The effect of thermal pre-incubation and exposure on sensitivity of zebrafish (**Danio rerio**) to copper and cadmium single and binary exposures

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Abstract

Zebrafish (Danio rerio) is a prominent model organism in a wide range of biological studies including toxicology. However, toxicological studies are often performed at species specific optimum temperature, and knowledge on the effect of different temperature regimes on the toxicity of metal ions is rather limited. To address this knowledge gap, present study investigates the effect of various thermal scenarios (simultaneous and sequential; acute and chronic) on the toxicity of Cu and Cd in zebrafish. For this purpose we assessed mortality and whole body metal burdens as indicators of toxicity and bioavailability, respectively, and whole body electrolyte concentrations and body condition as the indicators of physiological condition. Thermal pre-incubations (for 12 or 96 hours or 28 days) and subsequent metal ion exposures (for 10 days) were conducted at 17, 22, 25, 28, 32 and 34 °C. The metal exposures were performed at Cu concentrations of 1.2 μM and Cd concentrations of 0.2 μM, both singly and in binary mixtures. Irrespective of thermal treatments, Cu exposures resulted in greater mortality than Cd exposures at the given concentrations. Moreover, the Cu and Cd mixture indicated a synergistic effect. While acute pre-incubation for 12 or 96 hours at elevated temperatures increased mortality in the subsequent metal exposure at the optimum temperature (28 °C), pre-incubation at cold temperatures in this scenario appeared to increase tolerance towards the subsequent metal exposure. Chronic thermal pre-incubation of zebrafish to a range of temperatures for 28 days moderated the effect of temperature fluctuations on subsequent metal toxicity at the optimum temperature. Chronic thermal pre-incubation at a range of temperatures followed by metal exposure at the same temperature showed that environmental temperature variations (higher or lower than optimal temperature) coupled with metal exposure, led to increased mortality, furthermore, the highest whole body metal burdens were measured in this scenario. Nevertheless, neither the whole body burden of metals, nor the metal accumulation rate, were predictors of mortality, i.e. these two values were not higher in dead fish in comparison to those that survived the exposures. Finally, we observed a significant decrease in the whole body Na+ level of dead fish in comparison to fish which survived the exposure conditions, suggesting that survival depends on maintaining Na+ homeostasis under the applied multi-stress conditions. Overall, our results show that thermal pre-history and ambient temperature play an important role in determining the tolerance of zebrafish towards metal ion stress.

Keywords	Metal toxicity; Temperature; Survival; Metal burden; Electrolyte balance; multistressors.

The effect of thermal pre-incubation and exposure on sensitivity of zebrafish (*Danio rerio*) to copper and cadmium single and binary exposures

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Abstract

Zebrafish (*Danio rerio*) is a prominent model organism in a wide range of biological studies including toxicology. However, toxicological studies are often performed at species specific optimum temperature, and knowledge on the effect of different temperature regimes on the toxicity of metal ions is rather limited. To address this knowledge gap, present study investigates the effect of various thermal scenarios (simultaneous and sequential; acute and chronic) on the toxicity of Cu and Cd in zebrafish. For this purpose we assessed mortality and whole body metal burdens as indicators of toxicity and bioavailability, respectively, and whole body electrolyte concentrations and body condition as the indicators of physiological condition. Thermal pre-incubations (for 12 or 96 hours or 28 days) and subsequent metal ion exposures (for 10 days) were conducted at 17, 22, 25, 28, 32 and 34 °C. The metal exposures were performed at Cu concentrations of 1.2 µM and Cd concentrations of 0.2 µM, both singly and in binary mixtures. Irrespective of thermal treatments, Cu exposures resulted in greater mortality than Cd exposures at the given concentrations. Moreover, the Cu and Cd mixture indicated a synergistic effect. While acute pre-incubation for 12 or 96 hours at elevated temperatures increased mortality in the subsequent metal exposure at the optimum temperature (28 °C), pre-incubation at cold temperatures in this scenario appeared to increase tolerance towards the subsequent metal exposure. Chronic thermal pre-incubation of zebrafish to a range of temperatures for 28 days moderated the effect of temperature fluctuations on subsequent metal toxicity at the optimum temperature. Chronic thermal pre-incubation at a range of temperatures followed by metal exposure at the same temperature showed that environmental temperature variations (higher or lower than optimal temperature) coupled with metal exposure, led to increased mortality, furthermore, the highest whole body metal burdens were measured in this scenario. Nevertheless, neither the whole body burden of metals, nor the metal accumulation rate, were predictors of mortality, i.e. these two values were not higher in dead fish in comparison to those that survived the exposures. Finally, we observed a significant decrease in the whole body Na^+ level of dead fish in comparison to fish which survived the exposure conditions, suggesting that survival depends on maintaining Na^+ homeostasis under the applied multi-stress conditions. Overall, our results show that thermal pre-history and

ambient temperature play an important role in determining the tolerance of zebrafish towards metal ion stress.

Keywords: Metal toxicity, Temperature, Survival, Metal burden, Electrolyte balance, multistressors

1. Introduction

Zebrafish (*Danio rerio*) is a valuable vertebrate model organism in a wide range of biological research including ecotoxicology. Nonetheless, the effect of temperature is generally not considered in the setting of environmental quality standards, and ecotoxicity tests are mostly performed at species specific optimal temperature (Di Giulio and Hinton, 2008). Temperature is one of the most important environmental factors driving physiological and ecological dynamics with a wide ranging impact (Brett, 1971). Although the effect of temperature on the physiological metrics of zebrafish such as development (Kimmel et al., 1995; Santos et al., 2017), metabolism (Barrionuevo and Burggren, 1999; Sfakianakis et al., 2012) and behaviour (Pritchard et al., 2001; Lopez-Olmeda et al., 2006) has been studied in detail, the impact of different thermal regimes on the sensitivity of zebrafish towards metals as model toxicants is poorly documented.

In general, the effect of an increase in ambient temperature is known to increase metal toxicity to aquatic organisms. This effect is often ascribed to an increase in metabolic rate, which results in increased metal accumulation and consequent toxicity in fish and eels (Heugens et al., 2001; Sokolova and Lannig, 2008; Nichols and Playle, 2004; Yang and Chen, 1996; Macleod and Pessah, 1973; Kumar et al., 2018; Boughammoura et al., 2013). However, studies with other organisms, including *Daphnia* (Boeckman and Bidwell, 2006) and mussels (Mubiana and Blust, 2007), have reported no or a suppressive effect of increasing temperature on metal toxicity and uptake and also higher metal toxicity and accumulation in suboptimal temperatures for the organism. These cases draw attention to the importance of temperature as a determining factor towards a secondary stressor such as metal pollution and the fact that different thermal scenarios and the thermal pre-history of the organism can affect metal toxicity or tolerance. Thus, the effect of temperature on metal toxicity cannot be ascribed solely to its effect on metabolic rate; the temperature dependence of metal transport pathways, chemical parameters pertaining to the exposure medium and the intracellular fluid (*e.g.* diffusion coefficients), membrane permeability and systematic functions (such as ventilation or absorption from food) must also be considered (Sokolova and Lannig, 2008).

The body temperature of zebrafish, like other ectotherms, is governed by the temperature of its surrounding environment. The temperature of the natural habitat of zebrafish can range from 6 °C in winter to over 38 °C in summer (Spence et al., 2008) which are close to the thermal limits of zebrafish (Lopez-Olmeda and Sanchez-Vazquez, 2011). It is assumed that the thermal tolerance of zebrafish is proportional to the acclimation temperature. That

is, increasing the acclimation temperature can increase the highest and lowest temperatures that fish can tolerate and vice versa (Cortemeglia and Beitinger, 2005). Under laboratory conditions, zebrafish are mostly reared at 25-28 °C while 28.5 °C is known to be the optimal temperature for zebrafish reproduction and growth (Westerfield, 2000).

To date, several studies have investigated the impact of temperature acclimation and exposure regimes on metal toxicity to adult zebrafish as well as zebrafish embryos. For example, it has been reported that elevated temperatures increase the toxicity of Ni and reduce the hatching success rate of *Danio rerio* embryos (Scheil and Köhler, 2009). Hallare et al. (2005) exposed zebrafish embryos to Cd at different temperatures and monitored toxicological and developmental endpoints. They found that Cd induced mortality is higher at suboptimal low temperatures for zebrafish embryos in comparison to optimal and high temperature, and malformations were higher in both high and low temperatures compared to control groups (Hallare et al., 2005). In adult zebrafish, Cd toxicity and bioaccumulation were reported to increase by increasing the temperature; however, no link between Cd toxicity and liver oxidative damage was found (Vergauwen et al., 2013). Zheng et al. (2017) acclimated zebrafish (*Danio rerio*) to 26 ℃ and 34 ℃ for 4 days and subsequently exposed them to 0 or 200 µg/L (1.78 µM) of Cd for 7 days at the standard temperature (26 ℃). Based on their findings from promotor demethylation, gene transcription and enzymatic activity, the authors concluded that preheating-induced accumulation of transcripts via demethylation might invoke the rapid defence responses at post-transcriptional levels caused by subsequent Cd exposure and therefore reduced Cd toxicity in zebrafish.

In addition, there is an important knowledge gap concerning the possible interaction of different thermal scenarios and a secondary single or mixture metal stress, which is the typical situation in natural environments. Therefore, this study evaluates the combined effect of thermal and metal stress under a number of exposure scenarios. To this end, we have defined 3 scenarios, namely (1) a short temperature treatment followed by subsequent metal exposure for 10 days at the optimal temperature (Scenario 1), (2) the same as Scenario 1 but with a prolonged temperature acclimation (Scenario 2), and (3) temperature acclimation followed by metal exposure for 10 days at the temperature of pre-acclimation (Scenario 3). Survival and whole-body metal accumulation were measured as indicators of metal toxicity and bioaccumulation, respectively. In addition, whole-body essential electrolyte concentrations and the relative body condition factor were determined as measures of the physiological condition of the fish. The fish condition factor reflects the general well-being or robustness of an individual fish and is often calculated by the ratio of body weight to body length based on the assumption that fish are growing isometrically, hence the exponent on length is typically 3. In practice, the relative condition factor, K_{rel}, is calculated based on an empirical length-weight relationship and is the recommended condition index when exploring individuals within a certain study (Froese, 2006). By integrating the output of the mentioned assays we elucidate (1) the interaction between thermal pre-incubation and exposure on metal toxicity of zebrafish under multistress

conditions, (2) the relationship between whole-body burden of metal and the metal accumulation rate and mortality, and (3) the effect of applied temperature and metal exposure scenarios on the essential electrolyte composition of the organism.

2. Materials and methods

2.1. Ethical statement

All the experimental protocols of this study were approved by the Ethical Committee for Animal Testing (ECD) of the University of Antwerp and conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

2.2. Fish maintenance

Adult wild type zebrafish (*Danio rerio*) were obtained from the University of Antwerp zebrafish facility and were given 4 weeks to acclimate to laboratory conditions. They were stocked at 28 ℃ in 100 L glass aquariums (200 fish/aquarium) (Lawrence, 2007), filled with moderately hard water prepared following the U.S. Environmental Protection Agency protocol for preparation of synthetic freshwater (U.S. EPA, 2002; $NaHCO_3$: 96 mg/L; $CaSO_4.2H_2O$: 60 mg/L; $MgSO_4$: 60 mg/L; KCl 4 mg/L; pH: 7.54±0.09; water hardness: 80-100 mg/L $CaCO_3$). The pre-acclimation period as well as the exposure experiments were conducted in a temperature controlled chamber (Type WT15'/+5DU-WB, Weiss Technik, Reiskirchen-Lindenstruth, Germany) with a 14h light: 10h dark photoperiod (lights on at 8:00), the aquarium temperature was regulated by a supplemental microprocessor MP-heater (Aquatic Nature®, Roeselare, Belgium). Aquarium water was constantly aerated using a plastic air pipe, mechanically and biologically filtered, and ammonia, nitrite and nitrate levels were monitored using Tetratest (Tetra®, Melle, Germany) and always kept at the optimum levels for zebrafish (Lawrence, 2007).. The fish were fed once a day, ad libitum with Sera vipan® (Heinsberg, Germany) flakes and after 15 minutes the remaining food was removed.

2.3. Experimental setup

After the pre-acclimation period, fish were divided into the following 3 experimental groups:
1- Acute thermal pre-incubation: in this scenario zebrafish were subjected to 12 or 96 hours of thermal treatment at 17, 22, 25, 28 (control), 32 or 34 ℃. Subsequently, they were exposed to Cu 1.2 µM (*ca*. 75 µg/L Cu; as $CuSO_4.5H_2O$, Sigma-Aldrich®, MO, USA) or Cd 0.2 µM (*ca*. 25 µg/L Cd; as $CdCl_2.H_2O$, Merck KGaA®, Darmstadt, Germany) or their mixture for 10 days at 28 ℃. The metal ion concentrations in the exposure media were monitored by ICP-OES and the mean±SD values are given in Table 2. We denote these scenarios as Acute thermal 12 hours and Acute thermal 96 hours.
2- Chronic thermal pre-incubation: the same treatment as the Acute thermal scenarios was applied with one exception; the fish were acclimated to the 5 different temperatures for 28

days prior to the 10 day metal exposure at 28 °C. We denote this scenario as Chronic thermal.

