d) exposures, embryos and larvae of *Danio rerio* were exposed to a heavy metal salt, nickel chloride ($NiCl_2$), the insecticide chlorpyrifos (CHP) and their binary mixtures. Chlorpyrifos is an acetylcholine esterase inhibitor, which is likely to affect behaviour of the organism. $NiCl_2$ targets the active sites of enzymes and is regarded as an unspecific toxicant for aquatic organisms. Several endpoints, such as locomotor activity, morphological abnormalities, and mortality of *D. rerio* embryos and larvae were studied. During acute exposures to $\geqslant$0.25 mg/L of chlorpyrifos, locomotor activity tended to increase. However, this activity decreased significantly at $\geqslant$7.5 mg Ni/L. Subchronic exposures to CHP resulted in behavioural changes at much lower concentrations ($\geqslant$0.01 mg/L) and considerably earlier than the observed increase in morphological abnormalities and mortality (LC_{50} (10 d): 0.43 mg/L). Combined CHP and $NiCl_2$ mixtures led to an antagonistic deviation from the concept of independent action, in the case of locomotor activity. Compared to developmental or survival parameters, behaviour was the most sensitive endpoint for CHP exposure in this study; therefore we recommend this parameter to complement already established endpoints.

1. Introduction

In the environment, organisms are usually exposed not just to a single pollutant but rather to a mixture of these chemicals. Different concepts exist to describe the combined toxic effects of these compounds. For example, the concept of concentration addition is based on the assumption that components of a mixture have a common molecular target site and therefore show a "similar" mode of action. This implies that the toxicity remains constant when a compound is replaced, completely or partially, by an equally effective amount of another chemical. This concept can also be applied when pollutants exhibit different modes of action, but still lead to a common toxicological endpoint, e.g. mortality or inhibition of reproduction (Faust et al., 1996). Another concept used to describe chemical mixture toxicity is that of independent action. This is based on the assumption that substances acting in combination attack different target sites of an organism. Therefore, they should show a 'dissimilar' mode of action (Faust et al., 1996). Consequently, 'synergism', i.e. stronger effects than those expected from concentration addition, and 'antagonism', i.e. effects weaker than those predicted by the independent action model, may occur (Escher and Hermens, 2002).

An animal's behaviour integrates responses to internal (physiological) and external (environmental, social) factors and relates one organism to another (Evans, 1994). In this context, behavioural tests represent a sensitive method to detect effects of contaminants (Dell'Omo, 2002) as compared to conventional endpoints, such as mortality (e.g. Levin et al., 2003). Behavioural changes can be measured a short time after toxic chemical exposure (e.g. Lindsay and Vogt, 2004). Developmental parameters are regarded to be sensitive as well (e.g. Nagel, 2002). Nagel (2002) proposed the embryo test with *Danio rerio* (DarT) as replacement for the acute fish test with adult fish. In a prolonged embryo test, which lasted up to 96 h (Scheil et al., 2009), only a few effects of chlorpyrifos and $NiCl_2$ on developmental parameters were found, such as a decreased hatching rate due to $NiCl_2$ exposure. In this study, we extended the DarT test for up to 11 d to investigate whether prolonged exposure would reveal developmental and behavioural effects of test substances on *D. rerio*, or whether the results of the embryo test were representative for prolonged exposure as well.

For the mixed chemical experiments in the present study two dissimilarly acting compounds, the insecticide chlorpyrifos (CHP)

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and the heavy metal nickel, were chosen. Mixtures of pesticides and metals may occur in surface waters (e.g. near vineyards) as well as in agricultural areas near metal-processing industries. As nickel is also a naturally occurring trace metal (Duke, 1980) and highly soluble in water (Merck, 2004), co-occurrence of nickel and pesticides in surface waters is highly probable.

Nickel(II) chloride ($NiCl_2$) is of high environmental importance, because it is not biologically degradable and has been shown to exert long-term harmful effects to aquatic biota (Merck, 2004). Ni can act in an unspecific way on the active sites of enzymes and, furthermore, it can behave as an oxidative stressor and carcinogen. Environmental concentrations of this heavy metal range from 0.001 to 0.01 mg Ni/L (unpolluted Canadian rivers and lakes) up to 0.5 and 2 mg Ni/L (natural waters near industrial sites) with a maximum of 183 mg Ni/L near a nickel refinery in Sudbury, Ontario, Canada (Chau and Kulikovsky-Cordeiro, 1995; Kasprzak, 1987).

The insecticide chlorpyrifos is a broad-spectrum organophosphate compound (Kamrin, 1997), which forms the active ingredient in Dursban™ and Lorsban™ insecticides, which are among the most widely used insect control products (DowAgroSciences, 2008). They act on pests primarily as a contact poison, with some additional effectiveness as a stomach poison, and they are regarded as highly toxic to freshwater fish. CHP acts on the nervous system as an inhibitor of the enzyme acetylcholine esterase and accumulates in the tissues of aquatic organisms (Kamrin, 1997). The highest measured environmental concentrations of CHP were about 0.3 μg/L in surface waters in the United States (Gilliom et al., 2006).

To date, no studies concerning the effects of pesticide and metal mixtures on the behaviour and development of fish are available. Therefore, the aim of the present study is to quantify these effects by exposing early life stages of zebrafish (*D. rerio*) to the organophosphate insecticide chlorpyrifos and the heavy metal $NiCl_2$ in acute and subchronic tests. The following hypotheses were tested for embryos and larvae of zebrafish:

1. Acute exposure to $NiCl_2$ and/or CHP results in a higher locomotor activity (LA), indicating a possible avoidance reaction of the test organisms.
2. The toxicity expected by mixtures of CHP and $NiCl_2$ deviates from the concept of independent action.
3. Sensitivity to CHP, $NiCl_2$, and their mixture is exposure time-dependent for developmental and behavioural parameters.

