Acute and chronic sensitivity to copper of a promising ecotoxicological model species, the annual killifish **Nothobranchius furzeri**

Acute and chronic sensitivity to copper of a promising ecotoxicological model species, the annual killifish *Nothobranchius furzeri*.

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Abstract

Nothobranchius furzeri is a promising model for ecotoxicological research due to the species' short life cycle and the production of drought-resistant eggs. Although the species is an emerging vertebrate fish model for several fundamental as well as applied research domains, its potential for ecotoxicological research has not yet been tested.

The aim of this study was to characterise the acute and chronic sensitivity of this species to copper as compared to other model organisms. Effects of both acute and chronic copper exposure were tested on survival, life history and physiological traits. We report a 24h-LC_{50} of 53.93 µg Cu/L, which is situated within the sensitivity range of other model species such as Brook Trout, Fathead Minnow and Rainbow Trout. Moreover, in the full life cycle exposure, we show that an exposure concentration of 10.27 µg/L did not cause acute adverse effects (96h), but did cause mortality after prolonged exposure (3-4 weeks). Also chronic, sublethal effects were observed, such as a reduction in growth rate, delayed maturation and postponed reproduction. Based on our results, we define the NOEC at 6.68 µg Cu/L, making *N. furzeri* more sensitive to copper as compared to Brook Trout and Fathead Minnow. We found stimulatory effects on peak fecundity at subinhibitory levels of copper substances (hormesis). Finally, we found indications for detoxifying and copper-excreting mechanisms, demonstrating the ability of the fish to cope with this essential metal, even when exposed to stressful amounts. The successful application of current ecotoxicological protocols on *N. furzeri* and its sensitivity range comparable to that of other model organisms forms the basis to exploit this species in further ecotoxicological practices.

Keywords

- *Nothobranchius furzeri,* chronic toxicity, full lifespan, copper, fish model

Highlights

- Exceptional traits and high sensitivity make *N. furzeri* a suitable ecotox model
- *Nothobranchius* is more sensitive to acute copper exposure than other fish models
- Exposure to >10 µg/L affected chronic mortality, growth and maturation time

Abbreviations

- **REACH** Registration, Evaluation, Authorization and restriction of chemical substances
- **DT_{50}** Degradation time for 50% of the substance
- **OECD** Organisation for Economic Co-operation and Development
- **USEPA** United States Environmental Protection Agency
- **ELS** Early life stage
- **ICP-MS** Inductively Coupled Plasma Mass Spectrometry
- **MT** Metallothionein
- **MQ** Milli-Q
- **ACR** Acute to chronic ratio
- **NOEC** No observed effect concentration
- **LOEC** Lowest observed effect concentration
- **LC_{50}** Lethal concentration 50
- **F2** Second filial generation

Introduction

During the last two decades, studies on a wide array of environmentally hazardous chemicals have been carried out due to the European REACH legislation (European-Chemicals-Bureau 2005). Within this framework, relative sensitivity profiles of different species and populations have been determined for a range of strategically selected toxicants based on standard short-term toxicity tests. Exposure regimes may, however, be much longer in natural compared to laboratory settings and cumulative effects may emerge only in later stages of an organism's life cycle, or even in following generations. Moreover, not all calculated 'so called' safe concentrations of toxicants turn out to be safe in reality. For example, the safe concentration of methyl parathion on peppered catfish (*Corydoras paleatus*) had effects on fish metabolism, causing serious health problems later in life (Fanta et al. 2003). It is therefore advisable to also study the long-term and multigenerational effects of sublethal concentrations of toxicants in an environmentally relevant context.

As stated by the European Commission, long-term studies are required if the DT_{50} (50% degradation time, indicating persistence in the soil) of a compound is two days or more (Consommateurs 2002). According to the European Commission of Food Safety (European Commission 2002b), this means that almost every compound should be tested for its long-term impact on the ecosystem. Also, the USEPA (United States Environmental Protection Agency) states that fish life-cycle toxicity tests (OPPTS 850.1500) are necessary for the registration of compounds of which the estimated environmental concentrations are greater than one-tenth of the NOEC calculated in the fish ELS (early life stage) test (USEPA 1996).

As sublethal or transgenerational effects cannot be measured in, nor predicted by acute toxicity tests, current sensitivity profiles are incomplete. Prolonged exposure to non-lethal concentrations can indeed affect life history traits (De Boeck et al. 1997) such as growth (Hashemi et al. 2008a), maturation time (Koivisto and Ketola 1995) and fecundity (Brungs et al. 1976). Furthermore, the impact of toxicants on behavioural traits may have severe consequences for fitness as well as for inter- and

78 intraspecific interactions (Pyle and Mirza 2007). For example, Beyers and Farmer (2001) showed that
79 copper exposed Colorado Pikeminnows (*Ptychocheilus lucius*) have an impaired escape reaction when
80 presented to predator cues.

81 Currently, long-term ecotoxicological studies are mainly performed on invertebrates such as
82 oligochaetes (Bettinetti and Provini 2002), ostracods (Oleszczuk 2008), Cladocera (Wollenberger et al.
83 2000), Diptera (Watts et al. 2001) and damselflies (Janssens and Stoks 2013). A constraint with current
84 vertebrate models for long-term testing of chemicals is their high cost in terms of labour, money and
85 time. The traditional vertebrate model species, Zebrafish, is not well suited for such long-term
86 assessments since its generation time is too long (95-150 days; (Diekmann et al. 2004)) to perform
87 time- and cost-efficient long-term and multigenerational studies. Two other fish species that are
88 occasionally used for long-term toxicity testing are the Fathead Minnow (*Pimephales promelas*) and
89 Medaka (*Oryzias latipes*) (Ankley and Villeneuve 2006). Still, full life cycle experiments can take more
90 than a year with these species, making it labour-intensive and costly. Another limitation is the need
91 for a continuous culture of current fish models to provide sufficient numbers of replicates (Arenzon et
92 al. 2003). Thus, there is a need to develop new, efficient model species with short life cycles to lower
93 the threshold of performing long-term and life-cycle exposures (Ankley and Villeneuve 2006) on
94 vertebrates.