3- The 28 days chronic thermal pre-incubation was followed by 10 days of metal exposure at the temperature of acclimation. We denote this scenario as thermal Acclimation. An overview of the applied thermo-metal scenarios is presented in Table 1. The 10 day duration of metal exposure is sufficient to ensure attainment of the incipient lethal level in fish (Marr et al., 1998; Stubblefield et al., 1999; Liao et al., 2001; De Boeck et al., 2004).

The chemical speciation of Cu and Cd in the exposure medium was modelled using Visual MINTEQ 3.1 (Gustafsson, 2014). The LC50 value of the metals depends on the exposure conditions (such as pH, hardness and complexation). For example, in adult zebrafish, in water with similar composition as that used herein, i.e. hardness of 141 mg $CaCO_3$/L and pH 7.8, 96-h LC50 for Cu is reported to be 212 µg/L (3.33 µM) and that for Cd is 3822 µg/L (34 µM) (Alsop and Wood, 2011). The metal concentrations were chosen so that information on the time dependence of toxic effects could be observed for Cu in single metal exposures and for the Cu+Cd mixture; use of a concentration of Cd with low toxicity in single exposures is necessary to avoid rapid and complete mortality in mixture scenarios.

The thermo-metal exposures were conducted in polypropylene aquaria containing 7.5 L of exposure media. Each treatment comprised one aquarium containing 10 fish; each individual was considered to be independent, i.e. n = 10 per treatment (Colgrave and Ruxton, 2018). In the Chronic and Acclimation thermal scenarios the water temperature was increased or decreased gradually (1 °C/ 12 hours) to reach the target temperature, while in acute thermal scenarios the temperature was changed rapidly. The water was aerated but not filtered and it was replaced every second day with the water of the same metal concentration. The fish were not fed during the 10 days of metal exposure to prevent ambiguity in the uptake route as well as to avoid increased levels of ammonia and dissolved organic matter. Once a day, water samples were collected from each aquarium to determine the metal concentrations and DOC levels. Mortalities were recorded every 12 hours and dead fish were removed and kept frozen at -20 °C until the end of the experiment. The fish that were alive at the end of the experiment were euthanized by an overdose of tricaine methanesulfonate (MS-222) solution (Matthews and Varga, 2012).

Table 1. Overview of the applied temperature and metal treatments.

Scenario	Thermal pre-incubation at 17, 22, 25, 28, 32 or 34 °C	Metal exposure (Cu 1.2 µM, Cd 0.2 µM, or Cu 1.2 + Cd 0.2 µM)
Acute thermal pre-incubation	12 hours	10 days at 28 °C
	96 hours	
Chronic thermal pre-incubation	28 days	10 days at 28 °C
Thermal Acclimation	28 days	10 days at temperature of pre-incubation

To assess the effect of death and metal exposure on whole body Na^+ concentration, we conducted two complementary experiments. Firstly, 10 zebrafish were exposed to the mixture of Cu 1.2 µM and Cd 0.2 µM until they died. Thereafter, the carcass of the fish was collected from the water at time intervals of 0, 3, 6, 9 and 12 hours after death, respectively (2 fish were collected for each collection time) and the whole-body Na content was measured. These measurements are denoted as complementary experiment 1. In the second series of experiments (denoted as complementary experiment 2) 44 zebrafish were divided into 2 experimental groups: a control and Cu (1.2 µM) + Cd (0.2 µM) exposure for 96 hours. The live whole-body samples were collected every 6 hours (2 fish were collected at each collection time) and the whole-body Na content was measured. All metal exposure conditions, sampling and analytical procedures of these two experiments were identical to the main experiment and were conducted at control temperature of 28 °C.

2.4. Mortality and relative condition factor

Survival proportions were calculated using the product limit (Kaplan-Meier) method by GraphPad Prism 7.04 (GraphPad software, CA, USA). Mantel-Cox Log-rank test was performed to compare the survival curves between the different treatments within a specific scenario (Supplementary data, Fig S1).

In the present study we calculated the relative body condition factor, K_{rel} (dimensionless), via the following formula (Eq. 1):

$$K_{\mathrm{rel}} = \frac{W}{aL^{n}} \qquad (1)$$

Where *W* is the body weight (in grams) and *L* is the fork length (in centimetres) measured individually for each fish. Parameters *a* and *n* were obtained by regression analysis of the

weight-length relationship ($W=aL^n$) for 60 fish acclimated to 28°C at the start of the experiment (a = 0.0103 g cm^{-1}, n = 2.8016, R^2 = 0.7194).

2.5. Sample preparation and analysis

Water samples were collected every 24 hours from all containers before and after passage through an Acrodisc® 0.20 µm Supor Membrane syringe filter (Pall Life Sciences, Ville St. Laurent, QC) to measure total and dissolved metal and major ion concentrations. Samples were acidified to 2% H^+ with trace-metal-grade HNO_3 (69%) and analysed using ICP-OES (iCAP 6300 Duo, Thermo Scientific®). Part of the filtered water (collected prior to exposure water replacement) was acidified to pH 2 and used for DOC measurements with a TOC analyser (TOC-VCPH, Shimadzu).

The fish samples were weighed, and oven dried at 60 °C for at least 48 hours until constant weight, afterwards they were placed in a desiccator at room temperature and reweighed to obtain the dry weight. The samples were digested using a "hot block" (Environmental Express SC154®). For digestion, 2 mL nitric acid (HNO_3, 69%) was added to dried samples and (after 12 hours) they were placed in the hot block at 110 °C for 30 minutes. Subsequently samples were removed from the hot block and after cooling down 0.5 mL of hydrogen peroxide (H_2O_2, 30%) was added and digestion completed by heating at 110 °C for another 30 minutes. Metal concentrations in the fish digests were determined by inductively coupled plasma-mass spectrometry (7700x ICP-MS, Agilent Technologies®) with the quantification limits of 0.1 µg L^{-1} for copper and cadmium. All metal body concentrations were calculated on a dry weight basis. Concentrations of major cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) were determined using ICP-OES (iCAP 6300 Duo, Thermo Scientific®). Blanks and certificated reference material (SRM-2976, National institute of standards and technology, Gaithersburg, MD 20899, USA) were included in all series of metal analysis to validate the accuracy of the analytical procedure. Recoveries were within 4% of certified values.

2.6. Statistical analysis

The Log-rank (Mantel-Cox) test was used to compare the survival curves at different temperatures within a specific exposure. Prior to statistical analysis, all data were tested for normality of distribution using the D'Agostino-Pearson test. Two-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons test were done to test whether the K_{rel} differed significantly among different exposures (temperature and metal exposure as factors). Significant differences among whole body metal concentration (Cu and Cd) were studied using a nonparametric ANOVA, Kruskal–Wallis test followed by a multiple comparison Dunn's test. For each thermal scenario the average whole body electrolyte content of the pooled survived fish was compared with that of the pooled dead fish using student's t-test. The whole body electrolyte concentration among different temperatures in each scenario was compared using the Mann-Whitney test.

3. Results

3.1. Exposure medium characteristics

The average concentration of dissolved metals and major ions measured in the exposure medium during 10 days of metal exposure is presented in Table 2. Since measured dissolved Cu and Cd concentrations were consistently within ~4% of total metal concentrations, only dissolved concentrations are presented. During the 10 days of metal exposure the measured DOC levels were consistently between 1-2 ppm.

Table 2. Mean (±SD) concentrations of dissolved metals and major ions in the exposure medium during 10 days of metal exposure.

Exposure condition	Average measured concentration of dissolved metals and major ions (µM±SD)					
	Cu	Cd	Na	K	Ca	Mg
Cu only	1.2±0.05	<0.0008	1061.13±8.99	55.84±1.84	363.46±3.4	480.64±9.72
Cd only	0.02±0.003	0.21±0.007	1072.06±8.58	54.05±2.74	362.09±3.17	465.38±5.40
Cu+Cd	1.21±0.15	0.23±0.006	1068.27±10.27	53.52±2.23	362.88±2.42	457.97±5.80

The chemical speciation of Cu and Cd species in the exposure medium at standard temperature (Acute thermal and Chronic thermal scenario) is presented in Fig 1. In thermal acclimation scenario, the Cu and Cd free ion levels decreased slightly with increasing temperature, but the impact was negligible: <4 % and < 3% difference between the highest (34 °C) and lowest (17 °C) temperature for Cu and Cd respectively, i.e. temperature alterations did not significantly influence speciation modelling outcomes. DOC present in the water will tend to lower the free metal ion concentrations, however the speciation calculations based on the highest measured DOC level (2 ppm; modelled as fulvic acid) showed that this effect has no impact on the interpretation of the speciation results because the unsupported diffusive flux of free metal ion in the exposure medium was still greater than the estimated bio-uptake flux (Jansen et al., 2002). Thus we have excluded the DOC from the speciation modelling.

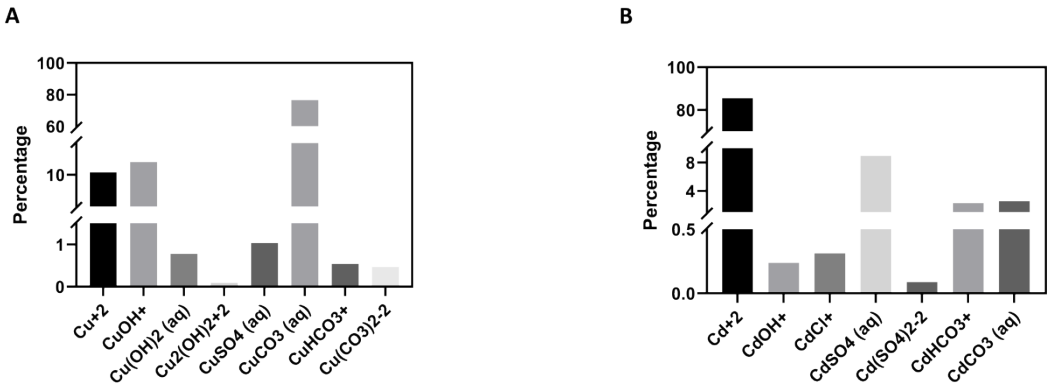

Fig. 1. The distribution of Cu and Cd species (% of total concentration in the exposure medium) in **(A)** Cu and **(B)** Cd exposures at 28 °C. The composition of Cu and Cd species in the mixture exposure was similar to that in the single exposures.

3.1. Mortality and condition factor

No mortality was recorded during the thermal pre-treatments. The overall survival under the thermo-metal treatments and the survival proportions as a function of time calculated based on Kaplan-Meier method are given in Fig. 2 and Fig. S1 respectively (outcomes of statistical significance, Mantel-cox test, are depicted in Fig S1). A greater mortality was observed for Cu than for Cd at the concentrations used herein (Fig. 2). Moreover, mortality rates in Cu+Cd exposures were always substantially higher than those observed in Cu or Cd single exposures at the control temperature of 28 °C in all scenarios. Since almost no mortality was observed in Cd single exposures, the observation that mortality in the mixture was always greater than that for Cu alone at almost all temperatures, suggests that the interaction of these two metals is synergistic. In the acute thermal scenario, toxicity of the metal mixture increased with increasing temperature of pre-incubation (Fig. 2A,B). In particular, mortality in the Cu+Cd exposure was significantly higher than that in the control group at 32 °C (p<0.05) in Acute 12 hours and 32 °C (p<0.01) and 34 °C (p<0.001) in Acute 96 hours scenario (Fig. S1). In the chronic thermal scenario, the highest toxicity of the metal mixture was noted at the lowest (17 °C) and highest (34 °C) temperature of pre-incubation in the mixture (Fig. 2C), However, the mortality was only significantly higher at 17 °C (p<0.05) compared to control group (Fig. S1). In the Acute and Chronic thermal scenarios no significant effect of pre-incubation temperature was observed on the toxicity of Cu or Cd in single exposures (Fig. 2 A, B, C and Fig S1). In the thermal acclimation scenario, toxicity was much higher at low temperatures compared to higher ones in both the single Cu and the binary Cu+Cd exposures (Fig. 2D). In this scenario, mortality in Cu single exposures was significantly higher at 17 °C (p<0.001), 22 °C (p<0.01) and 25 °C (p<0.01) compared to the control group (Fig. S1). Furthermore, in binary exposures, significantly higher mortality was observed at 17 °C (p<0.001), 22 °C (p<0.001), 25 °C (p<0.001), 32 °C (p<0.05) and 34 °C (p<0.05). Nevertheless, in agreement with the other two scenarios, Cd-exposed fish in the

thermal acclimation scenario showed no difference in sensitivity towards cadmium at different temperatures.