2. Materials and methods

2.1. Maintenance of test animals

Adult zebrafish (*D. rerio*, WIK strain, MPI for Developmental Biology, Tübingen) of both sexes were kept in 150–230 L aquaria with aerated and filtered water (50/50% mixture of tap and distilled water to achieve a conductivity of approximately 400 μS/cm) at a density of ⩽1/L. A temperature of 26±1 °C and a pH of ~8 were maintained, with a 12:12 h light:dark cycle without dimming. Dry flake food (Nutrafin Max, Hagen, Germany) and frozen crustaceans (*Moina* sp., Bosmidae) or midge larvae (MM Aquaristik, Germany) were given as food twice per day *ad libitum*.

2.2. Acquisition of eggs

The eggs for the tests were gathered with spawn traps placed on the bottom of each aquarium the evening before spawning. The spawn traps were removed from the aquaria in the morning (1 h after triggering the spawning via switching on the light). The eggs were transferred to Petri dishes containing reconstituted water (OECD, 1992, Guideline 203). Two to four hours after fertilization the fertilized eggs were separated and distributed over several Petri dishes containing test water (30 eggs per Petri dish). To prevent contamination with proliferating Protozoa, the eggs were transferred into new Petri dishes with fresh reconstituted water once after 24 h. The eggs were kept at a temperature of 26±1 °C with a 12:12 h light:dark cycle. Approximately half of the test water was exchanged every second day. The condition of the larvae was checked daily under a stereomicroscope for morphological abnormalities, mortality as well as behavioural anomalies.

Any studies involving experimental animals were conducted in accordance with national and institutional guidelines for the protection of animal welfare.

2.3. Acute exposure experiments with nickel chloride, chlorpyrifos and binary mixtures

For the acute exposure experiments (2 h exposure at an age of 5 d post fertilization, dpf), embryos and larvae were raised in glass Petri dishes with reconstituted water as described previously up to an age of 5 dpf. Malformed or inactive embryos and larvae were removed prior to the experiments. At 5 dpf, the larvae were exposed to the respective test chemical concentrations acutely for 2 h while measuring locomotor activity (procedure described in Section 2.5). Eight nominal concentrations for each substance were examined with two negative controls each. For Ni alone, concentrations of 0.25, 1, 2.5, 5, 7.5, 10, 12.5, and 15 mg Ni/L were tested and for CHP alone amounts of 0.0001, 0.001, 0.01, 0.1, 0.25, 0.5, 0.75 and 1 mg of CHP/L were used. The mixed chemical concentrations were chosen following the Box–Behnken design (Box and Behnken, 1960), aiming at a rational distribution of the data points over the response surface. Nine ratios of $NiCl_2$ to CHP, plus two negative controls, were tested: 0.5+0.1, 2.5+0.25, 5+0.5, 7.5+0.25, 7.5+1, 10+0.5, 12.5+0.75, 15+0.25 and 15+1 (mg Ni/L+mg CHP/L, respectively) (Fig. 1A).

2.4. Subchronic test with nickel chloride and/or chlorpyrifos

The subchronic test (exposure from ⩽1 hpf up to 11 dpf) was conducted according to the VMD Guidance Note "Ecotoxicity testing of medicines intended

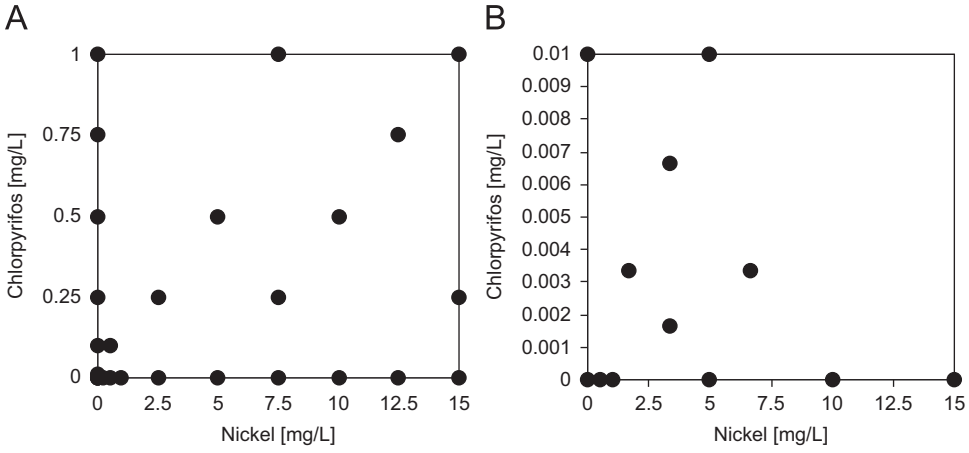

Fig. 1. Test design for acute (A) and subchronic (B) mixture experiments with nickel (mg/L) and chlorpyrifos (mg/L). For exact concentrations see text. Concentrations were chosen to be as evenly distributed in the Ni × CHP matrix as possible.

for use in fish farming'' (VMD, 1996) using a semi-static test design, with partly water exchange every second day. The exposure of the organisms to CHP, $NiCl_2$, and their mixture started at the time of fertilization ($\leqslant$1 h) and was terminated at an age of 11 d. Experiments were performed in glass Petri dishes (for CHP exposures and CHP/$NiCl_2$ mixtures) and in plastic Petri dishes (for $NiCl_2$ exposures) with 30 fertilized eggs in each dish, and three replicates per concentration. Glass or plastic Petri dishes were used to avoid possible interactions of the chemicals with the vessel. During the exposure time selected developmental endpoints were recorded daily, from 1 up to 11 dpf, including the rate of deformations and mortality. Four larvae from each replicate were randomly removed at regular intervals (5, 8, and 11 d post fertilization) for measurements of the locomotor activity (see Section 2.7). During behavioural measurements, the larvae remained exposed to the same solutions as for the respective subchronic exposures. No food was provided during the experiments, as zebrafish can live from their yolk sack up to 12 d after fertilization (Rombough, 2002). As we could observe no increased mortality in the control treatments, we assumed that the animals were well and not starving.

