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RESEARCH ARTICLE

Acute sensitivity of the killifish *Nothobranchius furzeri* to a combination of temperature and reference toxicants (cadmium, chlorpyrifos and 3,4-dichloroaniline)

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Abstract

Aquatic organisms of inland waters are often subjected to a combination of stressors. Yet, few experiments assess mixed stress effects beyond a select group of standard model organisms. We studied the joint toxicity of reference toxicants and increased temperature on the turquoise killifish, *Nothobranchius furzeri*, a promising model for ecotoxicological research due to the species' short life cycle and the production of drought-resistant eggs. The acute sensitivity of the larval stage (2dph) to three compounds (cadmium, 3,4-dichloroaniline and chlorpyrifos) was tested in combination with a temperature increase of 4 °C, mimicking global warming. Dose-response relationships were used to calculate 96h-LC_{50} of 0.28 mg/L (24 °C) and 0.39 mg/L (28 °C) for cadmium, 96h-LC_{50} of 9.75 mg/L (24 °C) and 6.61 mg/L (28 °C) for 3,4-dichloroaniline and 96h-LC_{50} of 15.4 μg/L (24 °C) and 14.2 μg/L (28 °C) for chlorpyrifos. After 24 h of exposure, the toxicity of all tested compounds was exacerbated under increased temperature. Furthermore, the interaction effect of cadmium and temperature could be predicted by the stress addition model (SAM). This suggests the applicability of the model for fish and at the same time indicates that the model could be suitable to predict effects of temperature-toxicant interactions.

Keywords *Nothobranchius furzeri* · Acute toxicity · Multiple stressors · Synergism · Fish model · Stress addition model

Introduction

Although organisms from inland waters often face a multitude of stressors simultaneously, few studies assess the effects of these stressors in a combined approach, especially for vertebrates (Jackson et al. 2016). Such research is highly relevant since most studies that have been undertaken do suggest strong interactive effects among pollutants and temperature (Moe et al. 2013; Patra et al. 2015). As contaminants are typically tested at the optimal culture temperature of the studied model organism, possible synergistic effects might be missed and predictions might underestimate the toxicity of a compound (Stoks et al. 2015).

Because of ongoing global warming, aquatic organisms are increasingly confronted with temperature extremes and overall higher average temperatures (IPCC 2014; Moe et al. 2013). Long-run climate outcome models predict a global average surface temperature increase of 2–4 °C by 2100 (IPCC 2014; Moss et al. 2010). From a physiological point of view, ambient temperatures influence metabolic rates and feeding activity, especially in ectothermic organisms (Sinclair et al. 2016). Combined with pollution, synergistic or antagonistic effects may arise from an altered uptake, detoxification or elimination of a toxicant (Holmstrup et al. 2010). Moreover, even when an increase in mean water temperature appears without direct effects, combined with chemical stress, it may prove to be harmful (Holmstrup et al. 2010). The effects of combined stressors may be highly variable and depend on the

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physico-chemical properties of the pollutant and the exposure temperature, as well as the life history strategy of the exposed species (Walker et al. 2012). Therefore, assays should screen effects on a wide range of taxa with differing sensitivities to identify suitable test organisms and to avoid underestimation of stressor interaction effects.

In this experiment, we tested the combined acute effects of a temperature increase of 4 °C and three chemical pollutants on the turquoise killifish *Nothobranchius furzeri* (Cyprinodontiformes) (Jubb 1971). This annual fish, endemic to temporary pools in south-eastern Africa, is an upcoming model organism in several research domains due to its interesting life history traits such as a fast growth, early maturation and the production of drought-resistant eggs (Cellerino et al. 2015; Genade et al. 2005). These characteristics can be translated into several advantages for ecotoxicological research, such as a simultaneous hatching of all experimental animals after inundation of the eggs, ensuring standardisation in time. Furthermore, as the drought-resistant eggs can be stored on dry peat in sealed petri dishes, they are easily transported without the need for a continuous culture of the test organism (Philippe et al. 2017). As *N. furzeri* is a new species in ecotoxicology and its sensitivity range is largely unknown, we will compare its sensitivity to other fish models.