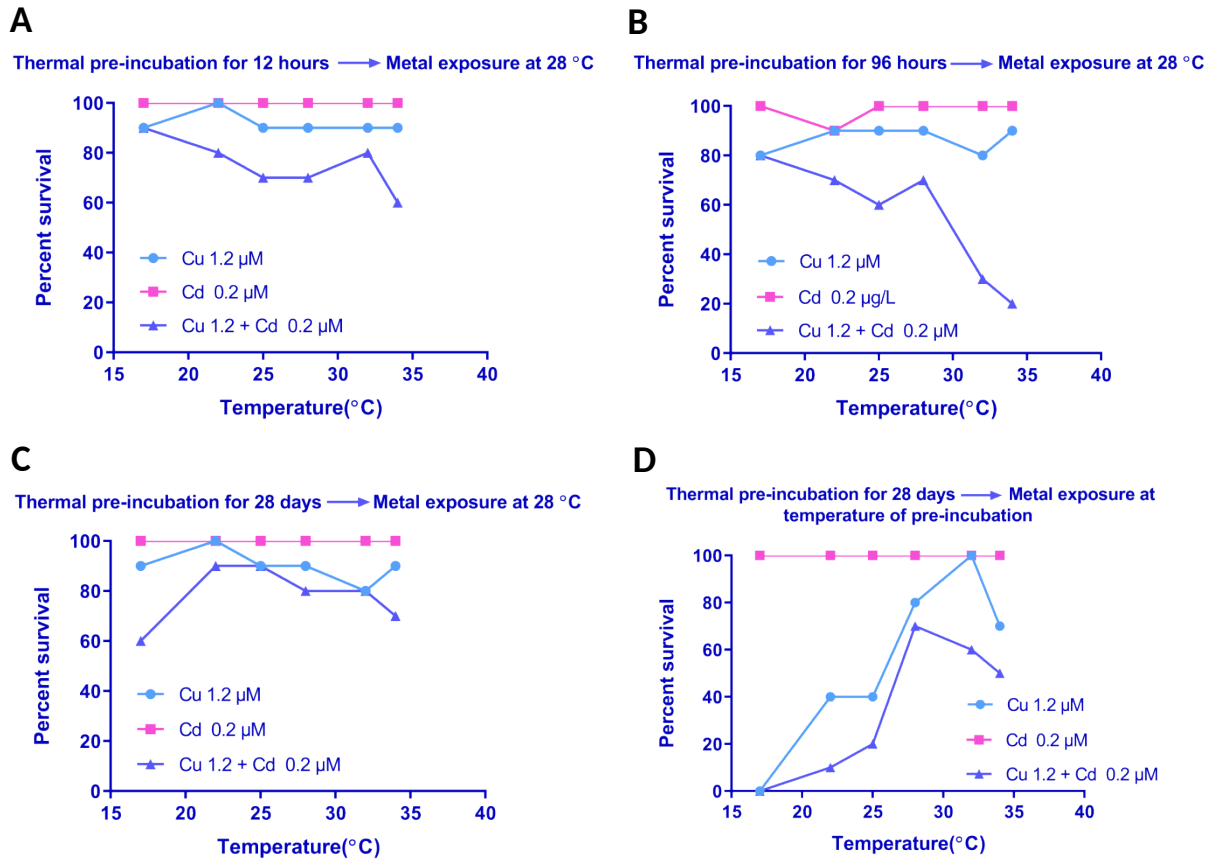

Fig. 2. The percentage survival of zebrafish subjected to various thermal conditions and 10 days of metal exposure (sample size, *n* = 10). (**A**) acute 12 hr thermal pre-incubation; (**B**) acute 96 hr thermal pre-incubation; (**C**) chronic 28 days thermal pre-incubation; and (**D**) thermal acclimation scenario.

The average body size of zebrafish across all the different thermal treatments for each scenario was measured as follows (weight (g) ± SD; length (cm) ± SD): Acute 12 hours (0.45±0.13; 3.71±0.20), Acute 96 hours (0.51±0.1; 3.8±0.16), Chronic (0.43±0.12; 3.61±0.18) and Acclimation (0.46±0.12; 3.65±0.16). Within each scenario, the body size was independent of thermal treatment. The relative condition factor, K_{rel}, of zebrafish in the 4 scenarios is shown in Fig. 3. In the acute thermal scenario (12 and 96 hr), no significant effect of applied temperature or metal treatments on K_{rel} was found (two-way ANOVA). In the chronic thermal scenario, the Cu+Cd binary exposure resulted in a significantly decreased K_{rel} in comparison with Cu or Cd single exposures(p<0.05; two-way ANOVA, Tukey's post-hoc test). In Cd exposed fish in the chronic thermal scenario, K_{rel} is higher at 22 °C in comparison to 34 °C (p<0.05). In the mixture of Cu+Cd , K_{rel} is significantly higher at 22 °C than at 32 and 34 °C (p<0.05). Copper exposed fish in the chronic thermal scenario had a higher K_{rel} at 17, 22 and 28 °C than at 34 °C (p<0.01, p<0.05 and p<0.001 respectively). The calculated K_{rel} values for three metal exposures at different temperatures in this scenario

demonstrated that the relative condition factor of Cu+Cd exposed fish at 28 °C is significantly lower than Cu (p<0.01) or Cd (p<0.05) exposed fish. Furthermore, at 32 °C the condition factor of Cd exposure is higher than Cu (p<0.05) or Cu+Cd (p<0.05) exposures. The values are comparable at other temperatures in the chronic thermal scenario.

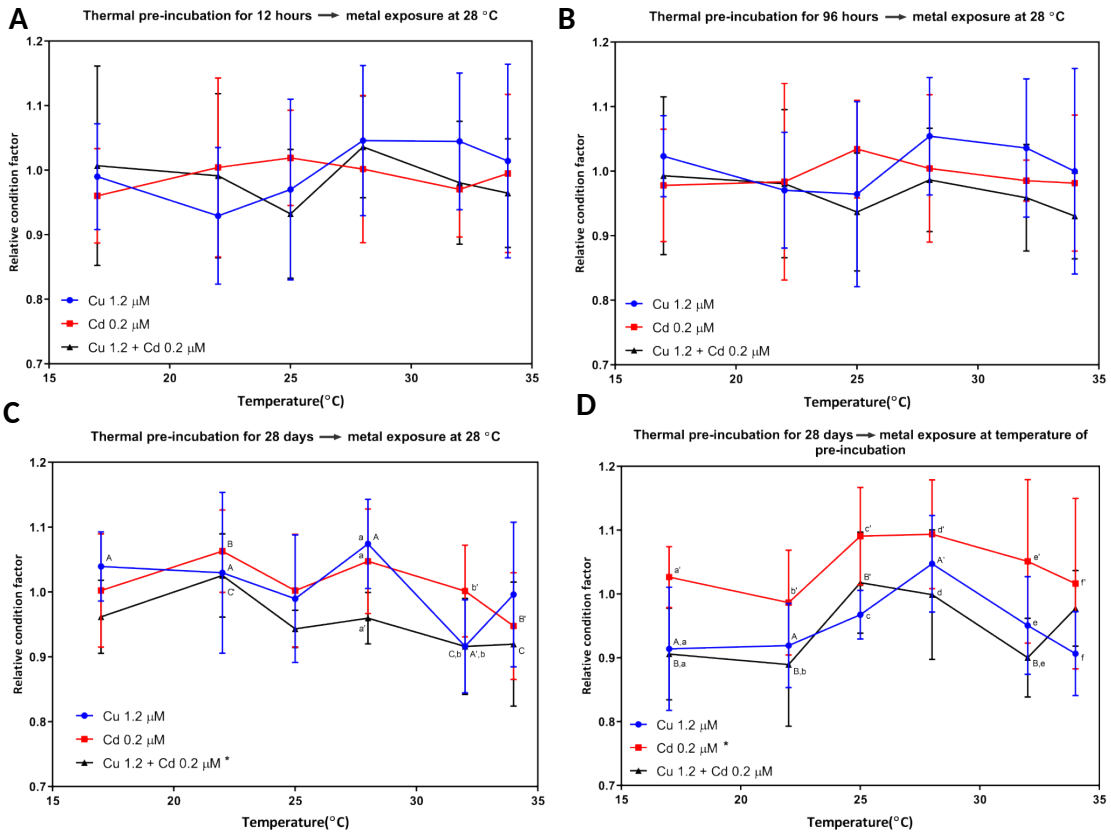

Fig. 3. Relative condition factor of zebrafish in (**A**) acute 12 hr thermal pre-incubation, (**B**) acute 96 hr thermal pre-incubation, (**C**) chronic 28 days thermal pre-incubation and (**D**) thermal acclimation scenarios. Values are means ± standard deviation; sample size, n = 10.
Same capital letters denote significant (p<0.05) difference between different temperatures within an exposure and same lower case letters denote significant difference at a specific temperature between different exposures (the letters with ‘ are significantly different than the letters without ‘); two way ANOVA, Tukey’s post-hoc test. * indicates a significant (p<0.05) effect of metal exposure on relative condition factor independent of thermal incubation.

In the thermal acclimation scenario the K_{rel} for the Cd exposure is significantly higher than the two other exposures (p<0.05). In Cd exposure, different temperatures have no significant effect on the relative condition factor but in Cu and Cu+Cd exposures the highest values were observed at 28 °C and 25 °C, respectively. Particularly, in Cu exposure K_{rel} was significantly higher at 28 °C than at 17 (p<0.01), 22 (p<0.05) and 34 °C (p<0.01). Additionally, in the Cu+Cd exposure in this scenario, the measured K_{rel} was significantly higher at 25 °C than at 17, 22 and 32 °C (p<0.05). By analysing the K_{rel} values of the metal exposures at

different temperatures, several differences were found in relative condition of Cd exposed fish and Cu and Cu+Cd exposures, as indicated in Fig. 3D.

3.2. Whole-body metal burden

The whole body accumulation of Cu and Cd in zebrafish after 10 days of metal exposure under the given scenarios are depicted in Table 3 and Table 4 respectively. The values were statistically analysed (Kruskal-Wallis test, Dunn's multiple comparison test) among different temperatures within a specific exposure and among different exposures within a specific temperature. The results indicated that in most, although not all, scenarios zebrafish accumulated more metals at 32℃ and 34℃ in comparison to the other 4 experimental temperatures. Statistically, in the Acute 12 hours scenario, in Cu single exposure the Cu accumulation level was significantly higher at 32℃ than at 17℃, 22℃, 25℃ and 28℃ (p<0.05). In mixture exposures, although the values were higher at 32 and 34 ℃, the effect was not significant (Table 3). Some significant differences was found in Cd levels in this scenario for both Cd single and Cu+Cd exposed fish but no consistent effect of temperature was observed, i.e. there were no significant trends in the Cd body burden as a function of temperature; Table 4. In Acute 96 hours scenario Cu levels were comparable in Cu+Cd exposures and significantly higher at 32 and 34 ℃ compared to 25 ℃ in Cu single exposure (p<0.05; Table 3). Similarly, Cd levels in this scenario were comparable in mixture exposures but in single Cd exposures the measured Cd level was significantly higher at 34 ℃ compared to 17 and 22 ℃ (p<0.01; Table 4). In the Chronic scenario, Cu accumulation in single Cu exposure was significantly higher at 34 ℃ than at 17, 22, 25 and 28 ℃ (p<0.001). In mixture exposure, Cu accumulation was significantly higher at 34 ℃ than 22 and 25 ℃ (p<0.05 and p<0.01 respectively; Table 3). The average whole body burden of Cd in Cd exposed fish in the chronic scenario was significantly higher at 32 and 34 ℃ compared to control temperature of 28 ℃ (p<0.05). Moreover, Cu+Cd exposed fish in this scenario had a significantly lower body burden at 25 ℃ compared to 17 and 22 ℃ (p<0.05; Table 4). Finally, in the thermal Acclimation scenario, both Cu and Cd accumulation increased with increasing temperature with one exception (Cd accumulation at 28℃). The outcomes of statistical analysis for the acclimation scenario showed that, in single Cu exposure, Cu accumulation is significantly higher at 32 and 34 ℃ compared to 22 ℃ (p<0.05). While in mixture exposure, Cu accumulation is significantly higher at 34 ℃ compare to 25 and 28 ℃ (p<0.05). The average whole body burden of Cd in Cd exposed zebrafish was significantly higher at 34 ℃ compared to 17, 25 and 28 ℃ (p<0.001, p<0.05, and p<0.05 respectively; Table 4). Comparison among different exposures within a specific temperature demonstrated that zebrafish accumulated more copper and cadmium in the thermal Acclimation scenario (particularly in warm temperatures *i.e.* 32℃ and 34℃) followed by the Chronic and Acute scenarios in Cu accumulation levels (Table 3) and Acute and Chronic scenarios in Cd accumulation levels (Table 4). The whole body burden of Cu and Cd in dead fish exposed to Cu+Cd in Acute thermal, Chronic thermal and thermal Acclimation scenarios is presented in Table S1 (Cu) and Table S2 (Cd). Additionally, the metal (Cu and Cd) accumulation *rate* of

surviving and dead fish is presented in Fig. 4 and S2. These results show that for dead fish (i) the whole body metal content generally increased with increasing lifetime, (ii) for a given treatment, the whole body burden (for both Cu and Cd) never exceeded the average whole body burden of the fish which survived the 10 days of metal exposure, and (iii) the Cu and Cd accumulation rate was generally comparable with that for surviving fish (Figs. 4 and S2).

Table 3. Whole-body copper concentration of live zebrafish after 10 days of metal exposure in Acute 12 hours, Acute 96 hours, Chronic and Acclimation scenarios (data for surviving fish). Values are means (given as µmol/g d.w.) ± standard deviation; sample size, *n*, values are given in parentheses. Statistically different means among exposures within specific temperature and among temperatures within specific exposure are denoted with different upper- and lower-case letters respectively ($p<0.05$) (values with no joint letters are significantly different).