Five nominal concentrations for each single substance were tested with one negative control each (for Ni alone: 0.5, 1, 5, 10, and 15 mg Ni/L and for CHP alone: 0.01, 0.1, 0.25, 0.5, and 1 mg CHP/L). The calculation of mixed concentrations was based on the LOECs (= 1 toxic unit, 1 TU) for the most sensitive parameter obtained in the single substance tests (LOECs for locomotor activity: 0.01 mg CHP/L, 10 mg Ni/L). In the mixed chemical experiment, combinations of the two substances were equal to either 0.5, 1, or 1.5 TU in a two-ray design with $\frac{1}{3}$ of the TU of chemical 1 and $\frac{2}{3}$ of the TU of chemical 2 combined and vice versa (see Fig. 1B). Five Ni/CHP combinations with one negative control were examined: 3.333+0.0017, 1.667+0.003, 6.667+0.003, 3.333+0.0067, 5+0.01 (mg Ni/L+mg CHP/L, respectively) (Fig. 1B). Approximately half of the respective test solutions were changed every second day.

Optimal conditions for the larvae were provided in control treatments (25.3 ± 0.8 °C, 7.94 ± 0.24 mg O_2/L, pH 7.99 ± 0.14, 640 ± 17 µS/cm; mean$\pm$SD, $n=6$). An increase in electric conductivity up to 719 ± 19 µS/cm (mean$\pm$SD, $n=13$) with increase in Ni salt concentration was detected, however, still within a tolerable range for the zebrafish embryos and larvae (Grabner, pers. comm., 2005).

2.5. Measurement of locomotor activity

Measurement of locomotor activity in acute and subchronic exposure experiments was performed with the Multispecies Freshwater Biomonitor® (MFB) (LimCo International, Germany), an online biomonitor for continuous and quantitative recording of the behaviour pattern of animals (Gerhardt et al., 1994) as described in Kienle et al. (2008). The behavioural signal of the animal was analysed by a fast Fourier transformation, resulting in a histogram of different signal frequencies (Gerhardt et al., 1994).

In summary, the test chambers were placed into glass aquaria ($20\times20\times15$ mm^3, 5 L) or polyethylene vessels ($208\times208\times64$ mm^3, 2.77 L) filled with 1.5 L (chlorpyrifos, Ni/CHP-Mixtures) or 2 L (nickel) of the respective solution. To eliminate disturbance from movement along the vessels, they were arranged in duplicate in a surrounding black basin with temperature-adjusted water (26±1 °C) and illuminated from above during the measurements (58 W neon light at 145 cm distance to the chambers). The larvae were transferred carefully into the chambers (one larva per chamber), the lid was closed and 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. The measurements were started after an acclimation time of 10 min and the behaviour of 11–12 larvae per treatment was continuously recorded for 2 h in intervals of 10 min. Each measurement was performed for 4 min. No food was provided to the larvae during the experiments.

2.6. Test substances

Chlorpyrifos (Sigma-Aldrich, Germany) was dissolved in reconstituted water (OECD, 1992, Guideline 203), which was constantly stirred for at least 4 h in order to prepare a stock solution of 1 mg/L at a water temperature of 45 °C and a pH of 8.0. Subsequently, the solution was kept at 35 °C overnight until use with constant stirring. Nickel(II) chloride hexahydrate ($NiCl_2\cdot6H_2O$) (Roth, Germany) was dissolved in reconstituted water in order to prepare a stock solution of 1 g Ni/L at pH 7.5. All test solutions were prepared directly before use.

2.7. Analytical conformation of test substance concentrations

The chlorpyrifos concentration was determined using gas chromatography–mass spectrometry (GC/MS) (HP5890 series II, Hewlett-Packard, Waldbronn, Germany) using a chlorpyrifos standard (Dr. Ehrenstorfer, Augsburg, Germany) at a concentration of 1 mg/L in reconstituted water. The substance was extracted from the aqueous, acidified solution with dichloromethane by shaking in a separating funnel for 3 min. Once the two phases had separated thoroughly, the solvent phase was dried using Na_2SO_4, and was then filled in a 50 mL rotovap bulb. Subsequently, the solvent was ablated in the rotating evaporator to a volume of 1 mL. The sample volume, containing the chlorpyrifos insecticide, was filled in GC–MS sampling vials and the concentration of insecticide was determined by GC/MS. The stock solution and highest test concentration of 1 mg/L was measured using an injection volume of 1 µL.

Analytical confirmation of nickel concentrations was performed by flame atomic absorption spectroscopy (F-AAS, Perkin-Elmer M1100, Waltham, MA, USA) at two characteristic wavelengths (232 and 341.5 nm) using a Tritisol nickel standard (1000 ± 2 mg nickel(II) chloride in water, Merck Darmstadt, Germany). The analysis was performed with an air/acetylene mixture at a flow rate of 2.5 L/min (C_2H_2) and 8 L/min (oxidant) and a gap width (monochromator) of 0.7H. For the calibration curve, nickel concentrations of 0.5, 1, 2, and 5 mg/L were diluted from the Tritisol standard in MilliQ® (18.2 mΩ/cm) (Millipore Corporation, Billerica, MA, USA). The stock solution for the tests (1000 mg Ni/L) and test dilutions of 0.5, 1, 5, 10, and 15 mg/L were measured for nickel concentrations using F-AAS.

2.8. Data analysis

Means of the percentage of time spent on locomotion were calculated for each larva separately for the first and the second hour in order to take into account early warning reactions and the decrease of locomotive activity over time. The data on the 'percentage of time spent on locomotion' were arcsin transformed, from proportional values, for statistical evaluation. As the data were only partially normally distributed (one sample—Kolmogorov–Smirnov-Test, SPSS 10.0.1, USA), non-parametric methods of statistical analysis were chosen. The data from all tests were analysed for significance by means of Friedman's ANOVA (Statistica 5.0, StatSoft, USA) with a subsequent Wilcoxon two-group test (JMP 4.0, SAS systems, USA) to detect differences between control and substance exposure treatments. A linear regression analysis was performed for acute and subchronic nickel and chlorpyrifos measurements, using the equation $y=ax+b$, with the locomotor activity (LA) as y and the toxicant concentration [CHP] or [Ni] as × (JMP 4.0, SAS systems, USA). In the regression equation a is the slope of the line and b the intercept. The MixTox Model (Jonker et al., 2005) was applied to calculate the type of responses to mixtures. Significance levels were defined as follows: $p<0.001$ highly significant: ***, $p<0.01$ strong significance: **, $p<0.05$ significant: * and $0.05<p>0.1$ tendency to be significant: (*). The LC_{50} after 10 d for CHP and the LC_{20} after 11 d for Ni were calculated using Table Curve™ 2D 5.1 (SYSTAT Software Inc., USA) Software.