The applied temperature stress in this experiment is in line with the predicted increase in temperature by 2100 under IPCC scenario A1FI (IPCC 2014). As reference toxicants, we selected three commonly found and well-studied compounds with different modes of action from different toxicological classes. Cadmium was chosen as a metal, 3,4-dichloroaniline as an aniline and chlorpyrifos as an organophosphorous pesticide. Cadmium (Cd) remains indefinitely persistent in the environment and is often used to determine an initial sensitivity range of non-model species. Cadmium imbalances the ion regulation in the gills, reduces the uptake of essential nutrients and induces oxidative stress by producing reactive oxygen species (Kumar and Singh 2010). Synergistic effects of cadmium exposure and temperature stress have been documented in various aquatic species, including water fleas (Heugens et al. 2003) and zebrafish (Vergauwen et al. 2013). In contrast, experiments on oysters and other bivalves showed temperature-independent or even antagonistic effects of both stressors (Holmstrup et al. 2010). 3,4-Dichloroaniline (3,4-DCA), an intermediate in the synthesis of several herbicides (Centre 2006), is a reference toxicant of the OECD (Organisation for Economic Co-operation and Development). Therefore, sensitivity of *N. furzeri* to this compound will allow us to determine its relative sensitivity compared to other ecotoxicological fish models. Anilines act as non-specific membrane irritants or metabolic inhibitors. Currently, information on interactive effects of 3,4-DCA and temperature is largely deficient (but see Freitas et al. (2016)) and the combination of stressors has not been studied in fish. Finally, chlorpyrifos (CPF), an organophosphorus pesticide acting as an acetylcholinesterase inhibitor (Ware 1983), was chosen because it has been used in research on combined effects of compounds and temperature stress (Dinh Van et al. 2014). Patra and colleagues exposed silver perch *(Bidyanus bidyanus)*, rainbowfish (*Melanotaenia duboulayi*) and western carp gudgeon (*Hypseleotris klunzingeri*) in an acute assay to a combination of chlorpyrifos and increased temperature and found synergistic effects in each fish species (Patra et al. 2015).

For each combination of compound and temperature, we performed a short-term exposure test of 14 days, based on OECD 'Early Life-stage Toxicity test Nr. 210', OECD 'Acute toxicity testing Nr. 203' and the protocol used by Shedd and co-workers (Shedd et al. 1999) in exploratory toxicity tests with the killifish *Nothobranchius guentheri*. The goal of such acute toxicity tests is to provide an immediate and low-cost indication of the relative sensitivity of a species to pollutants. Since mortality is commonly used as an objective endpoint in these studies, results such as the lethal concentration for 50% of the population over a certain time (LC_{50}) can be compared across species. Specifically, we hypothesise that any combination of chemicals and increased temperature will lead to synergistic effects. We expect that *N. furzeri* will show a comparable sensitivity to other fish model organisms, such as zebrafish, fathead minnow, rainbow trout and the congeneric *N. guentheri* (Shedd et al. 1999). Finally, we verify whether these results are consistent with the 'stress addition model' (SAM), a model that quantitatively predicts the synergistic direct effects of independent stressor combinations (Liess et al. 2016). The SAM was designed based on the results of several multiple stressor studies, but these did not include interactive effects between toxicants and temperature. Furthermore, while this model has potential in policy making, it has not been tested on fish so far. As such, we assess the applicability of the model for fish, as well as for temperature-toxicant interactions.

Material and methods

Maintenance of the test animals

We used *Nothobranchius furzeri* (strain NF2) from the Limpopo river basin in southern Mozambique. The population was reared for three generations under standardised laboratory conditions at 24 °C (Bartáková et al. 2013). The population was originally collected by scientists from the 'Czech Institute of Vertebrate Biology' in 2012 (collection permit 154/II/2009/DARPPE and sample export permit 049MP00518-A/09 issued by the Mozambican Ministry of Fisheries).

Larvae were hatched synchronically by inundating the eggs (stored in moist peat) with dechlorinated tap water (aged, aerated tap water, 400 mg/L $CaCO_3$) with a temperature of 12 °C. Afterwards, water temperature gradually converged to room temperature (22 °C) (Polačik et al. 2016). Forty-eight hours after hatching, each larva was transferred to an aerated 0.5-L jar and subjected to a specific toxicant-temperature treatment. The temperature in the hatching aquaria and experimental jars was kept constant by means of a bain-marie that was set at either 24 or 28 °C. All conditions were subjected to a 14 h:10 h light:dark regime. The experimental medium, prepared from dechlorinated tap water, was renewed every second day to maintain water quality and to minimise potential effects of compound degradation, as is common in literature (Grosell et al. 2007; Mebane et al. 2012).

Fish were fed *Artemia* nauplii twice a day (ad libitum). During the experiment, we kept dissolved oxygen levels above 80%, conductivity between 600 and 700 μS/cm, pH between 7.8 and 8.2 and hardness (as $CaCO_3$) between 350 and 450 mg/L, which lies within the range of optimal rearing conditions for *N. furzeri* (Polačik et al. 2016). Unlike the setups described in OECD guidelines, fish were exposed individually in separate glass jars, in order to minimise potentially confounding effects of social interaction such as competition for food and aggression.

Short-term exposure

All toxicant solutions were prepared with dechlorinated tap water. The cadmium source was $CdCl_2(H_2O)_{2.5}$ (Sigma, St. Louis, MO, USA). The nominal concentration range was selected around the 24h-LC_{50} concentration found for *N. guentheri* (4.2 mg/L) (Shedd et al. 1999). Fish were exposed to four concentrations (0.125, 0.5, 2 and 8 mg Cd/L) and a control at two temperatures (24 and 28 °C), resulting in a total of 10 treatments. Each treatment comprised 20 replicate fish.