95 The Turquoise Killifish *Nothobranchius furzeri* (Jubb 1981) is a member of the species-rich African
96 genus *Nothobranchius* (Wildekamp and Watters 1995) (74 species described; (Froese and Pauly 2014)).
97 *Nothobranchius* species only occur in temporary pools that dry out annually in the region between
98 South Sudan and South Africa. The life cycle of these annual fish is initiated by the inundation of the
99 pool at the start of the rainy season (Watters 2009). The embryos typically hatch within 12 hours,
100 followed by a rapid growth and early maturation and reproduction (3-4 weeks; (Valdesalici and
101 Cellerino 2003)). Fertilised eggs bridge the dry season in the sediment where they can remain viable in
102 a state of dormancy for several years. *Nothobranchius furzeri* occurs from the extreme south-west of

Zimbabwe (Gonarezhou National Park) to southern Mozambique (Wildekamp and Watters 1995). It is an emerging vertebrate fish model for fundamental (evolutionary biology; (Bartáková et al. 2013), ecology; (Pinceel et al. 2015)) as well as applied research (ageing; (Valenzano et al. 2015), neurology; (D'Angelo et al. 2014), genetics; (Reichwald et al. 2015)). It is one of the species with the shortest life cycle of about 10-12 weeks on average (adult to adult) compared to current fish-models namely, Zebrafish 14-22 weeks; (Diekmann et al. 2004), Fathead Minnow 15-22 weeks; (Parrott and Blunt 2005), and Medaka 22-29 weeks; (Seki et al. 2005), thus providing potential for whole-life cycle and multigenerational exposure studies. An additional asset of *N. furzeri* is the production of drought-resistant dormant eggs that can be stored on dry peat in sealed petri dishes, allowing for synchronous hatching of experimental individuals when needed (Cellerino et al. 2015). Although *N. furzeri* has not been used as a model species in ecotoxicology yet, Shedd *et al*. (Shedd et al. 1999) explored the use of the congeneric *N. guentheri* for acute toxicity testing. Their research clearly demonstrated the potential of *Nothobranchius* in short-term ecotoxicology, but species-specific characteristics related to culturing (breeding, male aggressiveness and hatching fraction) were suboptimal (Persoone et al. 2000). Recently, major progress has been made in the standardised culturing of *N. furzeri* and the storage and controlled hatching of the dormant eggs produced (Polačik et al. 2016). However, the potential for long-term exposure studies using *Nothobranchius* species remains unexplored.

For the launch of a new model species in ecotoxicology, it is important to compare its sensitivity to reference test substances with known model organisms (Arenzon et al. 2003). As the effects and underlying mechanisms of copper exposure have been studied extensively in various vertebrate (Eyckmans et al. 2010; Adeyemi and Klerks 2013; Soteropoulos et al. 2014) and invertebrate model organisms (Real et al. 2003; Prato et al. 2013; Naddy et al. 2015), this metal was selected to allow for a broad inter-species comparison. Copper is a trace element and an environmentally persistent toxic heavy metal that can interfere with intracellular protein mechanisms causing oxidative stress and effects on osmoregulation (Grosell et al. 2002; Eyckmans et al. 2010). Concentrations in the order of 10-150 µg Cu/L are acutely toxic to fish and effects are mainly caused by damage to the gills, resulting

in adverse effects on respiratory and ionoregulatory functions (Handy 2003). Sublethal exposure concentrations between 2 and 14 µg Cu/L are commonly tested with freshwater species (Brix et al. 2001), with the sensitivity being dependent on species, life stage and water quality. Sublethal copper concentrations can affect life history traits such as growth (De Boeck et al. 1997) and reproduction (Brungs et al. 1976), and cause physiological effects on several body systems, since copper does not target specific organs, but causes broader physiological adjustments and adaptations (Handy 2003).

In this study, we performed an acute and a chronic exposure experiment with *N. furzeri* to explore the sensitivity of this novel model organism to short- and long-term copper exposure. Furthermore, by complementing information on life history traits with data on bio-accumulation of copper and internal metallothionein concentrations, we will assess potential physiological defence mechanisms. Specifically, we expect that *N. furzeri* will show a comparable sensitivity to the congeneric N. guentheri (24h-LC_{50} 39 µg Cu/L) because of their phylogenetic proximity and similar life cycle (Shedd et al. 1999) which implies that the species would be more sensitive to copper than other fish model species. Second, we expect to find indications of stress at lower exposure concentrations in the chronic assay since the studied endpoints are sublethal and therefore often more sensitive than the assessment of mortality in the acute exposure. Finally, after a period of stress during the larval stage, chronic exposure to sublethal copper concentration could induce acclimation or defence mechanisms, such as a lower copper accumulation and an elevated metallothionein concentration in the tissue. Similar mechanisms appear to operate in other fish species such as Cyprinus carpio (Hashemi et al. 2008b; Hashemi et al. 2008c). While *N. furzeri* is considered a promising model species due to its short life cycle and drought-resistant eggs, our study will provide a first evaluation of the potential *of N. furzeri* for long-term ecotoxicological testing.

Material and methods

Maintenance of the test animals

In both the acute and chronic experiments, we used F_2 individuals from wild-caught fish of the natural population NF414 located in the Limpopo River basin in southern Mozambique (Bartáková et al. 2013) sampled by the Czech Institute of Vertebrate Biology in 2012. At the onset of both experiments, fish were hatched synchronically by inundating the eggs (stored in moist peat) using dechlorinated tap water at 12°C. Afterwards, water temperature gradually converged to room temperature (22°C). Larvae were transferred 48h post-hatching to 0.5-L jars and raised in dechlorinated tap water with a 14h:10h light-dark cycle at a constant temperature of 22°C in a climate controlled room. To ensure water quality remained constant, fish were transferred to jars with fresh medium every two days over the entire course of both experiments. Larvae were fed *ad libitum* with *Artemia* nauplii (Ocean Nutrition, Essen, Belgium) twice per day. In the third week (chronic exposure experiment), their *ad libitum* diet was complemented with chopped *Chironomus* larvae (Ocean Nutrition, Essen, Belgium). After the third week, they were fed *ad libitum* twice a day with frozen *Chironomus* larvae only. As the toxicity of copper depends on the water quality, we measured pH, temperature, conductivity and % dissolved oxygen every second day. Average values were 8.03 (se = 0.009) for pH, 21.38 °C (se = 0.093) for temperature, 610 µS/cm (se = 1.202) for conductivity, while oxygen concentration remained above 7mg/L (equalling >80% DO). These values were comparable between treatments. Water hardness was 400 mg $CaCO_3$, which corresponds to hard water.

Experimental setup – acute toxicity

Two days after hatching, larvae were exposed to copper nitrate ($Cu(NO_3)_2 \cdot 3H_2O$) (Sigma-Aldrich, St. Louis, MO, USA) prepared in dechlorinated tap water. The acute exposure was conducted using a protocol based on OECD Test Guideline 210 (OECD 1992), OECD Test Guideline 203 (OECD 1992) and the protocol employed by Shedd and coworkers (Shedd et al. 1999) on the sister species *N. guentheri*.

Specimens were continuously exposed to a range of six nominal concentrations between 3.5 µg Cu/L (concentration present in tap water) and 100 µg Cu/L during 14 days, based on the LC_{50} value of *N. guentheri* to copper (i.e. 39 µg Cu/L; (Shedd et al. 1999)). Per condition, ten fish were kept individually

in 0.5-L jars (n= 10). Stock solutions of copper nitrate were prepared in distilled water and added to the exposure water to produce nominal concentrations of 6.25 µg Cu/L, 12.5 µg Cu/L, 25 µg Cu/L, 50 µg Cu/L and 100 µg Cu/L.