Scenario		Exposure \ Temperature	17 °C	22 °C	25 °C	28 °C [(i)]	32 °C	34 °C
Acute	12 hours	*Cu*	$0.19 \pm 0.03^{A, a}$ (9)	$0.20 \pm 0.04^{A, a}$ (10)	$0.21 \pm 0.04^{A.B, a}$ (9)	$0.21 \pm 0.05^{A, a}$ (9)	$0.30 \pm 0.05^{A.B, b}$ (9)	$00.26 \pm 0.06^{A, a.b}$ (9)
		Cu+ Cd	$0.19 \pm 0.03^{A, a.b}$ (9)	$0.16 \pm 0.03^{A, b}$ (8)	$0.18 \pm 0.03^{A.B, a.b}$ (7)	$0.25 \pm 0.03^{A.B, a}$ (7)	$0.25 \pm 0.05^{A, a}$ (8)	$0.29 \pm 0.06^{A.B, a}$ (6)
	96 hours	*Cu*	$0.19 \pm 0.03^{A, a.b}$ (8)	$0.18 \pm 0.06^{A, a.b}$ (9)	$0.15 \pm 0.03^{B, b}$ (9)	$0.21 \pm 0.05^{A, a.b}$ (9)	$0.29 \pm 0.12^{A.B, a}$ (8)	$0.29 \pm 0.09^{A.B, a}$ (9)
		Cu+ Cd	$0.19 \pm 0.07^{A, a}$ (8)	$0.17 \pm 0.04^{A, a}$ (7)	$0.23 \pm 0.06^{A.B, a}$ (6)	$0.19 \pm 0.05^{A, a}$ (7)	$0.23 \pm 0.05^{A, a}$ (3)	$0.24 \pm 0.005^{A, a}$ (2)
Chronic		*Cu*	$0.22 \pm 0.04^{A, a}$ (9)	$0.23 \pm 0.05^{A, a}$ (10)	$0.23 \pm 0.03^{A.B, a}$ (9)	$0.23 \pm 0.03^{A.B, a}$ (9)	$0.28 \pm 0.05^{A.B, a.b}$ (8)	$0.33 \pm 0.04^{A.B, b}$ (9)
		Cu+ Cd	$0.21 \pm 0.01^{A, a.b}$ (6)	$0.20 \pm 0.03^{A, a}$ (9)	$0.18 \pm 0.03^{A.B, a}$ (9)	$0.21 \pm 0.03^{A, a.b}$ (8)	$0.24 \pm 0.04^{A, a.b}$ (8)	$0.27 \pm 0.02^{A, b}$ (7)
Acclimation		*Cu*	NA[(ii)]	$0.15 \pm 0.04^{A, a}$ (4)	$0.28 \pm 0.05^{A, a.b}$ (4)	$0.31 \pm 0.05^{B, a.b}$ (8)	$0.37 \pm 0.08^{B, b}$ (10)	$0.43 \pm 0.08^{B, b}$ (7)
		Cu+ Cd	NA[(ii)]	NA[(ii)]	$0.23 \pm 0.009^{A.B, a}$ (2)	$0.27 \pm 0.05^{A.B, a}$ (7)	$0.32 \pm 0.08^{A.B, a.b}$ (6)	$0.41 \pm 0.08^{B, b}$ (5)

[(i)] The average whole-body copper concentration for unexposed control fish (at 28°C) was: 0.077± 0.01 (µmol/g d.w. ± standard deviation).

[(ii)] Not applicable; no fish survived the exposure conditions or the statistical analysis is not applicable.

Table 4. Whole-body cadmium concentration of live zebrafish after 10 days of metal exposure in Acute 12 hours, Acute 96 hours, Chronic and Acclimation scenarios (data for surviving fish). Values are means (given as µmol/g d.w.) ± standard deviation; sample size, *n*, values are given in parentheses. Statistically different means among exposures within specific temperature and among temperatures within specific exposure are denoted with different upper and lower case letters respectively ($p<0.05$) (values with no joint letters are significantly different).

Scenario		Exposure \ Temperature	17 °C	22 °C	25 °C	28 °C [(i)]	32 °C	34 °C
Acute	12 hours	*Cd*	$0.026 \pm 0.013^{A.B,\ a.b.c}$ (10)	$0.019 \pm 0.005^{A.B.C,\ a.c}$ (10)	$0.018 \pm 0.005^{A,\ a}$ (10)	$0.033 \pm 0.009^{A.B,\ a.b.c}$ (10)	$0.041 \pm 0.012^{A.B,\ b}$ (10)	$0.036 \pm 0.013^{A.B,\ b.c}$ (10)
		Cu+ Cd	$0.019 \pm 0.011^{A,\ a.b}$ (9)	$0.01 \pm 0.002^{A,\ b}$ (8)	$0.02 \pm 0.008^{A.B,\ a.b}$ (7)	$0.025 \pm 0.006^{A,\ a}$ (7)	$0.019 \pm 0.007^{B,\ a.b}$ (8)	$0.032 \pm 0.011^{A.B,\ a}$ (6)
	96 hours	*Cd*	$0.024 \pm 0.007^{A.B,\ a}$ (10)	$0.027 \pm 0.014^{A.B.C.D,\ a}$ (9)	$0.029 \pm 0.008^{A.B,\ a.b}$ (10)	$0.30 \pm 0.006^{A.B,\ a.b}$ (10)	$0.047 \pm 0.022^{A.B,\ a.b}$ (10)	$0.058 \pm 0.009^{A.C,\ b}$ (10)
		Cu+ Cd	$0.014 \pm 0.005^{A,\ a}$ (8)	$0.009 \pm 0.005^{A,\ a}$ (7)	$0.017 \pm 0.009^{A,\ a}$ (6)	$0.025 \pm 0.005^{A.B,\ a}$ (7)	$0.031 \pm 0.006^{A.B,\ a}$ (3)	$0.04 \pm 0.01^{A.B.C,\ a}$ (2)
Chronic		*Cd*	$0.035 \pm 0.007^{B,\ a.b}$ (10)	$0.033 \pm 0.009^{B.D,\ a.b}$ (10)	$0.031 \pm 0.009^{A.B,\ a.b}$ (10)	$0.019 \pm 0.01^{A,\ b}$ (10)	$0.042 \pm 0.011^{A.B,\ a}$ (10)	$0.044 \pm 0.006^{A.B.C,\ a}$ (10)
		Cu+ Cd	$0.031 \pm 0.009^{A.B,\ a}$ (6)	$0.029 \pm 0.011^{B.C.D,\ a}$ (9)	$0.015 \pm 0.006^{A,\ b}$ (9)	$0.021 \pm 0.007^{A,\ a.b}$ (8)	$0.025 \pm 0.006^{A.B,\ a.b}$ (8)	$0.028 \pm 0.007^{B,\ a.b}$ (7)
Acclimation		*Cd*	$0.027 \pm 0.009^{A.B,\ a}$ (10)	$0.046 \pm 0.015^{D,\ a.b.c}$ (10)	$0.04 \pm 0.014^{B,\ a.b}$ (10)	$0.042 \pm 0.009^{B,\ a.b}$ (10)	$0.05 \pm 0.008^{A,\ b.c}$ (10)	$0.074 \pm 0.026^{C,\ c}$ (10)
		Cu+ Cd	NA[(ii)]	NA[(ii)]	$0.022 \pm 0.004^{A.B,\ a}$ (2)	$0.021 \pm 0.003^{A,\ a}$ (7)	$0.05 \pm 0.014^{A,\ a.b}$ (6)	$0.08 \pm 0.035^{C,\ b}$ (5)

[(i)] The whole-body cadmium concentration for unexposed control fish (at 28°C) was under the quantification limits (2.22×10^{-5} µmol/g d.w.) of ICP-MS.

[(ii)] Not applicable; no fish survived the exposure conditions or statistical analysis is not applicable.

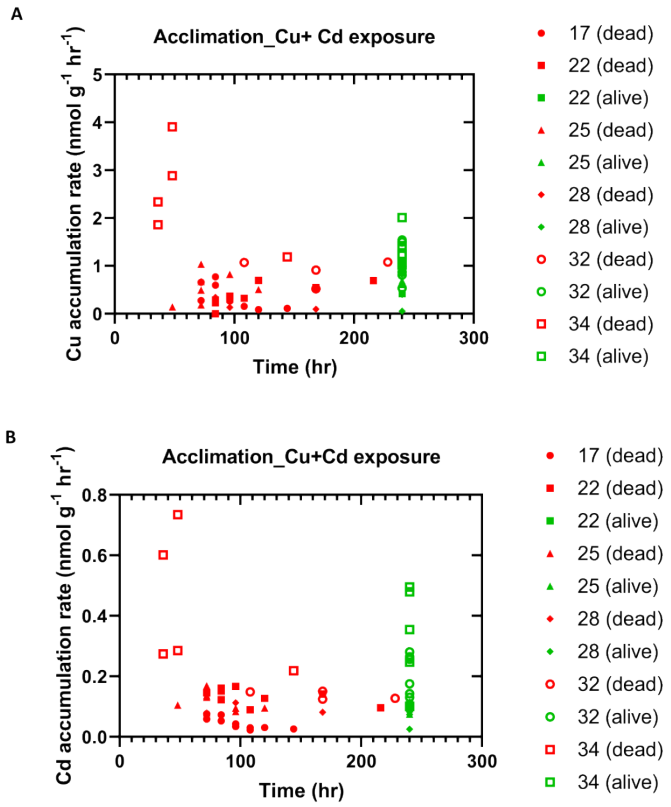

Fig. 4. Whole body Cu (**A**) and Cd (**B**) accumulation rate of dead and alive fish exposed to Cu+Cd in thermal Acclimation scenario. The accumulation rate is expressed as nmol per g d.w. per hr of metal exposure; the time on the *x*-axis corresponds to the time of death for dead fish, and 10 days for the surviving fish. The accumulation rate of copper was calculated after subtraction of the average whole body Cu concentration of unexposed control fish (0.077 µmol/g d.w.) from the initial value.

3.3. Electrolyte content

The whole body Na^+ content of the surviving and dead fish is presented in Fig. 5. While results showed a significant (p<0.05, t-test) drop in whole-body sodium concentration of dead fish in comparison to surviving fish, such an effect was not detected for the other major cations under the experimental conditions (Fig. S3). The results of complementary experiment (1) showed that although the whole-body Na level decreases somewhat over 12 hours (being the maximum time a dead fish in the exposure experiment stayed in the water), the effect is negligible (<10% loss compared to the whole-body Na content determined immediately after death) (Fig. S4).). The results of complementary experiment (2) demonstrated a significant reduction of whole-body Na content over the Cu+Cd exposure in comparison to the control group of that experiment (Fig. S5). Comparison

between the whole body electrolyte levels of zebrafish among different temperatures in each treatment did not indicate any significant trend in the effect of thermal conditions on electrolyte levels (Mann-Whitney test).

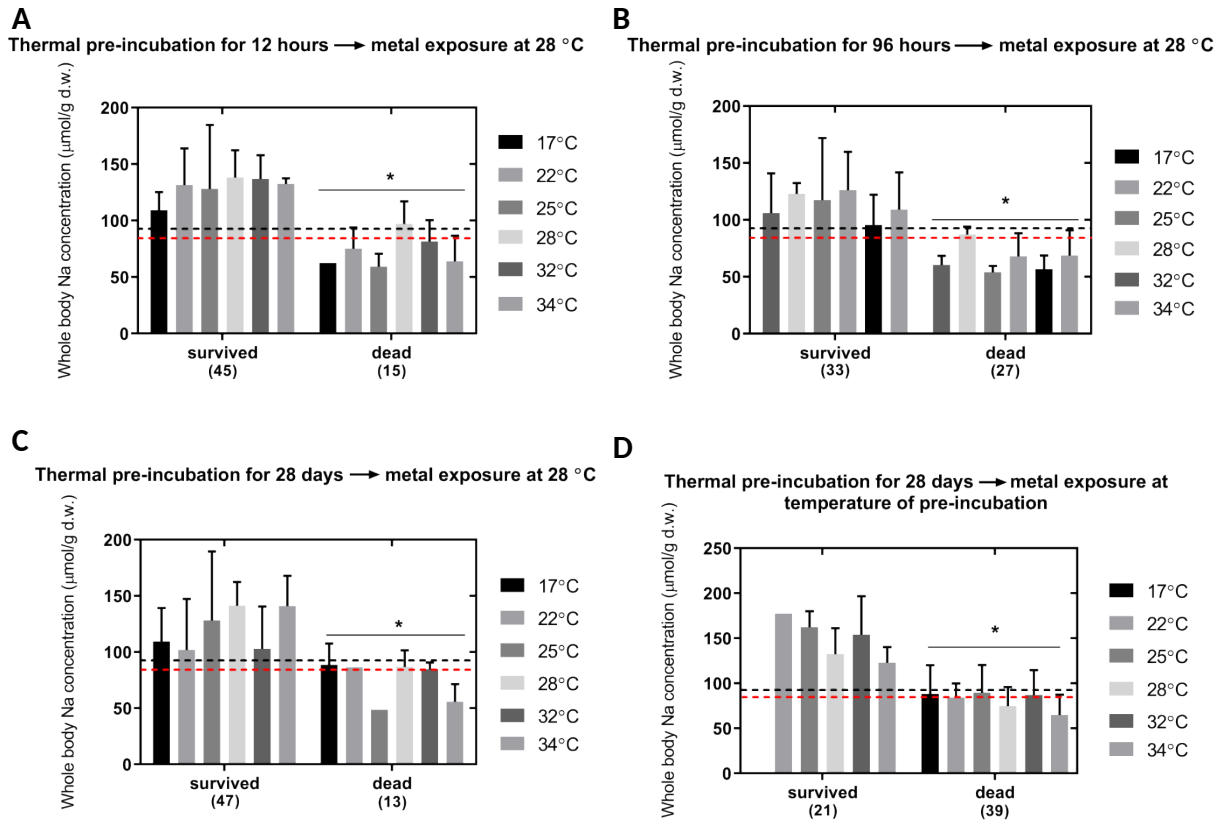

Fig. 5. Whole-body sodium concentration of zebrafish after 10 days of metal exposure (Cu+Cd) for the various thermal scenarios: (**A**) acute 12 hr thermal pre-incubation, (**B**) acute 96 hr thermal pre-incubation, (**C**) chronic 28 days thermal pre-incubation and (**D**) thermal acclimation, given as (µmol/g d.m.). All surviving fish in each scenario were considered to belong to the same group (irrespective of thermal treatment), the same approach was applied for dead fish and the whole body Na^+ concentration of these two groups was compared using t-test. Data for surviving and dead fish± SD; sample size, *n*, is given in parentheses in the x axis labels. *Denotes significant difference between the two compared groups, $p<0.05$. . Black and red dotted lines indicate the whole body Na^+ concentration measured at 0h and 12h following death respectively (complementary experiment 1).