For 3,4-DCA, we used pure 3,4-DCA (Sigma-Aldrich, St. Louis, MO, USA). The nominal concentration range was set around the 96h-LC_{50} concentration found for the zebrafish *Danio rerio* (3.76 mg/L) (Bichara et al. 2014). Fish were exposed to five nominal concentrations (1, 2, 4, 8 and 16 mg/L 3,4-DCA) and a control at two temperatures (24 and 28 °C), resulting in a total of 12 treatments. Every treatment at 24 °C was replicated 16 times, while the treatments at 28 °C were replicated 20 times. Due to the low solubility of 3,4-DCA, a stock solution (50 mg/L) was prepared and mechanically stirred for 2 days before each medium renewal.

Given the lack of reliable data, the nominal concentration range of chlorpyrifos (Sigma-Aldrich, St. Louis, MO, USA) was selected based on the results of a range-finding experiment. This experiment was conducted at 24 °C with eight replicates per treatment and showed that the concentration range between 0.2 and 25 μg/L was sublethal up to 72 h (see results range finding Table S4). Since chlorpyrifos is not soluble in water, a stock solution (125 mg/L) was prepared in ethanol and kept in the dark at 4 °C prior to the exposure. Fish were exposed to five concentrations (6.25, 12.5, 25, 50 and 100 μg/L), a solvent control (0.004% ethanol, as present in the highest CPF concentration) and a regular control at two temperatures (24 and 28 °C), resulting in a total of 14 treatments. Due to an unexpectedly low hatching rate, every condition at 24 °C was replicated only 10 times, while conditions at 28 °C were replicated 20 times.

All experiments and methods were approved by the ethical committee of KU Leuven (file numbers: P125/2015, P072/2016 and P173/2016).

Stress addition model

The stress addition model was used to predict the interaction effect between toxicant exposure and temperature rise, based on the mortality in the toxicant-only treatment and the magnitude of temperature stress in the control treatment at 28 °C. These effects were transferred into general stress levels that can be added to calculate the total interactive stress level. As this model was specifically designed for the assessment of acute mortality on the short term, we only use mortality data obtained after 24 h of exposure. An important assumption of the model is that it requires responses of 0 and 100% mortality as well as several intermediate mortality rates in the 'toxicant-only' treatment, to accurately predict effects of temperature-toxicant interactions. As such, we will be able to compare the effects predicted by the model to the actual obtained values.

Water analysis

A mixed water sample of four jars of each toxicant treatment was taken after the first refreshment and was analysed to verify realised compound concentrations. Concentrations of Cd were measured by inductively coupled plasma mass spectrometry (ICP-MS, Element XR, Thermofisher Scientific, Bremen, Germany) at the University of Antwerp (SPHERE). Concentrations of 3,4-DCA and chlorpyrifos were measured at the University of Ghent (Department of Crop Protection) by means of liquid chromatography (LC/MS/MS) with ESI (Waters ACQUITY UPLC, Xevo TQD mass spectrometer).

Data collection and statistical analysis

All statistical analyses were performed in R v3.2.3 (R Development Core Team, 2016). Mortality was monitored

Table 1 LC_{50} values over 96 h of exposure to different concentrations of cadmium at 24 and 28 °C, with the corresponding standard errors

24 °C			28 °C		
Time (h)	LC_{50} (mg/L)	Standard error	Time (h)	LC_{50} (mg/L)	Standard error
24	1.17	0.056	24	0.74	0.072
96	0.28	0.024	96	0.39	0.013

every 12 h as a binary response (0 = dead, 1 = alive). After 14 days, surviving fish were euthanised by submerging them in ice water. LC_{50} values were calculated with achieved concentration-response curves (Ritz et al. 2015) for mortality at 24 h, 48 h, 72 h, 96 h, one week and two weeks of exposure with the 'drm function' in the 'drc package' (version 3.0–1). Only responses after 24 and 96 h were reported in the main body of the manuscript but responses at other times points can be consulted in the supplementary materials (Table S1–S3). 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. The function survdiff (part of the package survival in R) was used to test for significant differences in survival between the control and solvent control in the chlorpyrifos exposure. SAM calculations were performed using the SPEAR calculator version 0.12.0 (Department System Ecotoxicology - Helmholtz Centre for Environmental Research (UFZ), Germany). The mortality caused by environmental stress (temperature only) was calculated using the mean mortality of all control fish at 28 °C over the three experiments.

Results

Mortality never exceeded 10% in the controls, which is a requirement of the OECD to consider the results of an experiment to be valid.