To measure the actual copper concentrations, a water sample (5 ml) was taken from a random replicate for every condition. Copper concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700x) at the ^{63}Cu line in the He mode using ^{72}Ge as internal standard (Devulder et al. 2013). Realised copper concentrations were 3.81 µg Cu/L in the control and 10.55, 15.13, 27.17, 53.23 and 103.23 µg Cu/L in the five exposure conditions respectively.

Experimental setup – chronic toxicity

For the chronic exposure experiments, the initial sample size was nine fish per condition. Fish were first kept individually in 0.5-L jars. Three weeks after the start of the experiment, fish were transferred individually to 2-L aquaria where they were housed until death. Similar to the acute exposure, this experiment was performed at 22°C in a climate controlled room.

Exposure concentrations needed to be sublethal to allow maturation, but at the same time high enough to induce stress. The six concentrations applied in this experiment were therefore based on the results of the acute exposure experiment. Thus, fish were exposed during their whole lifespan to nominal concentrations of 1.56, 3.125, 6.25, 12.5 and 25 µg Cu/L. With this exposure regime, we expected fish to show a gradient from normal growth and reproduction at the lowest concentrations to impaired performance at the highest concentrations. Realised copper concentrations were 3.74 (SE=0.299, n=37) in the control and 4.08 (SE=0.293, n=33), 4.88 (SE=0.306, n=34), 6.68 (SE=0.448, n=37), 10.27 (SE=0.570, n=36) and 19.38 (SE=0.893, n=21) µg Cu/L in the copper treatments.

Endpoints

In the acute experiment and during the larval/juvenile phase (first three weeks of exposure) of the chronic experiment, fish were inspected twice daily for mortality, loss of buoyancy and other aberrant

behaviour (e.g. swimming upside down). Size was measured at week 3, 7, 11 and 15. Body size was measured by photographing each fish individually in a Petri dish placed on millimetre paper, and the images were analysed digitally using the open source *Analysing Digital Images* software (Pickle 2008). Life history traits such as age at maturation, fecundity (i.e. the number of eggs produced per female per two weeks) and lifespan were assessed for all fish surviving to the reproductive phase. Age at maturation for males was defined as the age at which the first signs of nuptial coloration were visible. Females were coupled with older non-virgin (Reichard and Polačik 2010), non-experimental males to determine maturation as the first time the females produced eggs. This is necessary as early maturing females more easily produce their first eggs with an experienced male. After maturation, fish were mated randomly three times per week within each condition during two hours to assess female fecundity (Polačik et al. 2011). Females were coupled to spawn until they withheld from egg laying during three consecutive spawning events. Deceased fish were rinsed with dechlorinated tap water and stored dry at -20°C in a microcentrifuge tube (2 ml, Brand GMBH, Wertheim, Germany) for further analysis (bioaccumulation and concentration of metallothioneins).

At the tissue level, we measured the bioaccumulation of copper in all fish at the moment of death, as well as metallothionein (MT) concentration in fish older than three months. Fish were partially thawed, and wet weight was measured using a Mettler AT261 DeltaRange balance (precision of 0.01 mg) (Mettler-Toledo AG, Greifensee, Switzerland). Afterwards, fish older than three months were homogenised on ice with 4 ml of Milli-Q water with an Ultra-Turrax T3 homogenizer (IKA, Labor Technique, Shaufer, Germany) and subsamples were divided for copper analysis and MT analysis. The samples for MT analyses were kept in the freezer at −20°C until further analysis. Fish that died before reaching an age of three months, as well as all C4 and C5 fish, were only used for copper analysis due to the small amount of biological material, or due to few replicates. Samples for copper analysis were dried for five days at 60°C. Afterwards, they were weighed to calculate the dry weight of each fish. Samples were digested in 70% HNO_3 (50 – 600µl depending on the size of the fish). After 24h of digestion, samples were put at 110°C for 30min, then 50µl of H_2O_2 was added, and samples were put

back at 110°C for 30 minutes for further digestion. Finally, samples were diluted with 2 – 5 ml of Milli-Q grade water (Millipore, Bedford, MA, USA) and weighed again to calculate the relative concentration (g/L) of tissue in the samples. Due to the small size of the organisms and the unexpected relatively large error on the weighing of each cover glass on which larvae were dried, dry weight values could not always be determined accurately. Consequently, wet weight was used to calculate the relative concentration of copper in the whole body (µg Cu/g tissue). Total copper concentrations were measured using Inductively Coupled Plasma Mass Spectrometry on an Agilent Technologies Series 7700 Series ICP-MS. ICP multi-element standard solution IV (Merck, Darmstadt, Germany) was used to prepare calibration standards using a serial dilution. An ICP-MS autosampler (ASX-500 Series, Agilent Technologies, Machelen, Belgium) was used for automated sampling. During analysis, a nebuliser pump rate of 0.1rps was used to introduce the sample into the spray chamber. The internal diameter of the tubing (Tygon R3607, ISMATEC, Wertheim, Germany) used for sample uptake was 1.02mm. After the introduction of the sample into the spray chamber, a stabilisation time of 40s was used before actual measurement. The general plasma mode was used for analysis with the following parameters: 1550W RF Power, 10mm sample depth and 1.01L/min carrier gas. For MT determinations, tissue homogenates were centrifuged at 16000g at 4°C for 20 minutes, and the supernatant fraction was taken and kept at –20°C for further processing. Cytosols of the tissues of all the fish were analysed for total MT content (oxidized, as well as aggregated) following the procedure as described in Klein and co-workers (Klein et al. 1994). This method is specially developed to quantify Cu-containing metallothionein (De Boeck et al. 2003). Using 2-mercaptoethanol as a reducing agent and Zn^{2+} as a metal donor, oxidized MT is converted into native MT, that is subsequently quantified via Cd saturation. Acetonitrile was used to denaturate high molecular weight Cd-binding compounds and Cu bound to MT was removed with ammonium tetrathiomolybdate. Excessive tetrathiomolybdate and its Cu complexes were removed using DEAE-Sephacel (Sigma, St. Louis, MO, USA). Next, apothionein was saturated with ^{109}Cd-labeled $CdCl_2$ solution (Amersham Pharmacia Biotech, Buckinghamshire, England: 50 ppm of 37 MBq/mg Cd in 0.1 M HCl), and excessive Cd was bound to Chelex 100 (Bio-Rad, Munich,

Germany). The precipitate was removed by centrifugation and the supernatant counted for 1 min in a gamma counter (Minaxi , Canberra Packard, Frankfurt, Germany). The MT concentration was calculated assuming a molar ratio of Cd/MT of 7 (De Boeck et al. 2003).