3. Results

3.1. Measured concentrations

The retrieval rate of chlorpyrifos was 51.6% (nominal concentration 1 mg/L). Nickel retrieval rates were in the range of 101.6–104.7% of the nominal concentrations (see Table 1).

3.2. Behavioural toxicity in acute and subchronic exposures

Movement pattern: In control treatments, *D. rerio* larvae showed continuous locomotor movements without pauses, as suggested by the regular and constant peaks in the movement pattern over time (Fig. 2A). When exposed to CHP, the larvae showed a typical aberrant behaviour at CHP concentrations of 0.25 mg/L and higher. This abnormality consisted of paused jerky movements, as shown by the movement pattern in Fig. 2B.

Table 1
Nominal and measured nickel concentrations (mg/L) and retrieval rate of nominal concentrations (%); mean±SD of six replicate measurements (mean 232 and 341.5 nm).

Ni (mg/L) (nominal)	Ni (mg/L) (measured) (mean 232 and 341.5 nm)	Retrieval rate of nominal concentrations (%)
Control	0.00±0.00	–
0.5	0.52±0.06	104.2±12.7
1	1.02±0.06	102.1±5.9
5	5.08±0.18	101.6±3.6
10	10.47±0.92	104.7±9.2
15	15.42±0.47	102.8±3.1
1000	944.98±16.42	94.5±1.6

Muscular cramps could also be observed. No such effect was visible during $NiCl_2$ exposure.

Acute exposure to chlorpyrifos resulted in a slight concentration-dependent increase in locomotor activity. This was defined as the percentage of time the animal spent on locomotion (linear regression analysis: $p = 0.083$; LA = 0.581+0,068[CHP], $r^2 = 0.194$, $n = 130$) (Fig. 3B). A significant decrease in locomotor activity with increasing nickel concentration was detected ($p < 0.001$, LA = 0.702–0.0168[Ni], $r^2 = 0.188$, $n = 117$) (Fig. 3A). This resulted in a calculated LOEC of 7.5 mg Ni/L (significant difference vs. the control: $p < 0.001$, Friedman's ANOVA; $p = 0.005$, Wilcoxon test). When testing nickel in combination with low CHP concentrations (0.25 mg CHP/L) in acute exposures, the locomotor activity-decreasing effect of Ni dominated over CHP. Whenever Ni was combined with high CHP concentrations (1 mg/L), the activity-increasing effect of CHP dominated (Fig. 4). The calculation of the data with the MixTox model (Jonker et al., 2005) did not reveal any significant results for independent action. No mortality was observed in the acute exposure experiments.

In subchronic exposures, locomotor activity decreased significantly at an age of 5 d (linear regression analysis: Ni: $p = 0.008$, LA = 0.589–0.0130[Ni], $r^2 = 0.106$, $n = 65$, CHP: $p < 0.001$; LA = 0.849–0.778[CHP], $r^2 = 0.358$, $n = 64$) (Fig. 3A and B), resulting in significant differences to the control treatments at concentrations of $\geqslant 10$ mg Ni/L and $\geqslant 0.01$ mg CHP/L (Ni: $p < 0.001$, Friedman ANOVA; $p = 0.028$, Wilcoxon test; CHP: $p < 0.001$, Friedman ANOVA; $p = 0.013$, Wilcoxon test). Data analysis with the MixTox model revealed a significant antagonistic deviation from independent action ($p = 0.006$). The response surface for mixture exposures is displayed in Fig. 5.

3.3. Developmental toxicity in subchronic exposures

3.3.1. Deformations

D. rerio larvae exposed to CHP showed a significant increase in the percentage of individuals with morphological deformations from an age of 4 and 5 d onwards at both 0.25 and 0.5 mg CHP/L (Fig. 6). In these treatments the larvae suffered from an unnatural

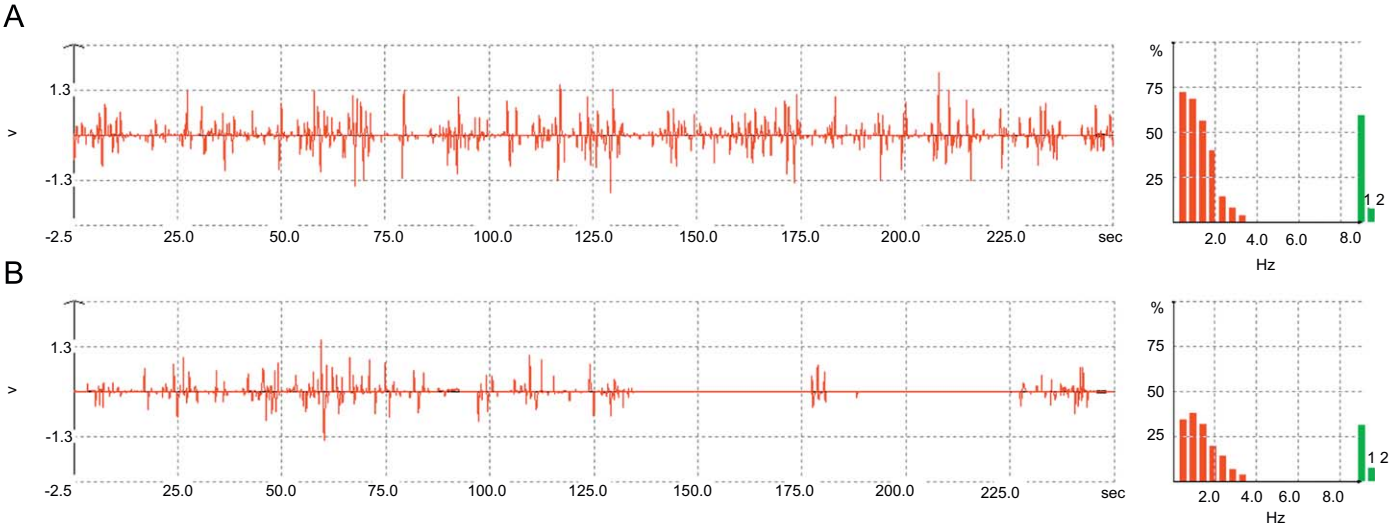

Fig. 2. Examples of spontaneous locomotor movement patterns (A) movement pattern (amplitude (V) vs. time (s)) (left) and fast Fourier transformation (FFT) histogram [activity in % of the time (250 s)] vs. frequency (Hz) (right) of a 5-d-old *Danio rerio* larva in control treatment showing continuous locomotor movements over time. (B) Movement pattern (left) and FFT histogram (right) of a 5-d-old *D. rerio* larva acutely exposed to 1 mg CHP/L, showing decreased locomotor activity with pauses.