Cadmium

Realised cadmium concentrations were 0.145, 0.562, 2.278 and 7.096 mg/L (89 to 116% of the nominal concentrations). LC_{50} values for cadmium ranged from 0.10 to 1.17 mg/L and decreased over time (Table S1). All LC_{50} values have SE of < 10% and are, therefore, considered reliable. After 24 h, the LC_{50} was slightly lower at 28 °C, compared to 24 °C (Table 1). Dose-response curves at 24 and 96 h are shown in Fig. 1.

The mortality due to temperature only, measured as the mortality in the control treatment at 28 °C, amounted to 1.7%. Using this environmental mortality and the toxicant-only mortality (data at 24 °C), the stress addition model predicted a dose-response curve for mortality due to Cd at 28 °C (Fig. 2). This predicted curve closely fits our data.

3,4-Dichloroaniline

Realised 3,4-DCA concentrations were 0.37, 1.53, 2.98, 6.59 and 11.94 mg/L (37 to 82% of the nominal concentrations). LC_{50} values for 3,4-DCA ranged from 4.84 to 39.53 mg/L (Table S2). All standard errors were relatively large. All LC_{50} values were lower at 28 °C, compared to 24 °C (Table S2). The 24h-LC_{50} at 24 °C could not be calculated because mortality in the highest exposure concentrations did not exceed 50% (Table 2). Dose-response curves at 24 and 96 h are shown in Fig. 3.

At 24 °C, mortality only reached 25% after 24 h and 81% after 96 h. For this reason, the SAM could not accurately predict the effect of combined stressors.

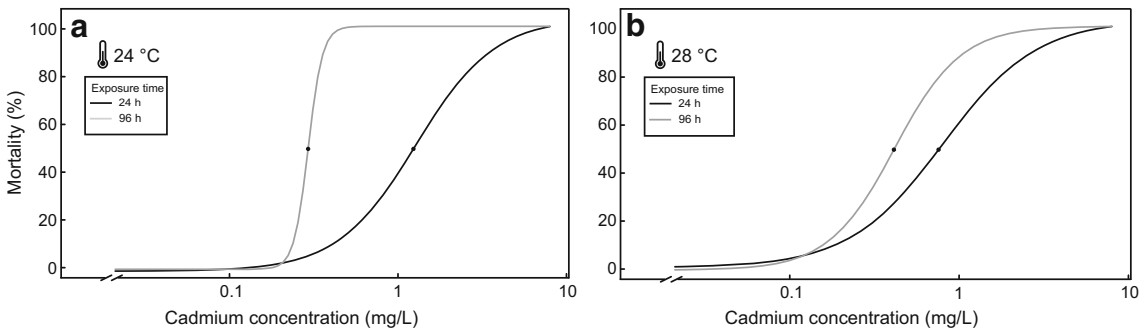

Fig. 1 Dose-response curves showing cumulative mortality of *Nothobranchius furzeri* in function of cadmium concentration (in mg/L) and in relation to exposure time at **a** 24 °C and **b** 28 °C. Dots indicate LC_{50} values. To improve the readability and interpretability of the figure, confidence intervals are not shown on the graphs. The SE of the LC_{50} values are reported in Table 1

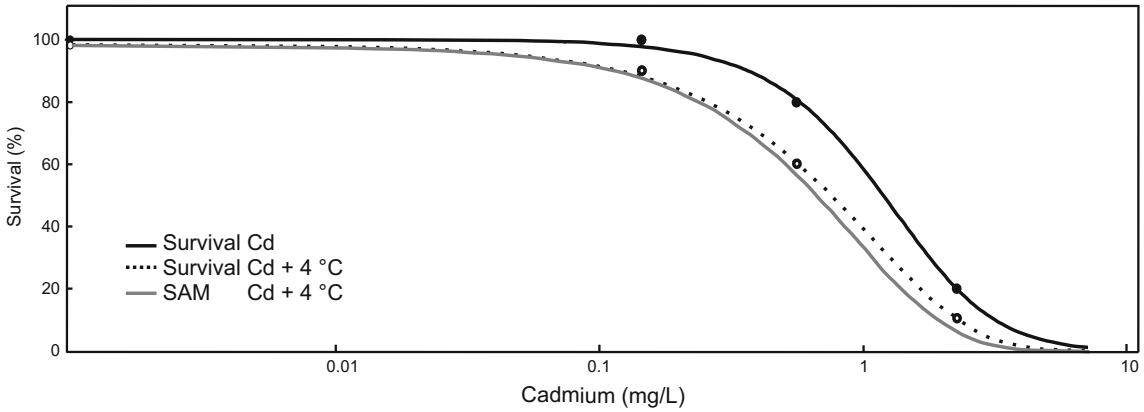

Fig. 2 Observed and predicted survival (%) of *Nothobranchius furzeri* exposed to cadmium and a temperature rise of + 4 °C. Dots indicate the observed mortality. The SAM curve predicts the interactive effect of cadmium exposure and environmental stress using the data on survival due to cadmium exposure only, combined with a 1.7% mortality due to the temperature rise

Chlorpyrifos

Achieved chlorpyrifos concentrations were 4.7, 9.9, 19.3, 39.1, and 79.6 μg/L (75 to 80% of the nominal concentrations). There was no difference in mortality between the controls and the solvent controls for both temperatures (24 °C: $\chi^2_{1,40} = 1$, $P = 0.317$; 28 °C $\chi^2_{1,2\ 0} = 1$, $P = 0.317$); therefore, these results were pooled into one control condition (Green and Wheeler 2013).