All experiments and methods were approved by the ethical committee of KU Leuven (file number: P101/2014)

Statistical analysis

Statistical analysis was performed in the statistical software R v3.2.3 (R Development Core Team, 2016). We used the packages *drc* (dose-response curve), *survival* (differences between survival plots), *lme4* (likelihood ratio test), *multcomp* and *lsmeans* (post-hoc tests), *car* (Anova), *stats* (generalized linear models) and *mass* (StepAIC).

LC_{50} values obtained from the acute exposure experiment were calculated with achieved dose-response curves (Ritz and Streibig 2005) for mortality at 24h, 48h, 72h, 96h, one week and two weeks of exposure (*drm* function, drc package). The standard error of every LC_{50} value indicates the precision of the calculated LC_{50} value and is, therefore, a measure for the reliability of the LC_{50} values (Ritz et al. 2015). We set the maximum cut-off at a SE < 10% for reliable LC_{50} values.

Given that fish suffering buoyancy problems consistently died shortly after that, we defined an additional measure as 'lethal damage' (i.e. dead fish or fish with buoyancy problems resulting in death). As the lethal damage was scored as 0 (healthy) and 1 (lethally damaged fish) and thus binomially distributed, we used a generalised linear model for the analysis of lethal damage in chronic exposure and added concentration as a fixed categorical predictor. Survival curves were constructed by plotting the survival ratio for every treatment with time (in days) and were compared between treatments and between sexes using the log-rank test survdiff (survival package). Size was analysed using generalised linear models with a gamma distribution and concentration as fixed factor. Maturation time was analysed for both sexes separately, as it was scored differently. For both males and females, a generalised linear model with gamma distribution and concentration as fixed categorical factor was

used. Fecundity (number of eggs per two weeks) was analysed using a likelihood ratio test (lme4 package) on a generalised linear mixed model with the Poisson distribution and concentration and size of the female at the time of egg laying, used as fixed factors and time (in weeks), as well as individual fish as random factors. As a second measure, peak fecundity was analysed as the maximal number of eggs produced in a two-week period for every female using a generalised linear model with using a Quasipoisson distribution, as there was significant overdispersion ($z=2.16$, $P=0.015$), adding concentration and size of the female as fixed factors. The size of the females was added to both fecundity models to correct for differences in length. Bio-accumulation of copper in the fish tissue was log (x+1) transformed and analysed using a general linear model with concentration as well as age at measurement as fixed factors. Metallothionein concentration in the tissue was analysed using a generalised linear model with gamma distribution and concentration and lifespan as fixed factors. Tukey's pairwise comparisons were used to identify significantly different treatments. The correlation between bio-accumulation of copper and metallothionein concentrations was calculated using a one-sided Pearson correlation (De Boeck et al. 2003; Isani and Carpenè 2014).

Results

Acute exposure

LC_{50} values at different time points during the exposure period are presented in Table 1, with their respective standard errors. The dose-response curves for time points, of up to a week are shown in Fig 1. The LC_{50} values from 24h up to 1 week were all estimated with high precision (standard error < 10%, except for SE at 72h of 11%) and ranged from 53.93 µg Cu/L to 28.98 µg Cu/L. The standard error of the 2-weeks-LC_{50} was much higher (26.4%) and the resulting LC_{50} value is therefore less reliable.

Table 1: LC_{50} values of Nothobranchius furzeri *during acute copper exposure with standard errors for different time points.*

Time	LC_{50} (µg Cu/L)	Standard error
24 hours	53.93	0.09
48 hours	55.99	6.30
72 hours	49.73	2.33
96 hours	38.49	3.28
1 week	28.98	1.42
2 weeks	17.60	4.65

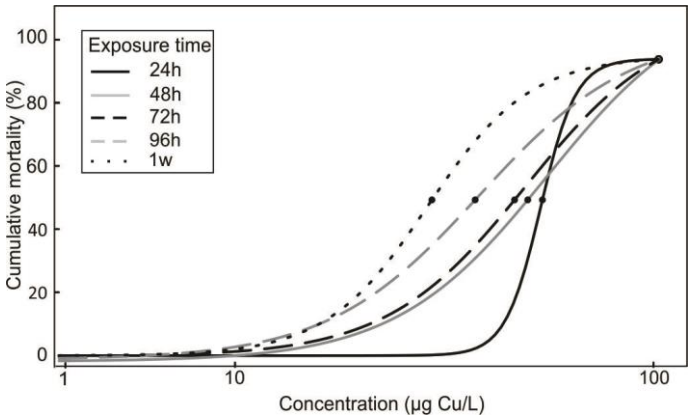


Figure 1: Dose-response curves showing cumulative mortality of Nothobranchius furzeri *as a function of copper concentration (in µg Cu/L) and in relation to exposure time. Dots indicate LC_{50} values.*

Table 2: Acute sensitivity to copper of freshwater fish species

Species	Species	Life stage	LC_{50} (µg Cu/L)	Time (h)	Hardness water (mg CaCO3/L)	T (°C)	Reference
N. furzeri	Turquoise Killifish	Larvae (48h old)	38.49	96	400	24	This study
N. guentheri	Redtail Notho	Larvae (48h old)	39	24	440	25	(Shedd et al. 1999)
P. promelas	Fathead Minnow	Larvae (12h old)	143-190	96	175	22	(Erickson et al. 1996)
O. mykiss	Rainbow Trout	Larvae (0.7g)	33.1	96	99	15	(Howarth and Sprague 1978)
O. mykiss	Rainbow Trout	Larvae (6,6 g)	298	96	361	15	(Howarth and Sprague 1978)
D. rerio	Zebrafish	Larvae (48h old)	149	96	141	28	(Alsop and Wood 2011)
C. carpio	Common Carp	Larvae (4g)	754	48	300	20	(Peres and Pihan 1991)

To compare the sensitivity of *N. furzeri* with other standard test organisms in ecotoxicology, we searched the ECOTOX database (USEPA) to find comparable short-term exposure tests in other fish species (Table 2).

Chronic exposure

Lethal damage and survival

Lethal damage in the first 24 hours ranged from 0 (in the control) to 37.5% (in C5). After six months, the lowest lethal damage was observed in C3 (50%) and the highest in C5 (100%) (Table S1). No

significant effect of copper on lethal damage was detected at 96h ($\chi^2_{5,42}$=2.27, *P*=0.810). Only after one month, copper had a significant effect on the lethal damage ($\chi^2_{5,42}$=13.86, *P*=0.017) and mortality ($\chi^2_{5,42}$=15.34, *P*=0.009). Fish that lost their ability to float died on average 16 days later.