4. Discussion

The assessment of metal toxicity in fish is often complicated by the concomitant complex effects of exposure water characteristics. Over the past years, the majority of the ecotoxicity studies have been conducted on the toxic effects of single or multiple metal exposures at a constant standard temperature. In the present study, we investigated the influence of altered thermal background (acute thermal pre-incubation and chronic thermal pre-incubation scenario) as well as the thermal background and environmental temperature interaction (thermal acclimation scenario) on metal toxicity tolerance in zebrafish using Cu and Cd as metal contaminants. To have a better understanding in an ecotoxicological point

of view we will discuss the metal toxicity first separately and independently of temperature and then we will examine their interaction.

4.1. Metal toxicity

Copper is one of the essential micronutrients contributing in a wide range of physiological processes in the body including formation of many enzymes and glycoproteins, cellular respiration, function of nervous system, erythropoiesis and melanin synthesis. Despite its vital role in biology, in levels just above the threshold of metabolic requirements, it could become toxic for the fish due to its high redox potential (Zhao et al., 2014). Copper compounds are widely used in industry and agriculture resulting in elevated Cu concentrations in the environment. Ionic copper (Cu^{2+}) is generally considered to be the main toxic form of Cu. Several pathways have been proposed for uptake of Cu including the high-affinity Cu transporter 1 (CTR1) and the low-affinity Cu transporter 2 (CTR2; mediates the release of Cu from intracellular vesicles, but is also expressed in low levels in the plasma membrane), apical Na^+ channel, divalent metal transporter (DMT1) and epithelial calcium channels (ECaC) (Alsop and Wood, 2011; Grosell, 2011; Grosell and Wood, 2002; Griffith, 2017; Zhao et al., 2014). In terms of osmoregulation, the main effect of Cu in the gills is impairment of the branchial Na^+ influx, mainly through effects on Na^+- K^+- ATPase (Lauren and McDonald, 1987, Playle et al., 1993). Cadmium, on the other hand, is a non-essential toxic element and therefore there is no Cd-specific transporter or pathway recognized in the cell membrane and Cd uptake generally occurs via replacing the essential cations in a competitive manner. It has been shown that at ecologically relevant exposure concentrations (10 nM or about 1 µg/L) the primary acute effect of Cd^{2+} is disruption of ion homeostasis, particularly Ca^{2+} (McGeer et al., 2011). The epithelial calcium channels (ECaC) and DMT1 have been proposed for Cd^{2+} uptake in fish gills (Verbost et al. 1987; Niyogi and Wood, 2003; Cooper et al., 2006). Moreover, it has been suggested that Zn transporting proteins (ZIPs) which are mostly known for intestinal uptake of Cd^{2+} (Klinck and Wood, 2011; Kwong and Niyogi, 2012) may also contribute in branchial uptake (specifically ZIP8) of Cd^{2+} (Komjarova and Bury, 2014).

In order to investigate the effect of metal toxicity irrespective of temperature, we consider the 28 °C treatment in all thermal scenarios as the reference for discussion. In our study, zebrafish showed almost no sensitivity towards the single Cd 0.2 µM exposure. This was expected due to our use of a relatively low Cd concentration (< 1% of the reported LC50 for Cd in adult zebrafish) (Alsop and Wood, 2011). The 1.2 µM Cu exposure caused some toxicity in zebrafish although the Cu induced mortality never reached 50 % of the population at the control temperature of 28 °C (Fig. 2). In general, Cd is found to be less toxic in cyprinids, such as zebrafish and carp, than salmonids (e.g. trout and salmon), while Cu is known be comparably toxic for both families (Alsop and Wood, 2011). Although the toxicity mechanisms of Cu and Cd in single metal ion exposures are well characterized, detailed knowledge of the mechanisms underlying the relatively high Cd tolerance of cyprinids compared to salmonids is scare. However, it may have a link to the differences in the

osmoregulation and ion handling mechanisms, leading to higher efficiency of cyprinids to maintain ion balance under Cd exposure. For example, a 6.4 µg/L (0.05 µM) chronic (up to 178 days) exposure to Cd was found to transitionally decrease the plasma Na^+, K^+, and Ca^{2+} in rainbow trout (*Salmo gairdneri*) (Giles, 1984). Similarly, chronic (up to 92 days) exposures to 0.87 µg/L (0.007 µM) and 8 µg/L (0.07 µM) of Cd was reported to significantly inhibit net Ca^{2+} and K^+ uptake respectively in Atlantic salmon (Salmo salar) alevins (Rombough and Garside, 1984). On the other hand, a 480 µg/L (4.27 µM) Cd exposure was needed to induce hypocalcemia in common carp (*Cyprinus carpio*) and 9.4 µg/L (0.08 µM) and 105 µg/L (0.93 µM) Cd exposures had no significant effect on plasma Ca^{2+} levels (Reynders et al., 2006). In another assay performed on adult zebrafish, 48 h exposure to 400 µg Cd/L (3.55 µM) had no significant effect on whole-body Na^+ and Ca^{2+} levels, whilst exposure to 4000 µg/L Cd (35.58 µM) significantly decreased the whole-body Na^+ levels with again no remarkable effect on Ca^{2+} levels (Alsop and Wood, 2011).

The results obtained in our study showed a synergistic effect of Cu+Cd in mixture exposures (Fig. 2, Fig. S1). Although information on the toxicity of Cu and Cd mixtures to adult zebrafish is limited, considering that many metals have common toxic mechanisms, *e.g.* inducing oxidative stress, additive or synergistic interactions are likely to occur. In a study on zebrafish (*Danio rerio*), Komjarova and Bury (2014) exposed adult zebrafish to 0.003 µM and 0.025 µM of Cd and 0.05 µM and 0.5 µM of Cu in single and mixture treatments for 48 hours. The authors measured metal accumulation in the gills and proposed common Cd and Cu uptake routes based on transcript levels of genes involved in Cu transport and accumulation of Cu and Cd in the gills. Another study on rainbow trout (*Oncorhynchus mykiss*) proposed that the biochemical interactions of Cu and Cd in the fish gill may lead to additive or more than additive toxicity in fish during acute waterborne exposure (Saibu et al., 2018). The possible mechanism of this additive effect associated with the endpoints of whole-body metal accumulation and electrolyte content is discussed below.

4.2. Effect of temperature on metal toxicity

In the Acute thermal scenario we aimed to elucidate the effect of an abrupt change in water temperature as a primary stressor on the zebrafish's sensitivity to a subsequent stressor, *i.e.* metal exposure. Many studies have addressed the question of whether a pre-exposure to a sub-lethal stressor can enhance the organism's stamina against a subsequent stressor of the same kind or of another type. For example, pre-exposure of zebrafish larvae to mild hypoxia, significantly improved their tolerance towards a secondary lethal hypoxia and lethal cold exposure (Long et al., 2015). Also, prior exposure to a low-level of ammonia was found to increase the survival time of common carp (*Cyprinus carpio*) during an extreme ammonia challenge (Shrivastava et al., 2016) and exposing least killifish (*Heterandria formosa*) to a low-level of Cu was reported to increase their survival time during a subsequent lethal Cu exposure in comparison to the control group (Adeyemi and Klerks, 2013).

For pre-exposures at cold temperatures, the results of the present study are in agreement with these literature reports. The Acute thermal scenario demonstrated that pre-exposing the zebrafish to cold temperatures (17 ℃, 22 ℃ and partially 25 ℃) for both 12 and 96 hr increased their tolerance to subsequent exposure to Cu+Cd mixture at 28 ℃. This protective effect can be linked to the stress-response mechanisms at two levels of biological organisation. Firstly, at the organismal level, the fish will undergo a series of biochemical and physiological changes in order to cope with the stress, these changes are mainly manifest by the rapid release of stress hormones, adrenaline (in short term) and cortisol (in longer term), resulting in alterations in blood chemistry and increase in blood glucose (Mommsen et al., 1999; Barton, 2002). Stress is an energy-demanding process, thus the high blood level of glucose will enhance the tolerance to the subsequent stressor (metal exposure). Secondly, at the cellular level, the stress response is mainly characterized by heat shock proteins (HSPs). HSPs are a group of highly conserved cellular proteins that are synthesized by cells under stressed conditions. The possible functions of HSPs on various aspects of fish physiology have been the subject of detailed studies (Iwama et al., 1998; Basu et al., 2002). Although the vital roles that HSPs play in the cell including the maintenance of protein integrity, preventing premature folding and aggregation of proteins, protein translocation, and mediating steroid and receptor binding have been clearly shown, knowledge about the mechanism underlying the relationships between the cellular stress response and the physiology of fishes under the stress conditions is limited (Iwama et al., 1999).

In contrast to cold temperatures, toxicity in mixture exposures increased with increasing temperature in the Acute thermal scenario and the highest toxicity was observed at 34 ℃ for both 12 and 96 hr of thermal challenge (Fig. 2 A, B, Fig. S1 A, B). That is, in the acute thermal scenarios, any protective effect of the prior thermal stress is outweighed by an increase in metal toxicity that follows the elevated pre-incubation temperatures. It is known that at higher temperatures, the potential increase of the metabolic rate may drive greater metal accumulation, thus leading to potentially greater toxicity (Sokolova and Lannig, 2008). Therefore, it is likely that the higher metabolic rate induced at higher pre-incubation temperature may carry over into the exposure period, thereby causing greater metal accumulation and toxicity. Maintaining aerobic metabolism capacity is thought to be one of the key processes in coping with thermal stress. By definition, lower and upper pejus temperatures represent the limits of the optimum hemolymph oxygenation and therefore, temperatures below or above these will disrupt the oxygen supply to the tissues (Portner and Peck, 2010). According to the previous evidence pejus limits may shift over time when the organism has the capacity to acclimate to the temperature change (Lucassen et al., 2006). Moreover, it has been established that rapid alterations of environmental temperatures (Acute thermal scenario) result in an "overshoot" in metabolism, while keeping the organism at the new temperature for a longer period (Chronic thermal scenario) can lead to an eventual stabilization of the metabolism. Therefore, acclimation to

a change in temperature tends to modulate the direct effect of this environmental variable on the ectothermic organism (Cairns et al., 1975). Accordingly, the results of the present study for the Chronic thermal scenario demonstrated that thermal adaptation of the zebrafish will reduce the effect of temperature fluctuations on metal toxicity (Fig. 2C, Fig. S1 C).

In the thermal Acclimation scenario we investigated the interactive effect of the exposure temperature and the metal toxicity. The survival curves indicated an optimum response pattern in temperature-toxicity relationship (Fig. 2D, Fig. S1D), that is, the metal toxicity is minimal at the species specific optimum temperature and toxicity increases in temperatures above or below this optimum temperature (Sokolova and Lannig, 2008). This trend partially occurred in the Cu single exposure in the thermal acclimation scenario as well, whilst in the other scenarios the effect of temperature on Cu toxicity in Cu single exposures was negligible (Fig. 2, Fig. S1). Numerous studies have reported the increased metal toxicity at elevated environmental temperatures due to the increased metabolic rates and concomitant increased energy demand. The increased energy demand results in elevated ventilation, and in turn, higher exposure to metal contamination which can lead to increased internal toxicant concentrations (Cairns et al., 1975; Heugens et al., 2001; Sokolova and Lannig, 2008; Teodorof et al., 2009). Furthermore, the decreased solubility of oxygen at elevated temperatures (Benson and Krause, 1980), coupled with decreased oxygen uptake efficiency due to the harmful effect of metal contamination on respiratory surfaces (Nonnotte et al., 1993) can exacerbate this effect.