LC_{50} values for chlorpyrifos ranged from 6.23 to 36.26 μg/L and decreased over time (Table S3). The 24h- and 96h-LC_{50} values all have small SE (< 10%), except for the 24h-LC_{50} at 28 °C. Again, all LC_{50} values are lower at 28 °C, compared to 24 °C (Table S3 and Table 3). Dose-response curves at 24 and 96 h are shown in Fig. 4.

Also, in this experiment, at 24 °C and after 24 h, mortality reached only 90% in the highest exposure concentration. Fitting these data in the SAM would result in wrong predictions.

To facilitate the interpretation of the calculated LC_{50} values for each compound, additional graphs are available in appendix (Fig. 5).

Interspecies comparison

One of the main goals of this study was to position *N. furzeri* on a sensitivity map, relative to other standard test organisms in ecotoxicology. We searched the ECOTOX database (USEPA), Google Scholar and Web of Science to find comparable short-term exposure tests in other fish species. LC_{50} values are reported in Table 4.

Discussion

Organisms are often simultaneously confronted with a multitude of physical and chemical stressors (Holmstrup et al. 2010). Still, when it comes to evaluating stressor effects, assays are typically conducted for each stressor separately (Noyes et al. 2009). The results of our experiments reveal that killifish respond differently to each of the three tested compounds when combined with an increase in temperature. Overall, the sensitivity of *N. furzeri* is in range with that of other fish species for 3,4-DCA and chlorpyrifos and much higher for cadmium.

Sensitivity to cadmium

Cadmium has been shown to be highly toxic at low concentrations, causing acute and chronic adverse effects on whole ecosystems (Cuypers et al. 2010). As fish appear to be more sensitive to acute cadmium pollution than other freshwater organisms (USEPA 2001), it is advisable to test the sensitivity of several fish species to determine a maximum admissible

Table 2 LC_{50} values of exposure to 3,4-DCA at 24 and 28 °C with the corresponding standard errors for different time intervals. Note that for 24 °C at 24 h, we were unable to fit the data with a sigmoidal dose-response curve and could not calculate the correspondent LC_{50} value

24 °C			28 °C		
Time (h)	LC_{50} (mg/L)	Standard error	Time (h)	LC_{50} (mg/L)	Standard error
24	/	/	24	8.11	13.16
96	9.752	14.53	96	6.61	7.89

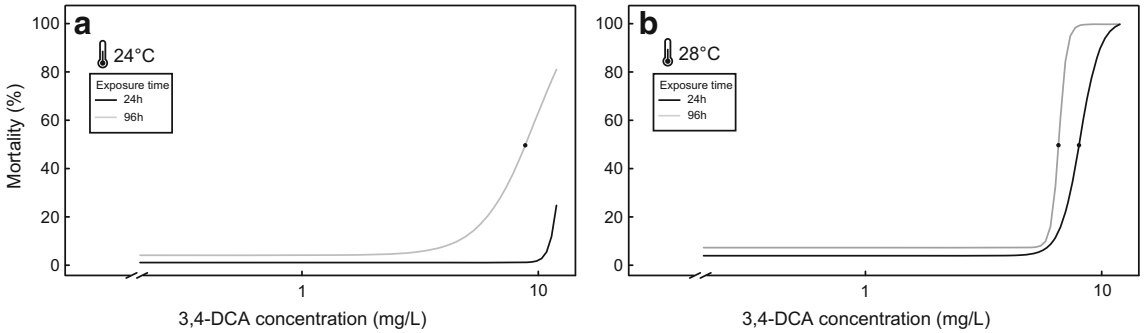

Fig. 3 Dose-response curves showing cumulative mortality of *Nothobranchius furzeri* in function of the 3,4-DCA concentration (in mg/L) and in relation to exposure time at **a** 24 °C and **b** 28 °C. Dots indicate LC_{50} values. To improve the readability and interpretability of the figure, confidence intervals are not shown on the graph