The lifespan curves (Fig. 2A) show that all C5 fish died after 27 days. After 32 days, 50% of the C4 fish died, while fish exposed to lower concentrations of copper had a minimum 50% survival of 97 days. The survival rate of C5 fish was significantly lower than that of control fish (χ^2=6.7, *P*=0.009), as well as of fish from other treatments (C1-C5 χ^2=9, *P*=0.003; C2-C5 and C3-C5 χ^2=10.6, *P*=0.001; C4-C5 χ^2=5.8, *P*=0.016).

Size

There was a significant effect of concentration during week three ($\chi^2_{5,34}$=40.7, *P*<0.001)) with C5 fish being smaller than all other fish (all *P*<0.001) (Fig. 2B), after which all C5 fish died. Also at week seven, there was an overall significant effect of concentration ($\chi^2_{4,25}$=10.2, *P*=0.040) with C4 fish tending to be smaller than C0 (z=2.58, *P*=0.071) and C3 (z=2.67, *P*=0.057). Copper did not affect size in week 11 ($\chi^2_{4,17}$=7.33, *P*=0.120) and week 15 ($\chi^2_{4,13}$=3.71, *P*=0.447).

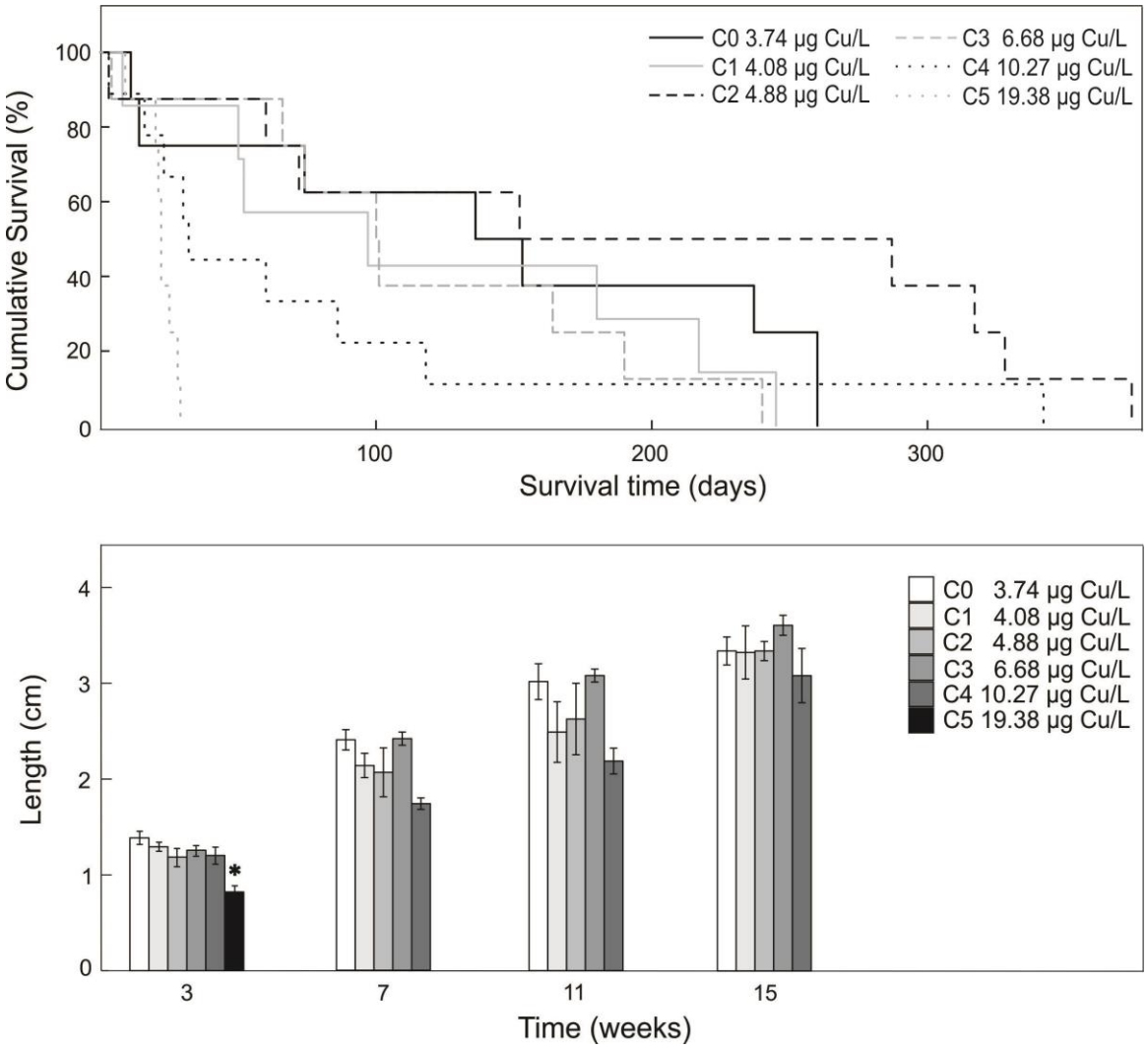


Figure 2: A) Survival distribution (time in days) of Nothobranchius furzeri in different copper treatments (C0 n=8; C1 n=7; C2 n=8; C3 n=8; C4 n=9; C5 n=8). B) Size (in cm) of Nothobranchius furzeri *exposed to different concentrations of copper at week 3, 7, 11 and 15. Asterisk indicates that C5 fish are smaller after three weeks at the significance level of P<0.05. Values are presented as mean ±SEM. Sample sizes are n=6; 6; 7; 7; 7; 7 in week 3, n=6; 6; 7; 7; 4 in week 7, n=5; 4; 5; 5; 3 in week 11 and n=5; 3; 3; 5; 2 in week 15*

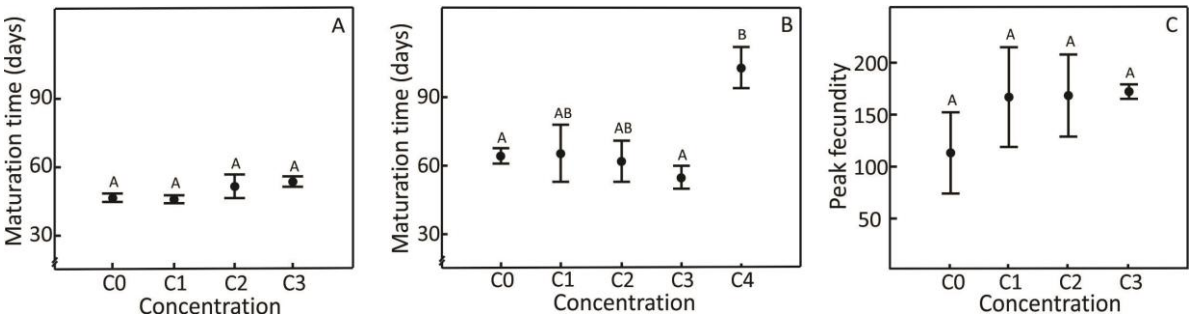
Maturation time

Fish that were unable to mature (i.e. all C5 fish, all C4 males) were excluded from the analysis. There was no effect of the copper exposure on male maturation in the C0 to C3 concentrations ($\chi^2_{3.9}$=5.30, *P*=0.151) (Fig. 3A). There was a significant effect of copper on female maturation time ($\chi^2_{4,6}$=18.6,

339 *P*<0.001) (Fig. 3B). The two surviving C4 females matured at an older age (mean of 103 days), which
340 significantly differed from C0 (*P*=0.015), C2 (*P*=0.028) and C3 (*P*=0.004).