On the other hand, the number of studies reporting an increase in metal toxicity by decreasing temperature is limited. In their study on zebrafish embryos, Hallare et al. (2005) reported higher Cd toxicity, decreased average heart rate and embryo hatchability in larvae reared at low temperature (21 ℃) in comparison to the control (26 ℃) and high (33 ℃) temperatures. Based on their findings from heat shock protein (hsp) induction, the authors proposed that this effect could be explained by the significantly higher expression of hsp (hsp70) in embryos at 26 ℃ and 33 ℃ compared to 21 ℃ (Hallare et al., 2005). In another report on the interactive effect of altered water temperature and water born Cd toxicity, it was demonstrated that the hepatic enzymes activities in Nile tilapia (*Oreochromis niloticus)* reared at 24-28 ℃ were optimized compared to those reared at 20 ℃ or 32 ℃. Moreover, it was shown in the same study that the haematological variables (red blood cell count, haematocrit value and haemoglobin levels) increased significantly with increasing water temperature from the lowest values at 20℃ up to 28 ℃, after which they decreased significantly at 32 ℃ in Cd-exposed fish (Abdel-Tawwab and Wafeek, 2017). In another study on *Daphnia magna*, chronic metal toxicity was found to be higher at 15 ℃, than at 25 ℃ or 20 ℃ (which is the temperature that is used in standard chronic toxicity tests). This observation was linked to the possible effect of temperature acclimation (physiological adjustment) and also the processes of detoxification and elimination which substantially increase with temperature (Pereira et al., 2017). Although the aforementioned observations

can partially explain the increased metal toxicity at reduced temperatures, there are more factors which may contribute to this effect, such as the reduced expression of metal-binding proteins at lower temperatures (Abdel-Tawwab and Wafeek, 2014) *e.g.* metallothioneins which play important and sometimes dominant roles in reducing metal toxicity (Roch and McCarter, 1984a; Roch and McCarter, 1984b) and the temperature dependent suppression of the immune system which is known to be one of the target organs of metal toxicity in fish (Zelikoff, 1993). Additionally, it is well documented that both lower and higher temperatures than the species specific standard temperature can induce oxidative stress in fish (Heise et al., 2006; Kammer et al., 2011; Fadhlaoui and Couture, 2016; Cheng et al., 2018). This effect, combined with the oxidative stress due to metal exposure can increase the overall toxicity and is in agreement with the results of present study in the thermal acclimation scenario wherein the mortality during metal exposures increased when the temperature diverged from the optimal thermal range for zebrafish (Fig.2D, Fig.S1).

4.3. Relative body condition factor alterations

Condition indices are widely used as morphological and physiological metrics to assess the health or quality of individual animals and are assumed to be related to fitness (Peig and Green, 2009). The starvation conditions during the 10 days of metal exposure will impact body condition. Therefore, the interpretation of body condition results herein is mainly based on the influence of applied thermo-metal treatments on the handling of energy reserves by zebrafish and its effect on K_{rel}. In the present study, the relative body condition, K_{rel}, of zebrafish in the Acute thermal scenario was not significantly affected by the experimental conditions (Fig. 3 A,B), suggesting that the 12 or 96 hr thermal challenge was too short to achieve a clear thermal and/or thermometal effect on body condition. In the Chronic thermal scenario, the K_{rel} was lower in warm temperatures (32 ℃ and 34 ℃) in comparison to the cold temperatures (17 ℃ and 22 ℃) in all of the 3 metal exposure scenarios (Fig. 3C). Several studies have reported a negative correlation between the ambient temperature and the condition indices due to the increased metabolic rates and decreased energy reserves (Bouchard and Guderley, 2003; Sappal et al., 2015a; Sappal et al., 2015b). The body condition of zebrafish in the thermal Acclimation scenario increased with increasing temperature in all 3 metal exposures up to intermediate temperatures (25 ℃ and 28 ℃), after which the values decrease with increasing temperature (Fig. 3D). Also, the K_{rel} of Cu+Cd exposed zebrafish in the Chronic thermal scenario and Cu and Cu+Cd exposed fish in the thermal Acclimation scenario was negatively affected by the metal exposure irrespective of temperature (Fig. 3 C,D). This is in accordance with the toxicity results of the present study which suggest that the increased toxicity and stress can potentiate the energy demand and in turn, increase the use of the energy stores. This effect, accompanied by the interrupted homeostatic and physiological functions due to multiple stress conditions can lead to decreased body conditions in fish.

4.4. Dynamics of Cu and Cd accumulation

In order to investigate the effect of metal contaminants on fish, it is important to consider their mechanisms of uptake and accumulation. The relationship between metal accumulation and toxicity endpoints is widely assessed in toxicology studies (Hogstrand et al., 1996; Muscatello et al., 2006; Tsai and Liao, 2006; Gobi et al., 2018). In the present study, the applied thermal scenarios significantly affected the whole-body burden of Cu and Cd in single as well as the binary exposures (Tables 3, 4). Overall, as illustrated in Fig. 6 the whole-body burdens increased at elevated temperatures. Higher metal accumulation at warmer temperatures has already been reported by many studies and has been assumed to be explained by increased metabolic rates at higher water temperatures (see section 4.2 for discussion). Moreover, for the treatments in which some fish survived the exposures, the highest metal body burdens in surviving fish were measured in the thermal Acclimation scenario. This observation highlights the more determinative impact of ambient temperature on metal accumulation as compared to an altered history of thermal conditions. In addition, during the 10 day metal exposure, elimination kinetics may also play an important role in determining the metal body burden. As the exposure time increases, the elimination rate is expected to increase until steady-state is reached. Although there is a lack of clear evidence about the effect of temperature on the elimination rate, generally, temperature is reported to have little or no influence on the elimination rate of many metals, especially, of non-essential metals in aquatic animals (Mubiana and Blust, 2007; Yang and Chen, 1996).

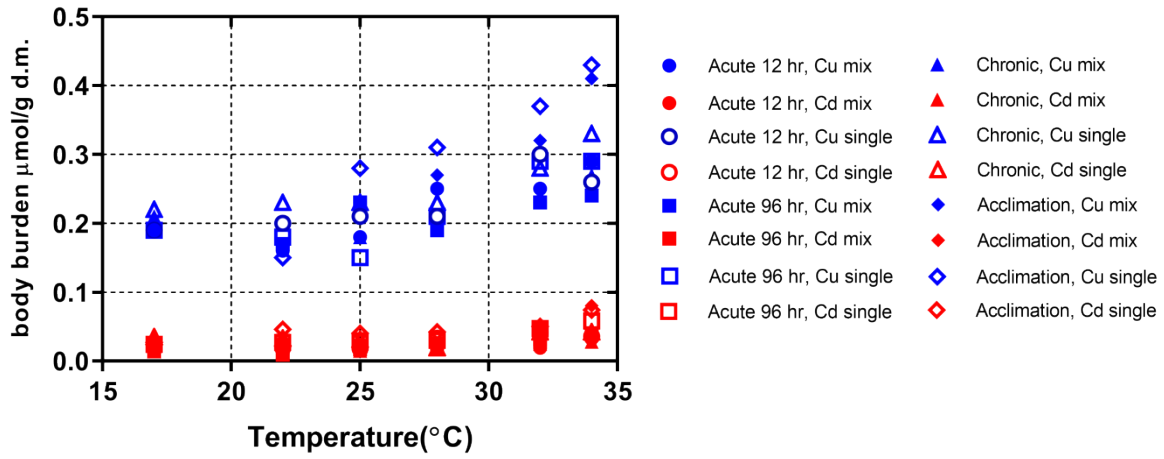

Fig. 6. Average whole-body metal burden of zebrafish after 10 days of metal exposure (data for surviving fish). The blue symbols correspond to the Cu data, and red symbols correspond to Cd; see legend for details of the exposure scenarios.

A comparison of Cu and Cd levels among single and mixture exposures reveals that the whole-body burden of Cd decreased in the presence of Cu in binary exposures while the body burden of Cu was hardly changed. The ratio of whole-body burdens of Cu and Cd in

single versus mixture exposures is illustrated in Fig. 7. There are several studies that report a reduction in body burden of Cd co-exposed with Cu in fish (Pelgrom et al., 1994; Komjarova and Blust, 2009; Komjarova and Bury, 2014). This effect could be due to Cu outcompeting Cd for the ECaC and DMT1 channels, which are known to be upregulated in the Cu+Cd mixture exposure in adult zebrafish (Komjarova and Bury, 2014) (see section 4.1 for relevant transporters of Cu and Cd), coupled with a Cu induced decrease in expression of ZIP8 transporters which are assumed to be involved in Cd uptake (Komjarova and Bury, 2014). At the cellular level, excess cytosolic Cu is mainly exported via ATP7A and ATP7B (Zhao et al., 2014). For Cd excretion there is no specific pathway, and Cd detoxification happens through metal binding moieties, such as metallothionein (MT) and glutathione (GSH) (Mason and Jenkins, 1995; Chowdhury et al., 2005). MT and GSH are also known to participate in regulation of intracellular Cu (Banci et al., 2010a,b). At the organismal level, Cu excretion in teleosts is mainly through the hepatobiliary system (Grosell et al., 1997, 2001). On the other hand, Cd excretion mainly occurs via mucosal sloughing in the gastrointestinal tract and also in a small proportion via urine, bile and gills (Chowdhury et al., 2004; Szebedinszky et al., 2001; Franklin et al., 2005). Therefore, it seems the elimination routes of these two metals are independent at the organismal level. Generally, Cd is known to have a long biological half-life in vertebrates compared to essential metals (e.g. Cu) which reflects the fact that they do not have any efficient pathways for Cd excretion. In a study on yellow perch (*Perca flavescens*), Kraemer et al. (2005) studied the elimination kinetics of Cu, Cd and Zn over a 75 days period and reported that Cd concentrations decreased most rapidly in the gills and gut (biological half lives were 18 and 37 d, respectively) and longer half-lives were observed in the liver (75 d) and kidney (52 d). Elimination of excess Cu by the liver and gut occurred much more rapidly, with estimated half-lives of Cu being 8 and 4 d, respectively. Accordingly, during the 10 day metal exposures used in the present work, elimination processes may have a more determinative impact on Cu accumulation as compared to Cd. Finally, despite the negligible effect of Cd on the body burden of Cu, and the lower body burden of Cd in the presence of Cu, we observed a greater toxicity in mixture exposures. This may be an indicator of a change in the nature of the overall handling pathway of Cu in the presence of Cd, and vice versa, perhaps explained by relatively more Cu being taken up via ECaC and DMT1 rather than by the apical Na^+ Channel and/or by changes in the intracellular metal speciation. However, further studies are needed to characterize the active pathways in these processes.

In a given treatment, the whole body metal content of the dead fish was always lower than the average whole body concentration of fish that survived the 10 days of metal exposure (Tables S1, S2, 3 and 4). Furthermore, the metal accumulation *rate* of Cu and Cd in dead fish was generally comparable with that of surviving fish in all scenarios (Fig. 4 and Fig. S2). These findings suggest that there is no link between mortality and metal accumulation rate.

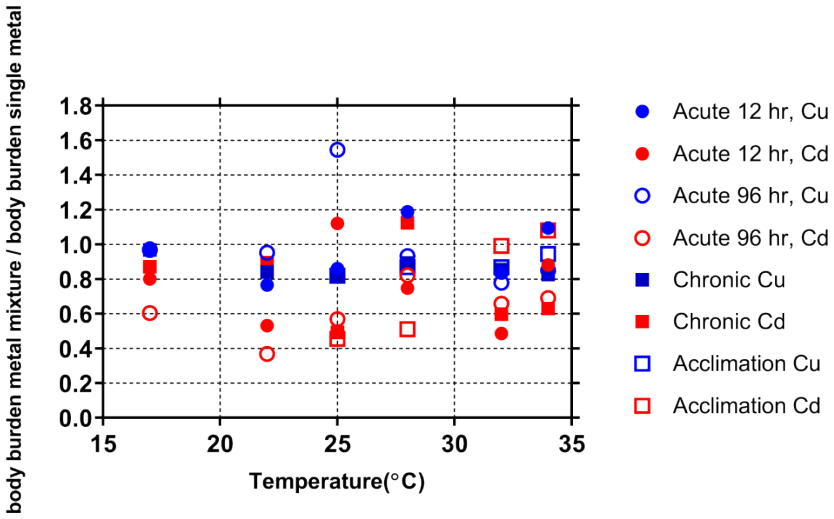

Fig.7. Ratio of whole-body metal burden of zebrafish after 10 days of metal exposure in mixture vs single treatments (data for surviving fish). The blue symbols correspond to the Cu data, and red symbols correspond to Cd; see legend for details of the exposure scenarios.

4.5. Electrolyte balance

In general, ionoregulatory disturbance is known to be one of the fundamental toxic mechanisms of many metals including Cu and Cd. Therefore, the ability to maintain osmoregulatory homeostasis under metal stress is crucial for fish survival. In the present study, we noted a significant drop in the whole-body Na^+ level of dead fish compared to surviving fish (Fig. 5) while the experimental conditions did not significantly affect the body content of the other major cations (K^+, Ca^{2+} and Mg^{2+}; Fig S3). Supplementary experiments verified that this effect is caused by the metal exposure (Fig. S4 and Fig.S5). Figure 5 indicates the portion of passive Na^+ loss post mortem observed in complementary experiment 1 versus the whole body Na^+ levels in surviving and dead fish. This comparison suggests that the passive Na^+ loss after death is negligible relative to the significant decrease in Na^+ levels in dead fish compared to fish which survived the exposures.