concentration without any adverse effect on the environment. The 96h-LC_{50} for *N. furzeri* (0.28 mg/L at 24 °C, 0.39 mg/L at 28 °C) is well below that of the congeneric *N. guentheri* (4.2 mg/L at 25 °C) (Shedd et al. 1999) and other frequently tested model species (*D. rerio*, 30.1 mg/L at 26 °C (Hallare et al. 2005); *P. promelas*, 4.8 mg/L at 20 °C (Suedel et al. 1996)), indicating a higher sensitivity to this compound. After 24 h, the LC_{50} value at 28 °C is lower than at 24 °C, which suggests the presence of a synergistic effect between cadmium exposure and an increased temperature at this time point. However, from 48 h onwards (Table S1), LC_{50} values are comparable or lower at 24 °C. The raw data also show comparable mortalities at both temperatures. This implies that, when looking at mortality, the higher cadmium toxicity at elevated temperatures could only be observed after 24 h. Synergisms between cadmium and temperature were found in zebrafish (Hallare et al. 2005), with larvae being severely deformed when jointly exposed to both stressors. Also, in juvenile mosquitofish (*Gambusia affinis*), the combination of cadmium and increased temperature caused spinal deformities (Sassi et al. 2010). Our results on the longer term (i.e. 96 h) are more in line with the temperature-independent effects of cadmium as demonstrated on oysters and bivalves (Holmstrup et al. 2010). However, as 100% mortality was reached very quickly in the two highest concentration treatments, it is possible that we failed to identify synergistic effects at intermediate concentrations between 0.5 and 2 mg Cd/L. Also, the studies mentioned above measured synergistic effects on sublethal endpoints (deformation), whereas we only scored mortality.

Sensitivity to 3,4-DCA

3,4-DCA is a widespread and persistent pollutant which enters the environment through the use of pesticides. LC_{50} values in both the 24 and 28 °C treatments showed high uncertainties, with 96h-LC_{50} of 9.75 and 6.61 mg/L at 24 and 28 °C, respectively. However, when looking at the trend in the LC_{50} values, there is a decrease in lethal concentration over time.

A first reason why dose-response curves cannot be fitted in a reliable way might be that the exposure concentrations are too low and mortality insufficient. Reliable LC_{50} values are obtained when a range contains a concentration that results in 100% mortality after a short time (24 to 96 h) while the control results in a low mortality (0 to 10%) over the whole exposure period. Since 90% mortality occurred after 24 h at 28 °C, higher exposure concentrations appear to be unnecessary. More likely, exposure to intermediate concentrations resulted in a low mortality (see Table S5) which, in turn, resulted in a lack of intermediate mortality data to fit reliable sigmoidal dose-response curves. A concentration range between 4 and 16 mg/L of 3,4-DCA would likely have resulted in more accurate dose-response curves.

The 96h-LC_{50} of 9.75 (24 °C) and the 96h-LC_{50} of 6.96 mg/L (28 °C) of *N. furzeri* are in range with the sensitivity of juvenile fathead minnow (7.57 mg/L) (Russom et al. 1997). Other species, such as perch (3.1 mg/L) (Schäfers and Nagel 1993) and rainbow trout (2.7 mg/L) (Crossland 1990), as well as zebrafish (3.76 mg/L) (Bichara et al. 2014), appear to be more sensitive to 3,4-DCA. Despite the high uncertainties for individual LC_{50} values, our results are consistent with a strong

Table 3 LC_{50} values of exposure to chlorpyrifos at 24 and 28 °C with the corresponding *P* values and standard errors for different time intervals

24 °C			28 °C		
Time (h)	LC_{50} (μg/L)	Standard error	Time (h)	LC_{50} (μg/L)	Standard error
24	36.262	0.283	24	18.344	26.35
96	15.395	0.655	96	14.223	1.046

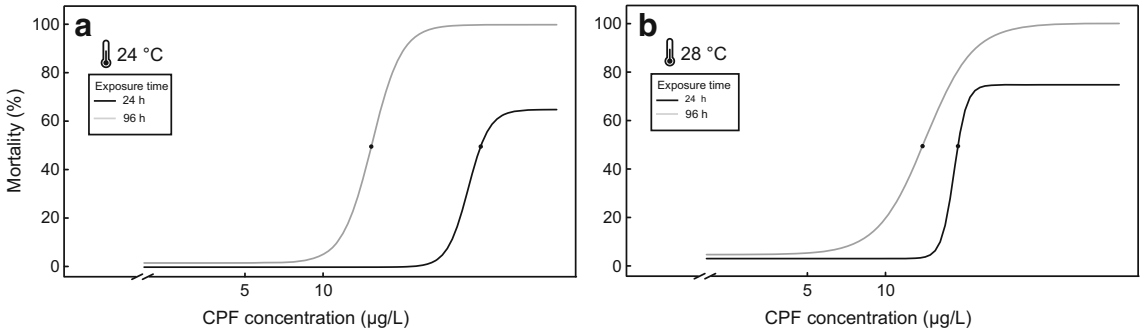

Fig. 4 Dose-response curves showing cumulative mortality of *Nothobranchius furzeri* in function of chlorpyrifos concentration (in µg/L) and in relation to exposure time at **a** 24 °C and **b** 28 °C. Dots indicate LC_{50} values. To improve the readability and interpretability of the figure, confidence intervals are not drawn of the graph. The SE of the LC_{50} values are reported in Table 3

synergism between 3,4-DCA and temperature. Since the exposure time to reach 100% mortality decreased by 50% under the highest 3,4-DCA concentration at +4 °C (see Table S2), our results imply a temperature-dependent toxicity at high concentration levels. Increased toxicity at higher temperature was also found in a study on tadpoles, where expression of thyroid hormone genes was upregulated in a synergistic way and resulted in an accelerated metamorphosis (Freitas et al. 2016).