341 **Fecundity**

342 The total egg laying period lasted between 25 and 38 weeks depending on the copper concentration.
343 C4-females were coupled with C0-males due to mortality of C4-males. One of the two surviving C4
344 females died a week after producing her first egg, and as such, fecundity data of C4 would only be
345 based on one female, producing eggs during 34 weeks. We therefore excluded the C4 treatment from
346 statistical analysis. When considering the whole egg laying period (generalised linear mixed model),
347 fecundity was positively affected by the length of the female (LRT=39.85, *P*<0.001), but was not
348 affected by copper exposure (LRT=1.439, *P*=0.697). However, we found peak fecundity was not
349 affected by length of the female ($F_{1,9}$=0.090, *P*=0.777) or copper exposure ($F_{3,9}$=0.475, *P*=0.713 (Fig.
350 3C).



351

352 *Figure 3: A) Maturation time of male* Nothobranchius furzeri *using age (in days) at which the first*
353 *signs of colouration appeared. Sample sizes are n=3; 3; 3; 4. B) Maturation time of female*
354 Nothobranchius furzeri using a*ge (in days) at which females produced their first eggs. Sample sizes*
355 *are n=3; 2; 2; 3; 2. C) Peak number of eggs produced by* Nothobranchius furzeri *exposed to different*
356 *copper treatments, measured as the maximal number of eggs laid during two weeks for every female.*
357 *C4 fish were excluded from statistics, as this treatment only had one female laying several eggs.*
358 *Sample sizes are n=3; 2; 2; 3; 2.Significant differences between treatments are indicated with*
359 *different letters and calculated at a significance level of P<0.05. Values are presented as mean ±SEM.*

Bio-accumulation and metallothioneins

Control fish had accumulated 0.12 to 0.18 µg Cu/g body weight at the time of death. Exposure concentration had an effect on copper accumulation, with increased copper levels at higher exposure concentrations ($F_{5,29}$=2.61, *P*=0.046) (Fig. 4A). The exposure time (i.e. lifespan) also had a significant effect on bio-accumulation of copper ($F_{6,29}$=17.5, *P*<0.001). Fish that survived the first weeks of exposure had lower accumulated copper concentrations. Bio-accumulation of C5 fish ranged between 5 and 46 µg Cu/g fish (mean of 20.86 Cu/g fish) with all fish dying within four weeks of experiment. C4 fish that died during the first 60 days of the experiment also showed an elevated accumulation. However, the C4 fish surviving for 88 days had reduced copper accumulation levels compared to C4 fish that died earlier (0.36 µg Cu/g fish). After 100 exposure days, fish from treatments C1, C2 and C3 showed similar copper concentrations as control fish of the same age (<0.25 µg Cu/g fish). The only C4 fish surviving longer than 100 days had whole body Cu levels of 3.13 µg Cu/g fish after 342 days.

As fish from C5 and C4 died at a relatively small size, they were not analysed for their MT concentration. Neither exposure concentration or age at measurement (i.e. lifespan) had a significant effect on MT concentration ($\chi^2_{3,12}$=0.587, *P*=0.899 and $\chi^2_{1,12}$=0.114, *P*=0.735 respectively) in C0 to C3 (Fig. S2). In general, whole body MT concentration ranged between 0.6 to 3.23 nmol MT/g homogenate (mean of 1.88 nmol MT/g homogenate). There was a significant positive correlation between accumulated copper and MT concentration (t=1.944, *P*=0.035) (Fig. 4B).