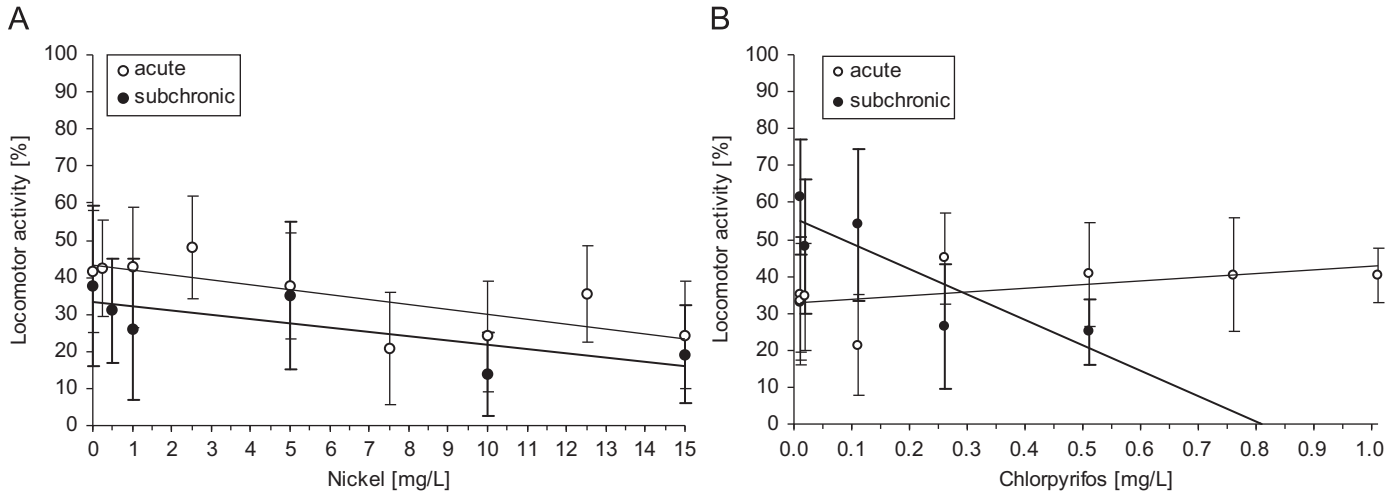

Fig. 3. Locomotor activity (percent of total time spent in locomotion) of 5-d-old *D. rerio* larvae acutely (2 h exposure at 5 dpf) and subchronically (from $\leqslant 1$ hpf up to 11 dpf) exposed to different nickel (A) and chlorpyrifos (B) concentrations (mg/L) in single exposures. Data of the second hour of measurement are displayed respectively.

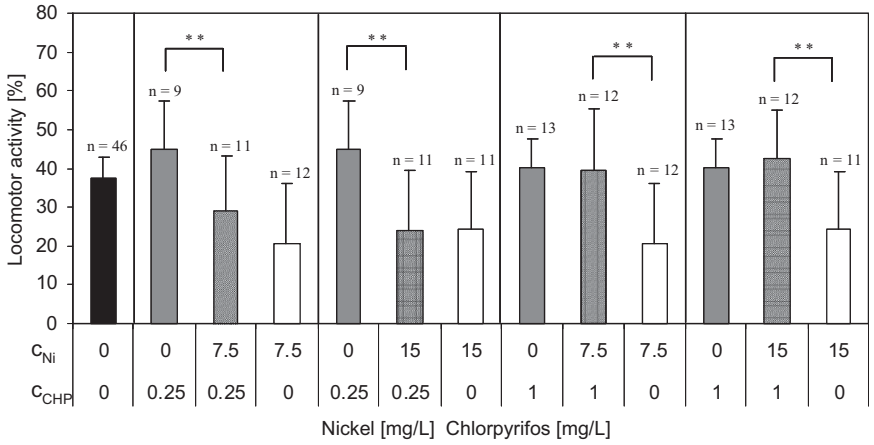

Fig. 4. Comparison of the locomotor activity (percent of total time spent in locomotion) of *D. rerio* acutely exposed to different nickel and chlorpyrifos concentrations (mg/L) single and in binary mixtures. Significant differences between treatments: $^{**}p<0.01$. Data of the second hour of measurement are displayed respectively.

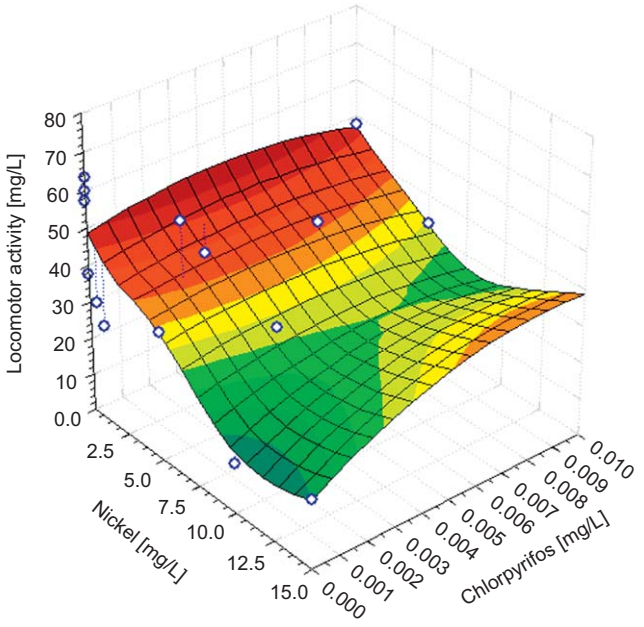

Fig. 5. Locomotor activity (percent of total time spent in locomotion) of 5-d-old *D. rerio* larvae exposed to different nickel and chlorpyrifos concentrations (mg/L) alone and in binary mixtures in subchronic exposure (from $\leqslant$1 hpf up to 11 dpf). Data of the second hour of measurement are displayed as surface plot with isobolic lines calculated on the basis of means.