It was not possible to further compare our results with other studies as, to our knowledge, this is the only study on the interactive effect of 3,4-DCA and temperature on fish.

Sensitivity to chlorpyrifos

The organophosphorus pesticide chlorpyrifos is one of the most widely used pesticides in the world. Since it is easily

Table 4 Acute sensitivity of *Nothobranchius furzeri* and other fish models to cadmium, 3,4-dichloroaniline and chlorpyrifos. Only exposure tests comparable to the set-up used in this experiment are shown. When possible, life stage of the organism, as well as temperature and hardness of the water, is indicated. *NR* not reported in source

Species	Time	LC_{50}	Life stage	Temperature (°C)	Hardness	Reference
Cadmium						
Nothobranchius furzeri	24 h	1.17 mg/L	Juvenile	24	400 mg/L $CaCO_3$	*
Nothobranchius furzeri	96 h	0.24 mg/L	Juvenile	24	400 mg/L $CaCO_3$	*
Nothobranchius furzeri	96 h	0.35 mg/L	Juvenile	28	400 mg/L $CaCO_3$	*
Nothobranchius guentheri	24 h	4.2 mg/L	Juvenile	25	40 mg/L $CaCO_3$	Shedd et al. 1999
Pimephales promelas	96 h	4.8 mg/L	Juvenile	20	60 mg/L $CaCO_3$	Suedel et al. 1996
Danio rerio	48 h	4.75 mg/L	Embryo	21	25 (Ca2+)	Hallare et al. 2005
Danio rerio	48 h	30.1 mg/L	Embryo	26	25 (Ca2+)	Hallare et al. 2005
Danio rerio	48 h	46.8 mg/L	Embryo	33	25 (Ca2+)	Hallare et al. 2005
3,4-DCA						
Nothobranchius furzeri	96 h	9.75 mg/L	Juvenile	24	400 mg/L $CaCO_3$	*
Nothobranchius furzeri	96 h	6.61 mg/L	Juvenile	28	400 mg/L $CaCO_3$	*
Perca fluviatilis	96 h	3.1 mg/L	Juvenile	20	23.8°dH	Schäfers and Nagel 1993
Pimephales promelas	96 h	7.57 mg/L	Juvenile	25	NR	Russom et al. 1997
Oncorhynchus mykiss	96 h	2.7 mg/L	Juvenile	NR	NR	Crossland 1990
Danio rerio	96 h	3.76 mg/L	Embryo	26	NR	Bichara et al. 2014
Chlorpyrifos						
Nothobranchius furzeri	96 h	15.4 µg/L	Juvenile	24	400 mg/L $CaCO_3$	*
Nothobranchius furzeri	96 h	14.2 µg/L	Juvenile	28	400 mg/L $CaCO_3$	*
Danio rerio	10 days	430 µg/L	Embryo	25	NR	Kienle et al. 2009
Gasterosteus aculeatus	96 h	8.5 µg/L	Adult	NR	NR	Giesy et al. 1999
Lepomis macrochirus	96 h	5.3 µg/L	Juvenile	23	NR	Mehler et al. 2008
Oncorhynchus mykiss	96 h	24 µg/L	NR	NR	NR	Deb and Das 2013

*This study

washed away with surface waters, non-target organisms are often exposed unintentionally (Patra et al. 2015). Chlorpyrifos acts as an acetylcholinesterase inhibitor, which is highly temperature dependent. For this reason, combined effects of chlorpyrifos and temperature stress have been researched extensively (Bednarska et al. 2009; Janssens and Stoks 2013; Patra et al. 2009). In this study, we found indications of additive effects between both stressors in *N. furzeri*. On the short term (< 96 h), the combination of chlorpyrifos and + 4 °C resulted in lower LC_{50} values indicating an increased chlorpyrifos toxicity at a higher temperature. From 96 h onwards, however, LC_{50} values of both temperature treatments were comparable. Even though the LC_{50} values were comparable for both temperatures from 96 h onwards, mortality exceeded 70% after a week when exposed to 12.5 μg/L at 28 °C, compared to 45% at 24 °C (see Table S2). This further suggests a synergism between both stressors, most likely caused by the temperature-dependent inhibition of acetylcholinesterase by chlorpyrifos (Gordon 2005). Patra et al. (2015) also found that the toxicity of chlorpyrifos increased at higher temperatures in four fish species (Patra et al. 2015) and Humphrey and Klumpp (2003) reported a similar observation in rainbow trout (Humphrey and Klumpp 2003). Also, for invertebrates, this synergism appears to occur and has, for instance, been described in damselflies (Dinh Van et al. 2014) and midges (Lydy et al. 1999). This temperature-dependent toxicity is likely a feature of most organophosphorus pesticides since they all have a similar mode of action (Costa 2006).