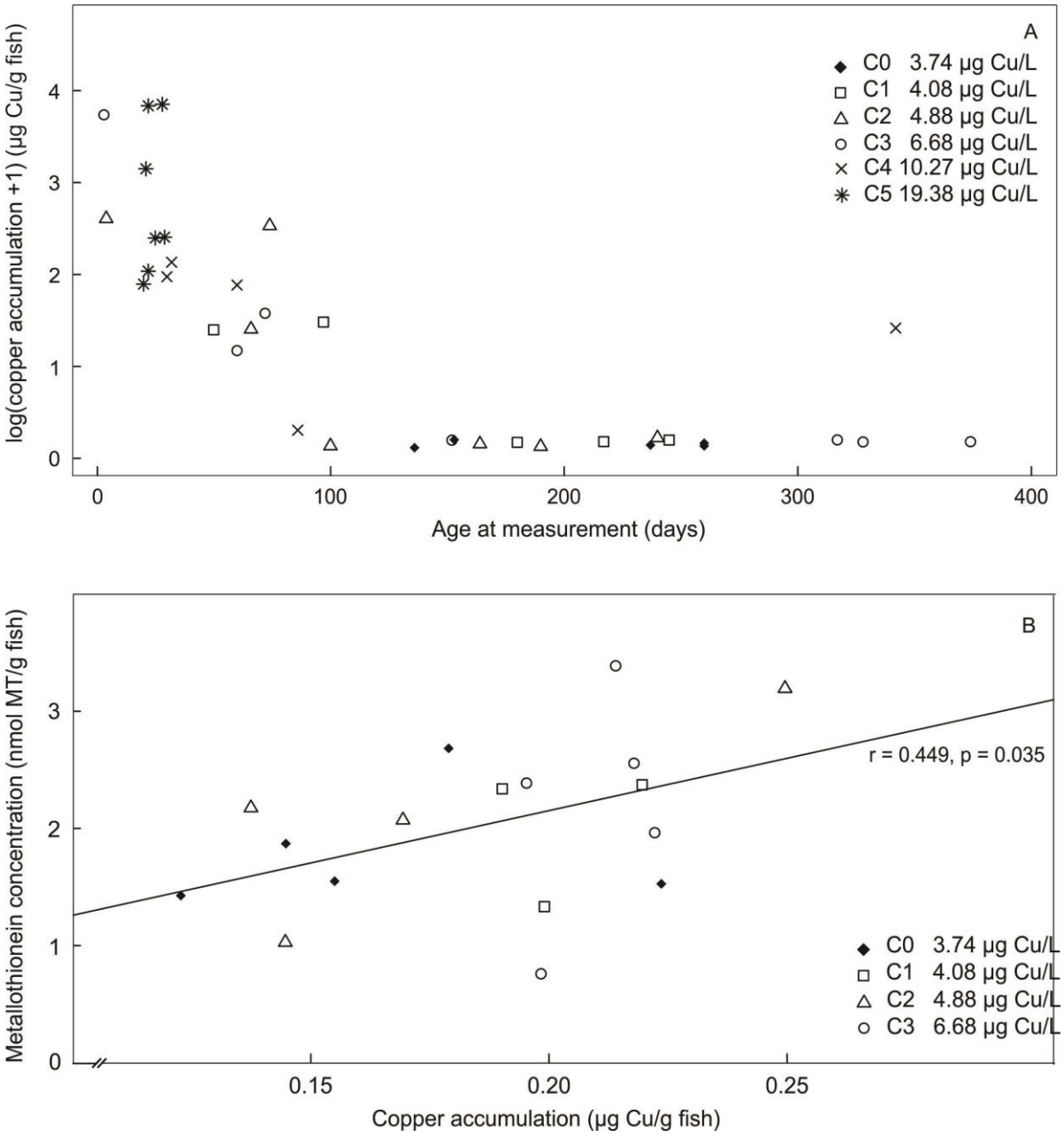


Figure 4: A) Accumulation of copper (log(x+1)-transformed) in the whole body homogenate of Nothobranchius furzeri *from different copper treatments at time of death. Sample sizes are n=5; 5; 7; 7; 5; 7. B) Correlation between total copper accumulation and MT concentration in the whole body homogenate of Nothobranchius furzeri from different copper treatments at time of death. Pearson's product-moment correlation r = 0.449 , p= 0.035.*

Discussion

While *Nothobranchius furzeri* is a well-established model system in ecological, evolutionary and ageing research (Cellerino et al. 2015), its use within ecotoxicological research remained unexplored. Due to its unique life history characteristics, *N. furzeri* might allow for time-and cost-efficient long-term and multigenerational studies, but the latter remains untested. Here, we set out to test the practical feasibility of *N. furzeri* as a model species for ecotoxicological research and explored its sensitivity to an important stressor as compared to other model fish species. Specifically, we investigated the effects of acute and chronic exposure to copper on life history and physiological traits. By doing this, we have the first impression of the species' acute and chronic sensitivity range, and protocols based on other model species could now be adapted and tested.

Acute exposure

Interspecific comparison of the acute sensitivity to chemicals is difficult because responses can depend on size, life stage and water variables. Compared to the congeneric *N. guentheri* (24h-LC_{50} value of 39 µg Cu/L), *N. furzeri* seemed to be more tolerant to copper, as the 24h-LC_{50} value of 54 µg Cu/L was higher in *N. furzeri*. When compared with Zebrafish and Fathead Minnow, *N. furzeri* appears to be more sensitive. As *O. mykiss* has a comparable LC_{50} value in soft water and it is known that soft water increases copper toxicity (Howarth and Sprague 1978), we can say that *N. furzeri* is likely also more sensitive to copper than *O. mykiss.* In general, we can conclude that both Nothobranchius species seem to be more sensitive to copper than other reported model organisms. This could be due to the nature of their habitat. Residing in temporary pools, these species have adapted their life history to deal with time stress which possibly trades off with tolerance to other stressors such as metals. Because of their higher sensitivity, *N. furzeri* and *guentheri* could be complementary model species to other fish models.

Chronic exposure - NOEC

In general, *N. furzeri* showed an increased lethal damage, decreased lifespan, slower maturation and reduced fecundity when chronically exposed to copper concentrations of 10.27 µg Cu/L and higher. Based on our results, we defined the NOEC at 6.68 µg Cu/L (C3). As chronic studies, in general, are rare,

410 we can only compare the NOEC value with the few values reported for freshwater fish, where test
411 concentrations varied between 2 µg Cu/L and 14 µg Cu/L (Brix et al. 2001). The NOEC values of 9.4 µg/L
412 for brook trout (*Salvelinus fontinalis*) (McKim and Benoit 1974) and 9.49 µg/L for rainbow trout
413 (*Oncorhynchus mykiss*) (Hansen et al. 2002) are only slightly lower than the C4 concentration in this
414 experiment clearly affecting mortality, growth and maturation time in *N. furzeri*.

415 *Chronic exposure – mortality and lethal damage*

416 When evaluating the applied endpoints in our study, loss of buoyancy (failure of larvae to maintain a
417 functional swim bladder) was a good predictor of early death. Although the test started with healthy,
418 buoyant larvae, an increasing fraction of the control fish lost their ability to float with time. However,
419 this buoyancy problem occurred much faster in the highest copper concentration C5, with 38%
420 suffering this condition only six hours after the start of the exposure. Loss of buoyancy was also
421 observed in Common Carp (*Cyprinus carpio*) exposed to copper (Stouthart et al. 1996). In Common
422 Carp and Gibel Carp (*Carassius auratus gibelio*), copper exposure led to cell swelling in the gills, an
423 effect seen more often in copper-exposed fish. This led to increasing diffusion distances for oxygen at
424 the gills, resulting in reduced respiration rates, reduced oxygen pressure and pH in the arterial blood
425 and a reduction of haemoglobin saturation (De Boeck et al. 2006), all of which could affect swim
426 bladder inflation.

427 Furthermore, our results highlight the importance of long-term tests, which thus far have not been
428 performed in *Nothobranchius*. We found delayed mortality effects of copper exposure which would
429 not have been observed in standard toxicity tests that typically only last for 96 h. At the highest
430 concentration (C5), no significant mortality was observed after 96h exposure. However, all individuals
431 died after four weeks, compared to only 25% in the control. Also at the second highest concentration
432 (C4), delayed effects were present with 78% and 89% of the fish dying before reaching respectively
433 three months and six months of age, contrary to 38% and 63% in the control after three and six months.

The measured mortality rate under control conditions was in line with our expectations since the short lived *N. furzeri* typically has a life-span of <6 months in the laboratory (Polačik et al. 2016).

Chronic exposure – Life history

Nothobranchius furzeri exposed to the highest copper concentration (C5) were on average 5.6mm or 40% smaller than the controls after three weeks. As all C5 fish died after four weeks, this smaller size is a good indicator of stress and early mortality. Moreover, we also found a trend, although less pronounced, towards a smaller size of C4 fish, suggesting an increased energy expenditure for survival which resulted in reduced growth. This is in compliance with the reduced growth in Common Carp exposed to copper, where energy was redirected from growth to homeostasis (Hashemi et al. 2008a), inducing protective metallothionein and restoring ion transport capacities (Hashemi et al. 2008c).