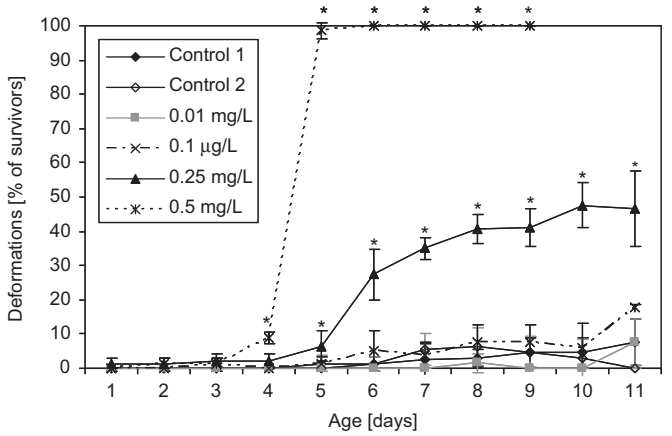

Fig. 6. Deformations (% of survivors) of *D. rerio* larvae exposed to different chlorpyrifos concentrations (mg/L) (mean $\pm$ SD; number of larvae per replicate: 30 (days 0–5); 26 (days 6–8); 22 (days 9–11), 3 replicates each experiment). Significant differences to control treatment: $^{*}p<0.05$. Only the deformations of surviving larvae are displayed.

bending of the spine (100%, 0.5 mg CHP/L, day 7, $n = 24$) in concentrations of 0.25 and 0.5 mg CHP/L. Additionally, heart oedema (23.3%, 0.5 mg CHP/L, day 7, $n = 24$) and other deformations of the spine occurred, apart from bending (5.4%, 0.5 mg CHP/L, day 7, $n = 24$) (Fig. 7A–D). In contrast, $NiCl_2$ did not induce morphological deformations. Also, in the subchronic test with binary mixtures of CHP and $NiCl_2$, which comprised only CHP concentrations of 0.01 mg/L and lower, no spine deformations were observed.

3.3.2. Mortality

Up to an age of 12 d, no increased mortality occurred in the control treatments. Mortality increased significantly at 8 dpf and 0.5 mg CHP/L ($p = 0.034$, Friedman's ANOVA; $p = 0.046$, Wilcoxon test) up to 100% mortality at 10 dpf, with a calculated LC_{50} of 0.43 mg CHP/L. In the case of $NiCl_2$, mortality increased significantly at 11 dpf and 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%, resulting in a calculated LC_{20} of 9.5 mg Ni/L. In the subchronic test with binary mixtures of CHP and $NiCl_2$, no increased mortality was observed due to the lower concentration range tested.

4. Discussion

This study represents the first approach to investigate behavioural and developmental effects of mixtures of pesticides and metals on fish. We aimed at quantifying this aspect in acute as well as subchronic exposures with early life stages of zebrafish (*D. rerio*) using the organophosphate insecticide chlorpyrifos and the heavy metal nickel chloride.

4.1. Measured concentrations

The detection rates for nickel were about 100% of the nominal concentrations (101.6–104.7%). This corresponds well with the

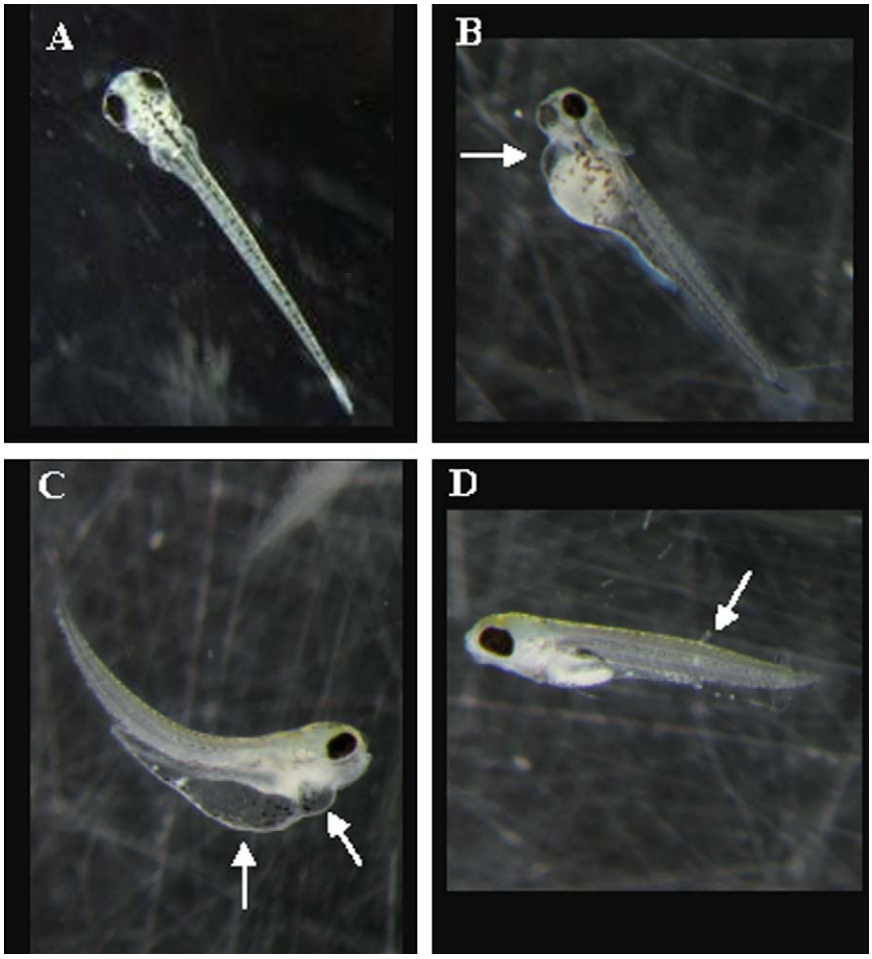

Fig. 7. Selected morphological malformations: (A) 5-d-old control larva of *Danio rerio*, (B) 5-d-old larva exposed to chlorpyrifos (CHP) with heart oedema, (C) 6-d-old larva exposed to CHP with heart oedema and abnormal bending of the spine, (D) 5-d-old larva exposed to CHP with deformations of the spine.