After 96 h of exposure, the LC_{50} values of chlorpyrifos for *N. furzeri* (15.4 and 14.2 μg/L) are comparable with the LC_{50} values reported for three-spined stickleback (8.5 μg/L) (Giesy et al. 1999), rainbow trout (24 μg/L) (Deb and Das 2013) and bluegill (5.2 μg/L) (Mehler et al. 2008). In contrast, *N. furzeri* larvae appear to be more sensitive than zebrafish embryos (430 μg/L) (Kienle et al. 2009).

SAM

The stress addition model (SAM) predicts combined effects of stressors, such as pollution and higher temperatures, based on their individual effects (Liess et al. 2016). The prediction of such effects can be useful for integrated environmental risk assessment. However, due to the novelty of the model, its validation for predicting the effects of stressor combinations on a broad range of organisms is ongoing. The combined effects of cadmium and temperature stress on *N. furzeri* matched the predictions made by the SAM. As this model was not tested on fish thus far, our results are highly relevant in that they at least offer some support to the applicability of the model for fish. Furthermore, our study delivers a proof of principle that the model can also be fitted to responses to a combination of temperature and toxicant stress. It is, however, essential to follow the assumptions of the model (i.e. 0%, intermediate and 100% mortality), which in this case resulted in the exclusion of data on 3,4-DCA and chlorpyrifos exposure. Future studies should aim to consolidate our findings and validate the model for other organisms and stressor combinations to reinforce its applicability for risk assessment and policy making.

The potential of *Nothobranchius furzeri* as a model species

The results of our study suggest that *N. furzeri* could be a valuable new model organism in ecotoxicology. Previous results obtained with copper already suggested its usefulness for the study of both acute and chronic effects (Philippe et al. 2017). After studying the acute sensitivity of *N. furzeri* to reference toxicants with different modes of action, it appears that the sensitivity of the species is in range with, or higher than, that of other model species, depending on the studied compound. A major advantage of using this fish species as a model for acute toxicity screening is the fact that it produces drought-resistant eggs. This enables researchers to store eggs or to obtain them from a supplier and eliminates the need for costly and time-consuming on-site cultures. Moreover, the embryos can be stored for several months up to a year until hatchlings are needed (Shedd et al. 1999).

Although acute tests, like in this study, provide quick and reliable indications of acute sensitivity, their ecological relevance is low because of the high toxicant concentrations used (Nagel 2001). Sublethal, lifelong and multigenerational tests are far more realistic representations of the natural situation and are considered to be more sensitive alternatives compared to acute tests (Walker et al. 2012). However, these experiments are very time consuming and costly with current model species such as zebrafish or medaka. The unique characteristics of the annual killifish *N. furzeri* (fast growth, early maturation, short life span) could drastically lower these costs (Polačik et al. 2016). Future studies that assess the potential chronic effects of combined stressors would be essential to assess harmful effects that may be delayed and may not appear during a standard 24-to-96-h test (Philippe et al. 2017). Prolonging exposure for just one month would enable researchers to measure sublethal endpoints, such as growth and maturation time, in *N. furzeri*. Measuring such endpoints in more traditional fish model species would at least require two or three additional months of exposure.

Conclusion

The joint effects of toxicants and increased temperature on *N. furzeri* varied with the type of pollutant. We found indications of synergisms between the combined stressors for all tested pollutants. Compared to other commonly used model

organisms, *N. furzeri* appeared to be more sensitive to cadmium whereas its sensitivity to 3,4-DCA and chlorpyrifos was in range with or higher than the sensitivity of other fish models. Furthermore, at 24 h, the interaction effect of cadmium and temperature could be predicted by the SAM.

Overall, our results imply that *N. furzeri* could be a valuable fish model in ecotoxicology. Advantages compared to other fish test organisms include the production of long-lived drought-resistant eggs and the fastest maturation time of 18 days known in vertebrates (Blažek et al. 2013). Combined, these traits facilitate time-efficient chronic exposure tests, which is essential for toxicants with delayed effects, but also to study long lasting effects within and among generations.

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Compliance with ethical standards All experiments and methods were approved by the ethical committee of KU Leuven (file numbers: P125/2015, P072/2016 and P173/2016).

Conflict of interest The authors declare that they have no conflict of interest.

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