We found similar patterns for age at maturation, a relevant and efficient endpoint in ecotoxicological studies since it gives a first indication of the reproductive potential (Khan et al. 1992). C5 fish did not mature at all, as they all died within the first month of exposure. Two C4 females were able to mature, yet at a slower rate, maturing at 103 days compared to 55.5 days for control fish. Such a retarding effect of copper on maturation was also found in cladocerans (Koivisto and Ketola 1995), mysids (Gentile et al. 1983) and amphipods (Prato et al. 2013). It must be noted that age at maturation was substantially higher in control fish than the typical age at maturation of fewer than three weeks (Polačik et al. 2016). A number of factors, inherent to our experimental set-up, may have contributed to this. We housed fish at 22.5°C in 2L containers of which the water was renewed every second day. The total water volume and water renewal frequency have an influence on fish growth and their maturation time (Polačik et al. 2016). This separate housing set-up was, however, inherent to our design and water renewal frequency was chosen in order to guarantee general fish health (unpublished data). A lower water temperature also decreases metabolic rates and thus prolongs maturation time. A water temperature of minimally 24°C should be considered for future experiments to benefit from the fast growth and maturation of the species. The mean maturation time of *N. furzeri* in the control

treatment of 47 days for males and 64 days for females is still fast compared to e.g. Zebrafish at 14-22 weeks (Diekmann et al. 2004).

Despite the strong link of reproduction with fitness and ecological relevance, this trait is rarely considered in fish as examining the effects of pollutants on reproduction is complex and time-consuming. Especially in *N. furzeri*, where most energy is invested in reproduction from maturation onwards (Blažek et al. 2013), evaluating this trait is of utmost importance. However, we could not statistically show effects of copper exposure on fecundity, as C5 fish did not survive until maturation and C4 consisted of two females, of which one died after producing her first egg. Nevertheless, concentrations of 10.27 µg/L and more affect fecundity indirectly, as most of the fish do not survive to produce offspring.

Chronic exposure – defence mechanisms

As we found effects on size, maturation and absent or retarded egg laying in C4 fish, it is possible that there was an energy drainage towards defence and copper-excreting mechanisms in this treatment (De Boeck et al. 1997). Fish have been shown to produce metal binding proteins such as metallothionein (De Boeck et al. 2003) and protect themselves against oxidative stress (Eyckmans et al. 2010). Furthermore, sublethal copper concentrations can induce a downregulation in copper uptake across the gills and a re-distribution of the newly acquired copper to the liver for excretion as defence mechanisms (Buckley et al. 1996; Grosell et al. 1996; Grosell et al. 1997). Prior exposure to copper can activate these defence mechanisms in advance and enhance resistance to copper (Hashemi et al. 2008b). However, these processes take time and seem to depend on tissue copper accumulation rates (De Boeck et al. 2010), sodium turnover rates (Grosell et al. 2002; De Boeck et al. 2010) and MT induction rates rather than on absolute levels. Consistently, we indeed found that fish that died the earliest showed the highest copper accumulation rates (mostly C5 and C4) while fish that had slightly lower accumulation rates survived longer. This results in a reduced copper accumulation over time in fish that survived. This could mean that fish surviving the first exposure weeks developed defence

mechanisms to reduce copper intake (selective accumulation of incoming metals), redistribute metals and use tissue dilution with growth, or increase copper excretion. However, there appear to be limits to such defence mechanisms and the speed at which they develop, as indicated by the early mortality in C5 fish. Moreover, copper accumulation in the one surviving C4 fish, unlike in fish from intermediate concentrations (C1-C3), never returned to levels as low as in control fish. As we only have results for fish at the time of death, we cannot say which defence mechanisms were induced in the surviving fish or whether copper levels were even higher at the onset of exposure. In Gibel Carp, however, the rate of sodium loss seemed to be the major contributing factor indicating that the capacity to keep ion homeostasis is detrimental for survival (De Boeck et al. 2010).

Regrettably, fish from C5 and C4 could not be analysed for MT concentration, therefore we cannot say whether MT induction took place in these fish. In C0-C3, copper accumulation was low and also MT induction appeared to be low. Therefore, there was no relationship between MT induction and survival, although a correlation was seen between tissue copper and tissue MT (independent of exposure concentration), indicating that MT was present to detoxify the tissue copper levels. It is possible that fish in exposure treatments C1-C3 initially also had elevated copper bio-accumulation, but succeeded in adapting their physiology resulting in a higher excretion, but this is impossible to confirm based on our data.

C4 fish could also almost reduce their copper accumulation to control levels, but to be able to do so, they seemed to invest a lot of energy in defence mechanisms. This energy could not be used for other essential body functions, eventually leading to a slower maturation and a depressed fecundity. However, we cannot directly compare the bio-accumulation signal between concentration treatments as all fish were measured on different days, adding the effect of age. Therefore it would be interesting to quantify the bio-accumulation at a fixed time point during the experiment.

Taken all together, *N. furzeri* appears to be an appropriate candidate model organism for full life cycle exposure studies on vertebrates for the following reasons. Firstly, we have shown that *N. furzeri* is

509 more sensitive to copper than other tested fish species (except for the congeneric *N. guentheri*). This
510 is a relevant finding since ecotoxicological tests should incorporate testing on sensitive species to
511 assess the maximal impact of a toxicant (Forbes and Calow 2002). Secondly, background mortality was
512 low, sufficient *N. furzeri* individuals reached maturity to assess reproduction and offspring in this set-
513 up.

514 Finally, with a maturation time of 10 weeks in our experimental design, a life cycle test could be
515 performed within 14 weeks, a much shorter timeframe compared to other life cycle experiments using
516 available fish model organisms. However, the acute and chronic sensitivity of *N. furzeri* to different
517 compounds should be further studied to position the species' sensitivity among currently used model
518 organisms.

519 ## Conclusions

520 The acute sensitivity range of *N. furzeri* was similar to that of common model species. The lack of
521 measured effects after 96h in the highest Cu concentration, while 100% mortality was reached after
522 four weeks of exposure emphasises the importance of long-term tests. Also at the second highest
523 concentration, exposed fish revealed no effects after 21 days, yet 78% died before maturation.
524 Moreover, surviving fish grew slower, matured later and had a postponed reproduction. These results
525 advocate for prolonged exposure tests to set safe concentrations of toxicants accurately.
526 *Nothobranchius furzeri* proved to be more sensitive than Brook Trout and Fathead Minnow for copper.
527 Furthermore, we found indications for detoxifying and copper-excreting mechanisms which
528 demonstrates the ability of *N. furzeri* to cope with high concentrations of copper.
529 The adaptations of this species to its habitat have led to interesting characteristics ideal for chronic
530 ecotoxicological testing. Because of the fast life cycle, this model has shown to be suited for full lifespan
531 experiments. The next step towards this functional model species is the determination of the
532 sensitivity of the species to a range of well-studied toxicants with different modes of action and a
533 comparison of its sensitivity range with currently established fish model species. In this way,

Nothobranchius furzeri could be developed as a valuable ecotoxicological model for chronic toxicity testing.

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