Our findings complement the research of Veltman et al. (2014) who developed a model to predict the acute metal toxicity towards freshwater organisms from a generic sodium loss-mortality curve, established based on empirical data for five aquatic species and different exposure durations of two metals (*i.e.*, Cu and Ag), suggesting that mortality is significantly related to sodium loss. Many studies have reported that exposure to Cu reduces Na^+ uptake and increases Na^+ loss at fish gills, with death occurring due to the decreased blood Na^+ levels (Lauren and McDonald, 1986; Wood, 1992; Paquin et al., 2002). However, in their study on adult and larval zebrafish, Alsop and Wood (2011) reported that the decreased whole-body Na^+ levels observed following up to 48 hrs of Cu exposure cannot be explained by the inhibiting effect of Cu on Na^+ uptake. On the other hand, while the main toxic effect of Cd in terms of osmoregulation is assumed to be impairment of Ca^{2+} metabolism. Alsop

and Wood (2011) in the same study noted that although the Cd exposure (up to 48 hrs) reduced the Ca^{2+} uptake, it did not lead to a suppressive impact on whole-body Ca^{2+} levels. Rather, they found a decrease in whole-body Na^{+} levels subsequent to the Cd exposure. Furthermore, Cd has been demonstrated to suppress the ATPase enzyme system due to its high affinity for sulfhydryl groups of these enzymes, Suresh et al. (1995) reported decreased activity of muscle Na^{+}-K^{+}-ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase after short term exposure of common carp (*Cyprinus carpio*) fry and fingerlings to acute or subacute Cd concentrations together with loss of Na^{+}, K^{+} and Ca^{2+}.

Finally, several studies have investigated whether temperature is a determinative factor in osmoregulatory balance. For example, a study on rainbow trout (*Oncorhynchus mykiss*) revealed that an acute thermal challenge at 13 °C and 18 °C significantly increases the water flux rate over the gills compared to the control temperature of 8 °C, but no correlation was found between the net Na^{+} loss rate and the temperature challenge (Onukwufor and Wood, 2018). In another investigation, Fiess et al. (2007) exposed Mozambique tilapia (*Oreochromis mossambicus*) to different water salinities and temperatures, and reported no effect of altered temperature on Na^{+}-K^{+}-ATPase activity. In the present work, we observed no consistent effect of different temperature challenges on the Na^{+} content of surviving or dead fish (Fig. 4). Overall, our findings revealed a strong relationship between mortality and whole-body Na^{+} concentration suggesting that survival, under the applied multi-stress conditions, strongly depends on the capability to maintain Na^{+} homeostasis.

5. Conclusion

Our study highlights the interplay between temperature regimes and the toxicity of metal ions to zebrafish. Both thermal pre-treatments and ambient temperature variations are found to affect zebrafish survival upon exposure to Cu, Cd, and mixtures thereof. Our study also showed that the whole body metal burden is not a predictor of toxicity, i.e. there was no link between body burden and mortality. There was also no relationship found between the metal accumulation rate and mortality. Moreover, following exposure to Cu+Cd mixtures, we observed a significant drop in the whole body Na^{+} level of dead fish as compared to those that survived, irrespective of the thermal regime. This observation suggests that, under the applied multi-stress conditions, survival might be dependent on the capacity of the organism to maintain its Na^{+} balance.

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Supplementary information

The effect of thermal pre-incubation and exposure on sensitivity of zebrafish (*Danio rerio*) to copper and cadmium single and binary exposures

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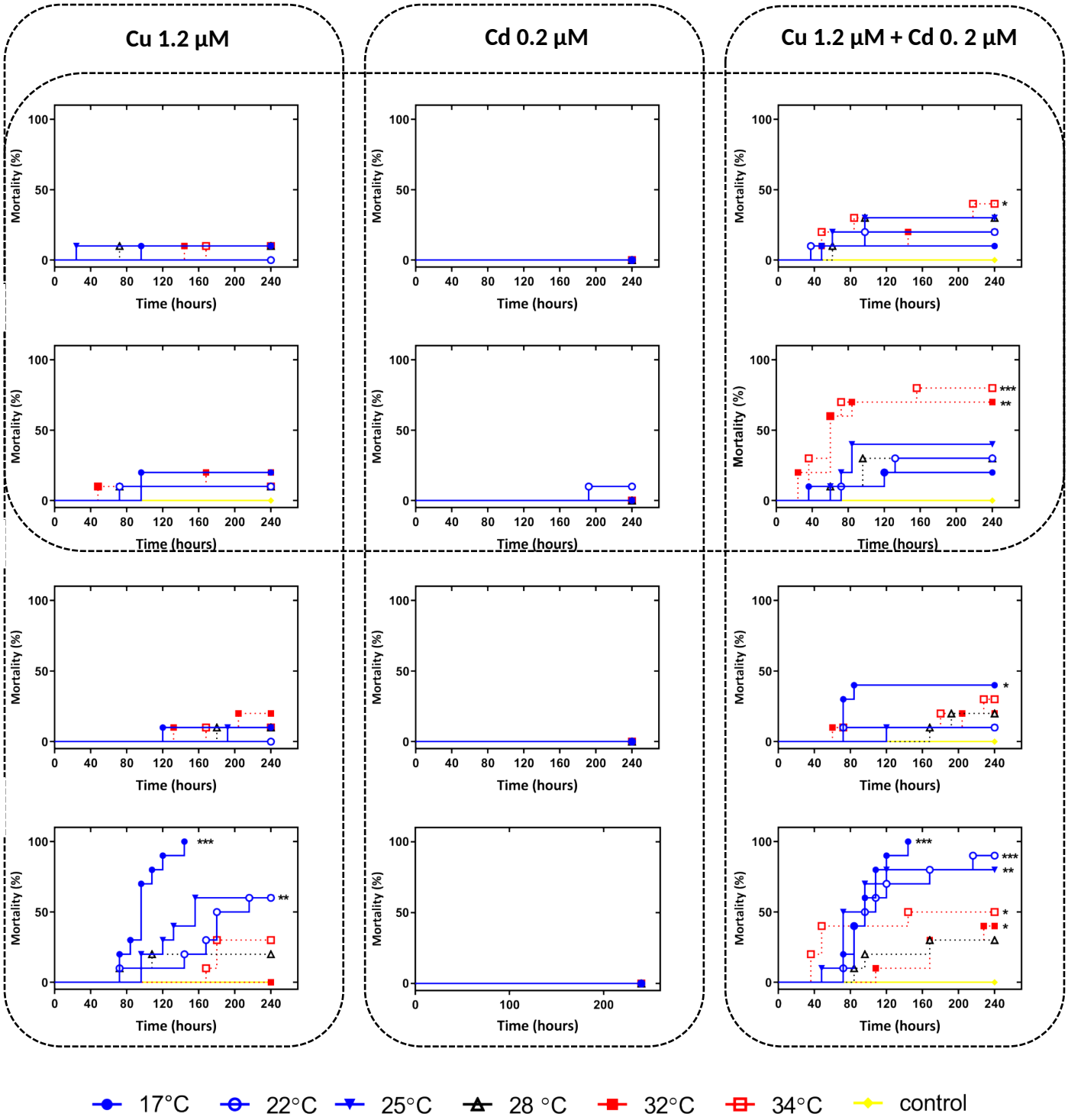


Fig. S1. Percent mortality for zebrafish during a 10 day metal exposure following several thermal regimes: (**A**) Acute 12 hr thermal pre-incubation (**B**) Acute 96 hr thermal pre-incubation (**C**) Chronic 28 days thermal pre-incubation and (**D**) thermal acclimation scenarios. * Indicates significant difference between treatment and control group of the same scenario (*, $p<0.05$; **, $p< 0.01$; ***, $p<0.001$).

Table S1. Whole body copper concentration of the dead zebrafish during the Cu+Cd exposure in Acute 12 hours, Acute 96 hours, Chronic and Acclimation scenarios. Data are given as µmol/g d.w. for each individual fish and the numbers in brackets are the time of death in hours.

Scenario		Temperature	Whole body Cu concentration µmol/g d.w. (time to death/hr)[i]
Acute	12 hours	17 °C	0.07 (48)
		22 °C	0.1 (36), 0.1 (96)
		25 °C	0.15 (48), 0.13 (60), 0.09 (96)
		28 °C	0.14 (60), 0.16 (96), 0.17 (96)
		32 °C	0.13 (48), 0.15 (114)
		34 °C	0.16 (48), 0.16 (48), 0.23 (84), 0.27 (216)
	96 hours	17 °C	0.12 (36), 0.15 (120)
		22 °C	0.12 (72), 0.13 (120), 0.13 (132)
		25 °C	0.13 (60), 0.13 (72), 0.12 (84), 0.11 (84)
		28 °C	0.13 (60), 0.15 (96), 0.16 (96)
		32 °C	0.12 (24), 0.14 (24), 0.10 (60), 0.15 (60), 0.13 (60), 0.14 (60), 0.13 (84)
		34 °C	0.11 (36), 0.12 (36), 0.09 (36), 0.11 (60), 0.14 (60), 0.11 (60), 0.15 (72), 0.19 (156)
Chronic		17 °C	0.12 (72), 0.11 (72), 0.13 (72), 0.15 (84)
		22 °C	0.1 (72)
		25 °C	0.11 (120)
		28 °C	0.15 (168), 0.12 (192)
		32 °C	0.1 (60), 0.13 (204)
		34 °C	0.14 (72), 0.16 (180), 0.24 (228)
Acclimation		17 °C	0.09 (72), 0.12 (72), 0.12 (84), 0.14 (84), 0.10 (96), 0.11 (96), 0.09 (108), 0.09 (108),0.08 (120), 0.09 (144)
		22 °C	0.07 (72), 0.09 (84), 0.07 (84), 0.10 (84), 0.11 (96),0.11 (108), 0.16 (120), 0.17 (168), 0.22 (216)
		25 °C	0.08 (48), 0.09 (72), 0.15 (72), 0.12 (72), 0.11 (72), 0.10 (96), 0.15 (96), 0.13 (120)
		28 °C	0.1 (84), 0.09 (96), 0.09 (168)
		32 °C	0.12 (108), 0.23 (168), 0.16 (168), 0.32 (228)
		34 °C	0.14 (36), 0.16 (36), 0.21 (48), 0.26 (48), 0.25 (144)

[i] The average whole-body copper concentration for unexposed control fish (at 28°C) was: 0.077± 0.01 (µmol/g d.w. ± standard deviation).

Table S2. Whole body cadmium concentration of the dead zebrafish during the Cu+Cd exposure in Acute 12 hours, Acute 96 hours, Chronic and Acclimation scenarios. Data are given as µmol/g d.w. for each individual fish and the numbers in brackets are the time of death in hours.

Scenario		Temperature	Whole body Cd concentration µmol/g d.w. (time to death/hr)[(i)]
Acute	12 hours	17 °C	0.014 (48)
		22 °C	0.004 (36), 0.005 (96)
		25 °C	0.009 (48), 0.01 (60), 0.009 (96)
		28 °C	0.007 (60), 0.014 (96), 0.014 (96)
		32 °C	0.013 (48), 0.025 (114)
		34 °C	0.013 (48), 0.009 (48), 0.016 (84), 0.024 (216)
	96 hours	17 °C	0.005 (36), 0.009 (120)
		22 °C	0.006 (72), 0.008 (120), 0.007 (132)
		25 °C	0.012 (60), 0.015 (72), 0.005 (84), 0.012 (84)
		28 °C	0.015 (60), 0.011 (96), 0.013 (96)
		32 °C	0.004 (24), 0.005 (24), 0.004 (60), 0.008 (60), 0.006 (60), 0.005 (60), 0.009 (84)
		34 °C	0.005 (36), 0.008 (36), 0.003 (36), 0.005 (60), 0.007 (60), 0.009 (60), 0.016 (72), 0.019 (156)
Chronic		17 °C	0.011 (72), 0.015 (72), 0.03 (72), 0.024 (84)
		22 °C	0.008 (72)
		25 °C	0.008 (120)
		28 °C	0.017 (168), 0.01 (192)
		32 °C	0.008 (60), 0.024 (204)
		34 °C	0.009 (72), 0.013 (180), 0.024 (228)
Acclimation		17 °C	0.004 (72), 0.005 (72), 0.004 (84), 0.006 (84), 0.004 (96), 0.003 (96), 0.003 (108), 0.002 (108), 0.003 (120), 0.003 (144)
		22 °C	0.01 (72), 0.013 (84), 0.01 (84), 0.012 (84), 0.016 (96), 0.009 (108), 0.015 (120), 0.023 (168), 0.02 (216)
		25 °C	0.005 (48), 0.012 (72), 0.009 (72), 0.005 (72), 0.009 (72), 0.008 (96), 0.009 (96), 0.011 (120)
		28 °C	0.006 (84), 0.01 (96), 0.013 (168)
		32 °C	0.016 (108), 0.02 (168), 0.025 (168), 0.029 (228)
		34 °C	0.009 (36), 0.021 (36), 0.013 (48), 0.035 (48), 0.031 (144)

[(i)] The whole-body cadmium concentration for unexposed control fish (at 28°C) was under the quantification limits (2.22×10^{-5} µmol/g d.w.) of ICP-MS.