good water solubility of nickel(II) chloride of 2540 g/L (Merck, 2004). The lower detection rate of chlorpyrifos (51.6%) may be explained by the partial solubility of the added pesticide, even though a water solubility of 2 mg/L at 25 °C was expected (Kamrin, 1997) and no suspended particles could be observed. Adhesion of the pesticide to the surface of the glass bottles could also have played a role.

4.2. Mixture toxicity of CHP and $NiCl_2$ regarding acute and subchronic behavioural effects

In acute exposures, locomotor activity decreased significantly at 7.5 mg Ni/L but tended to increase with increase in chlorpyrifos concentrations as well as in binary mixtures containing $NiCl_2$ (Fig. 3). However, the observed increase in locomotor activity with increase in CHP concentrations and in binary mixtures also containing Ni suggests the lack of an avoidance reaction, but an increase in activity due to the effects of CHP on the nervous system, causing muscular cramps (Kamrin, 1997). This was observed after an acute exposure time of 2 h. A first escape response to CHP, indicated by a higher degree of locomotor activity compared to the control, would only be possible when the larvae were able to detect the substance by means of their chemosensory systems before the effects on the nervous system occurred. This could not be verified, as no literature data were available concerning this matter. Due to the reasons stated above, our hypothesis, stating that 'Acute exposure to $NiCl_2$ and/or CHP results in a higher locomotor activity indicating a possible avoidance reaction of the test organisms' could not be proven for the investigated substances and mixtures.

Generally, CHP was shown to be much more toxic than Ni in our tests (effects at $\geqslant$0.25 and $\geqslant$0.01 mg/L in acute and subchronic tests, respectively, with Ni at $\geqslant$7.5–10 mg/L). Because of the different modes of action of CHP (AChE inhibitor) and $NiCl_2$ (enzyme function, respiratory mechanism), it is likely that both substances act completely independent of each other. For acute exposures, the two substances elicited different results regarding locomotor activity (an increase was observed with CHP and a significant decrease with Ni exposure). This made it quite difficult to judge mixture toxicity. In subchronic exposures, a significant antagonistic deviation from the independent action concept could be detected.

Examples of the effects of binary mixtures on aquatic organisms can be found in the literature. Atrazine and organophosphate insecticides, both having different modes of action, showed a synergistic effect on *Chironomus tentans* (Pape-Lindstrom and Lydy, 1997) whereas mixtures of Ni and chromium (1:1 ratio with LC_{50} concentration) (96 h-LC_{50} 16.46 mg/L (nickel) and 13.58 mg/L (chromium)) elicited an additive effect in guppies (*Poecilia reticulata*) (Khangarot and Ray, 1990). Binary pesticide–metal mixtures (copper–malathion, cadmium–malathion, cadmium–dichlorvos) showed synergistic effects on the marine microcrustacean *Tigriopus brevicornis* (Copepoda) (Forget et al., 1999).

However, no studies concerning the effects of metal and pesticide mixtures have been found in the literature so far for zebrafish.

Altogether, our hypothesis 'The toxicity expected by mixtures of CHP and $NiCl_2$ deviates from the concept of independent action' could not easily be proven for mixtures of CHP and $NiCl_2$ in acute exposures, as both substances elicited different behavioural responses; however, an antagonistic deviation from independent action could be proven for subchronic exposures regarding locomotor activity.

4.3. Time dependency of CHP, Ni, and mixture toxicity in subchronic exposures

The most pronounced behavioural effects occurred at an age of 5 d for single substance, as well as for mixture exposures. Compared to acute exposures, the subchronic test was much more sensitive to CHP concerning locomotor activity (0.25 mg/L (acute) vs. 0.01 mg/L (subchronic)), whereas the response levels for Ni exposure were quite similar in acute and subchronic exposures (7.5 mg/L (acute) vs. 10 mg/L (subchronic)). Owing to the fact that subchronic exposures with nickel were carried out in plastic Petri dishes and subchronic exposures with chlorpyrifos and mixtures of chlorpyrifos and nickel chloride in glass vessels, different adsorption behaviour of nickel to the surface of the glass vessels could have played a role in mixture toxicity. However, as the concentration of nickel in the mixtures was much higher (1 TU = 10 mg/L) compared to chlorpyrifos (1 TU = 0.01 mg/L), the probable difference in adsorption seems to be of minor importance.

Thirty-day-old Japanese medaka (*Oryzias latipes*) exposed to chlorpyrifos displayed different behavioural and morphological symptoms like loss of equilibrium. These included hypoactivity, underreactivity to startle response, haemorrhage in the caudal area, and deformities (scoliosis and/or lordosis, forward pointing of the pectoral fins) (Rice et al., 1997). These symptoms were consistent with the three general modes of action response syndromes (hyperactivity, hypoactivity, and physical deformity) Drummond and Russom (1990) mentioned for categorizing a range of investigated neurotoxic chemicals, with each syndrome or sign of stress indicating a different mode of action. At higher CHP concentrations, a shorter time until initial onset of morphological effects and mortality was observed. The symptoms of CHP toxicity observed in *D. rerio* embryos and larvae, in the present study, were qualitatively similar to the results in those studies with respect to behavioural responses (hypoactivity, Figs. 2 and 3) as well as to deformation types, such as lordosis, a sustained, abnormal spinal curvature with a convex form of the dorsal surface (Rice et al., 1997) (Fig. 7C).

A significant increase in deformations could only be observed for subchronic exposure to chlorpyrifos (Fig. 6), whereas no deformations were observed in exposures with $NiCl_2$ and binary mixtures, most likely due to the low CHP concentrations used (max. 0.01 mg/L) in the mixture experiments. In the subchronic test, mortality increased with exposure time in higher concentration levels (0.5 mg CHP/L, 10 mg Ni/L). However, in mixture experiments no increased mortality was observed also due to the reason stated above. In a prolonged embryo test with zebrafish, Scheil et al. (2009) did not observe any increase in mortality within the exposure period of 96 h at concentrations of up to 1 mg CHP/L as well as in mixtures of Ni and CHP. Exposure to 100 ng CHP/L from 1–5 dpf was found to lead to elevated mortality rates in zebrafish from 20 to 38 weeks of age (Levin et al., 2003). In 30-d-old Japanese medaka (*Oryzias latipes*), the 48-h LC50 for chlorpyrifos was 0.25 mg/L (Rice et al., 1997). From our results and those in the cited literature, it becomes clear that at these low concentrations of CHP no mortality should be expected.

As shown above, there were toxic effects of CHP on the monitored parameters: locomotor activity, deformations, and mortality, which increased with exposure time for development and survival. No significant differences in behaviour were recorded after prolonged exposures to Ni and CHP at 8 and 11 dpf. Our hypothesis that 'Sensitivity to CHP, Ni and binary mixtures of these chemicals is exposure time-dependent for developmental and behavioural parameters' was therefore proved true.

4.4. Comparison of test systems related to their sensitivity towards different endpoints

The reaction of an organism to pollutants occurs on a biochemical as well as on an individual level. On a biochemical level, e.g. with nerve poisons, a physiological reaction already must have taken place to elicit a behavioural response. Similar effects on behaviour occur within certain groups of action, e.g. muscular cramps occur with AChE inhibitors; hence, in this case, for example, it is possible to conclude effects on a biochemical or histological level. In the following interpretation, the methods used in this study shall be compared with previously used test routines for their sensitivity to different endpoints.

Parallel to this work, stress protein (hsp 70) and histopathological investigations have been conducted in the presence of nickel chloride and chlorpyrifos using prolonged embryo tests with zebrafish (Scheil et al., 2009). Hatching rate decreased significantly with increase in Ni concentrations, but no effects on deformations and mortality were observed in the prolonged embryo test up to an age of 96 h in concentrations of up to 15 mg Ni/L and 1 mg CHP/L. The hsp70 level tended to be higher than in the control level (1 mg Ni/L), indicating an increase in stress protein production, and significantly lower at $\geqslant$10 mg Ni/L, indicating a pathological response. With CHP, an increased hsp70 level occurred only at 0.1 mg/L. Histopathological effects were obvious in the gut at $\geqslant$20 mg Ni/L and in liver, gut, pancreas, and skin at $\geqslant$0.6 mg CHP/L (Scheil et al., 2009).

These results show that the sensitivity of the test systems can be varied in the presence of different substances. Concerning the metal Ni, the prolonged embryo test revealed effects on the hatching rate earlier, but in the same concentration range as in the other test systems. Considering the organophosphate, chlorpyrifos showed no morphological effects in the prolonged embryo test up to concentrations of 1 mg CHP/L, whereas with longer exposure duration such effects occurred already at lower concentration levels. Behavioural effects in the subchronic test occurred at lower CHP concentrations of 0.01 mg/L.

It can be concluded that behaviour was the most sensitive parameter for the acetylcholinesterase inhibitor chlorpyrifos but was equally sensitive to the other parameters for nickel chloride exposure. This shows that it depends very much on the substance and its mode of action which parameter reacts first. Also the exposure time is important, e.g. if one only looks at the prolonged embryo test for CHP exposure results, no risk for fish might be concluded. However, in the subchronic test nearly all relevant effects occurred after the test duration of 96 h for this test.

4.5. Environmental relevance

The effects of CHP and $NiCl_2$ on *D. rerio* larvae were tested within the range of environmentally relevant concentrations: 0.0003 mg CHP/L in different surface waters in the USA (Gilliom et al., 2006); 0.001–0.01 mg Ni/L in unpolluted Canadian rivers

and lakes, 0.5–2 mg Ni/L in natural waters near industrial sites with a maximum of 183 mg/L (Chau and Kulikovsky-Cordeiro, 1995; Kasprzak, 1987).

Hence, an acute risk of chlorpyrifos for fish, even if they were much more susceptible to pollutants than the zebrafish, can presumably be excluded. But subchronic effects are more relevant, especially regarding behaviour, as they occur already at lower concentrations. Exposure of zebrafish larvae to CHP can, although occurring only over a short time, influence the behaviour up to the adult stage (Levin et al., 2003), which might also be true for other fish species. In the environment, a decreased activity caused by exposure to nickel and/or chlorpyrifos could, on the one hand, lead to an easier capture of fish larvae by predators. However, their diminished activity could make them less recognizable for predators, as observed with mummichog larvae (*Fundulus heteroclitus*) (Zhou and Weis, 1999). Additionally, food-searching behaviour could also be affected due to the diminished activity with subsequent negative impacts on their growth and fitness. Acute or subchronic exposure (at high concentrations) could provide an easier capture of fish larvae in the wild due to the toxicant-related cramps and jerky movements, as has already been observed with juvenile (21–32 d old) medaka (*Oryzias latipes*) (Carlson et al., 1998). They also would have only a low chance of escape, since the inhibition of acetylcholine esterase also influences perception (Kamrin, 1997).

The highest concentrations we tested for CHP are unlikely in natural surface waters because of the low solubility and the fast adsorption of CHP on sediment particles. However, as shown in our study, subchronic effects can occur at very low concentrations and therefore make the observed effects environmentally relevant. It has to be kept in mind that more sensitive species than the zebrafish might be at a much greater risk from CHP exposures at lower concentrations than those discussed in this study.

5. Conclusions

The effects of nickel and chlorpyrifos on critical parameters, including deformations, mortality and locomotor activity, were dependent on exposure time in subchronic exposures. Behaviour was the most sensitive parameter for the acetylcholinesterase inhibitor chlorpyrifos in subchronic exposures. In acute exposures, the two substances elicited different behavioural results. Our results show that it depends on the investigated substance and its respective mode of action which endpoint reacts first and in which concentration range.

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