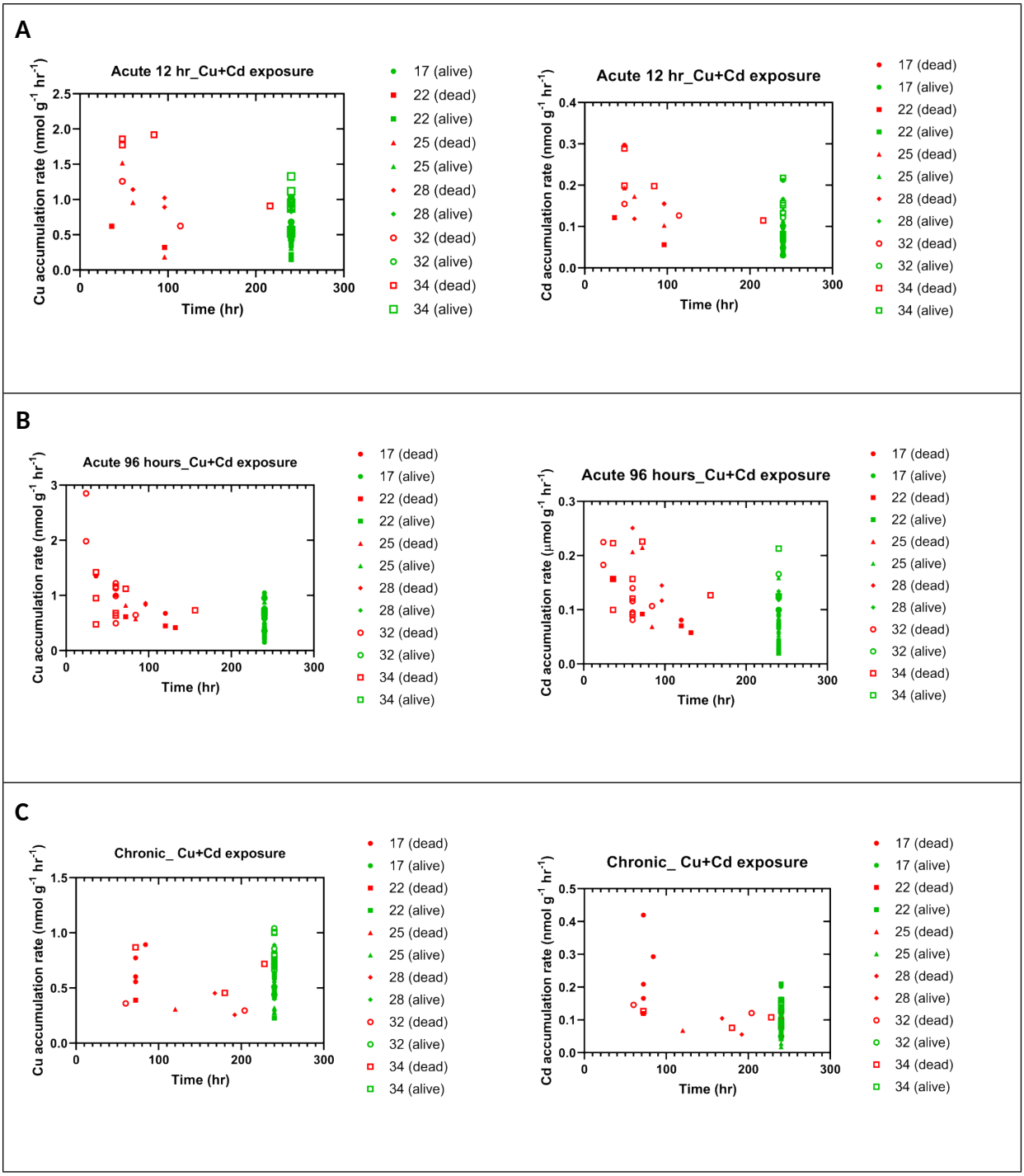
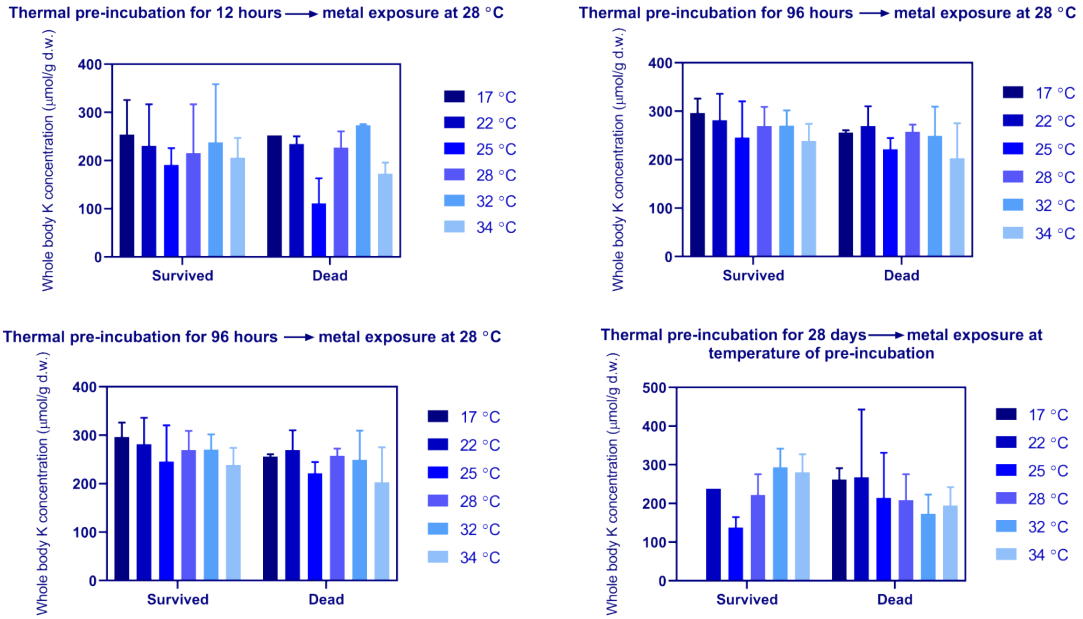
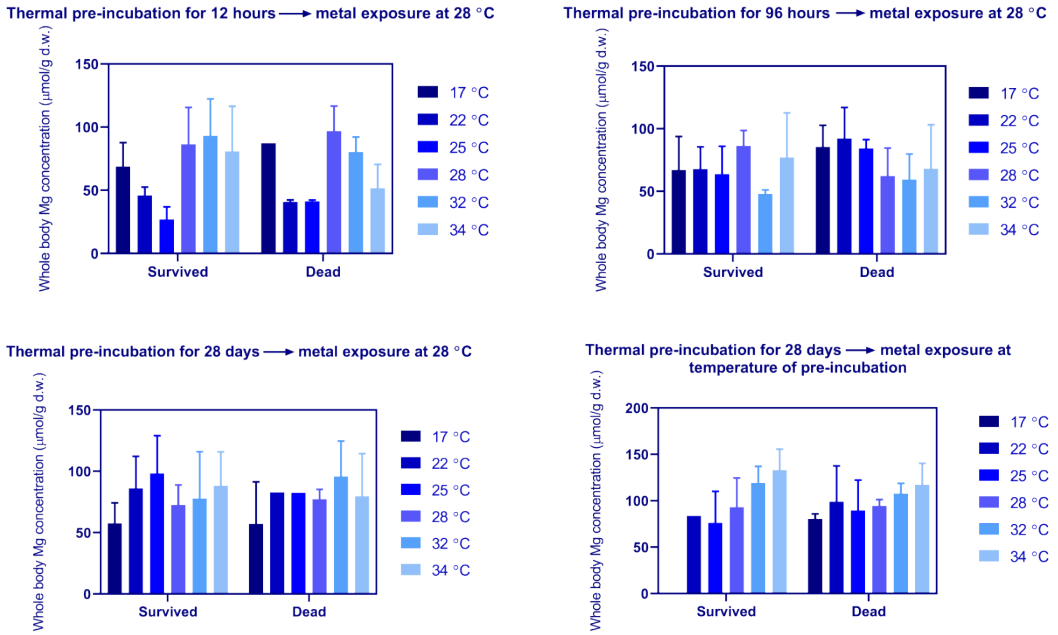


Fig. S2. Whole body Cu and Cd accumulation rate of dead and alive fish exposed to Cu+Cd in **(A)** Acute thermal 12 hours, **(B)** Acute thermal 96 hours and **(C)** Chronic thermal scenario. The accumulation rate is expressed as nmol per g d.w. per hr of metal exposure; the time on the *x*-axis corresponds to the time of death for dead fish, and 10 days for the alive (surviving) fish. The accumulation rate of copper was calculated after subtraction of the average whole body Cu concentration of unexposed control fish (0.077 µmol/g d.w.) from the initial value.

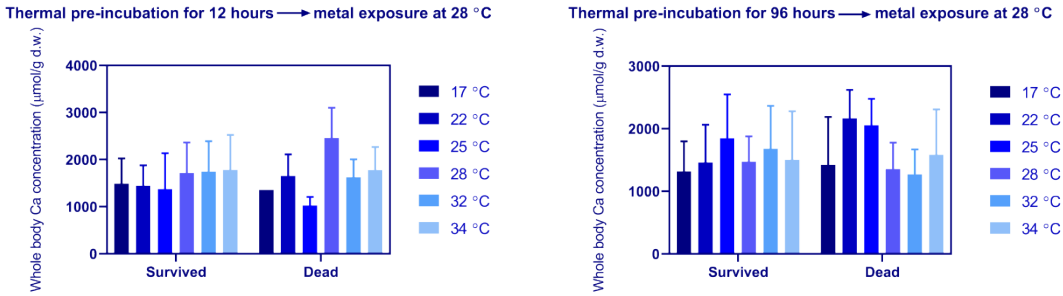
A


Thermal pre-incubation for 12 hours ⟶ metal exposure at 28 °C
Whole body K concentration (µmol/g d.w.)
400
300
200
100
0
Survived
Dead
17 °C
22 °C
25 °C
28 °C
32 °C
34 °C
Thermal pre-incubation for 96 hours ⟶ metal exposure at 28 °C
Whole body K concentration (µmol/g d.w.)
400
300
200
100
0
Survived
Dead
17 °C
22 °C
25 °C
28 °C
32 °C
34 °C
Thermal pre-incubation for 96 hours ⟶ metal exposure at 28 °C
Whole body K concentration (µmol/g d.w.)
400
300
200
100
0
Survived
Dead
17 °C
22 °C
25 °C
28 °C
32 °C
34 °C
Thermal pre-incubation for 28 days ⟶ metal exposure at temperature of pre-incubation
Whole body K concentration (µmol/g d.w.)
500
400
300
200
100
0
Survived
Dead
17 °C
22 °C
25 °C
28 °C
32 °C
34 °C

B


Thermal pre-incubation for 12 hours ⟶ metal exposure at 28 °C
Whole body Mg concentration (µmol/g d.w.)
150
100
50
0
Survived
Dead
17 °C
22 °C
25 °C
28 °C
32 °C
34 °C
Thermal pre-incubation for 96 hours ⟶ metal exposure at 28 °C
Whole body Mg concentration (µmol/g d.w.)
150
100
50
0
Survived
Dead
17 °C
22 °C
25 °C
28 °C
32 °C
34 °C
Thermal pre-incubation for 28 days ⟶ metal exposure at 28 °C
Whole body Mg concentration (µmol/g d.w.)
150
100
50
0
Survived
Dead
17 °C
22 °C
25 °C
28 °C
32 °C
34 °C
Thermal pre-incubation for 28 days ⟶ metal exposure at temperature of pre-incubation
Whole body Mg concentration (µmol/g d.w.)
200
150
100
50
0
Survived
Dead
17 °C
22 °C
25 °C
28 °C
32 °C
34 °C

C


Thermal pre-incubation for 12 hours ⟶ metal exposure at 28 °C
Whole body Ca concentration (µmol/g d.w.)
4000
3000
2000
1000
0
Survived
Dead
17 °C
22 °C
25 °C
28 °C
32 °C
34 °C
Thermal pre-incubation for 96 hours ⟶ metal exposure at 28 °C
Whole body Ca concentration (µmol/g d.w.)
3000
2000
1000
0
Survived
Dead
17 °C
22 °C
25 °C
28 °C
32 °C
34 °C

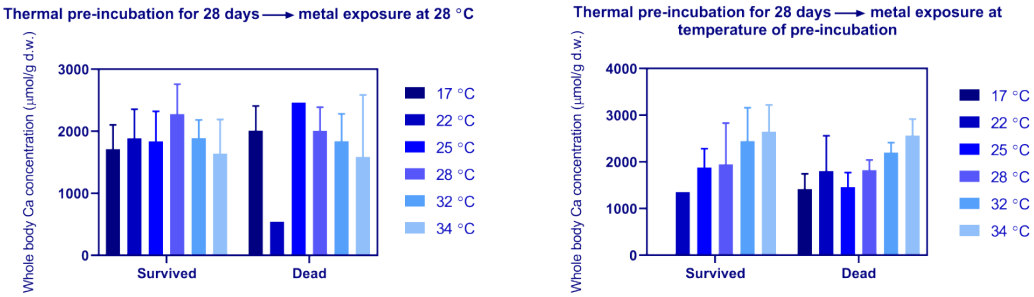


Fig S3. Whole body concentration of **(A)** K, **(B)** Mg and **(C)** Ca in dead and survived fish under the given thermal treatments and after 10 days of Cu+Cd exposure. Data are presented as mean±SD.

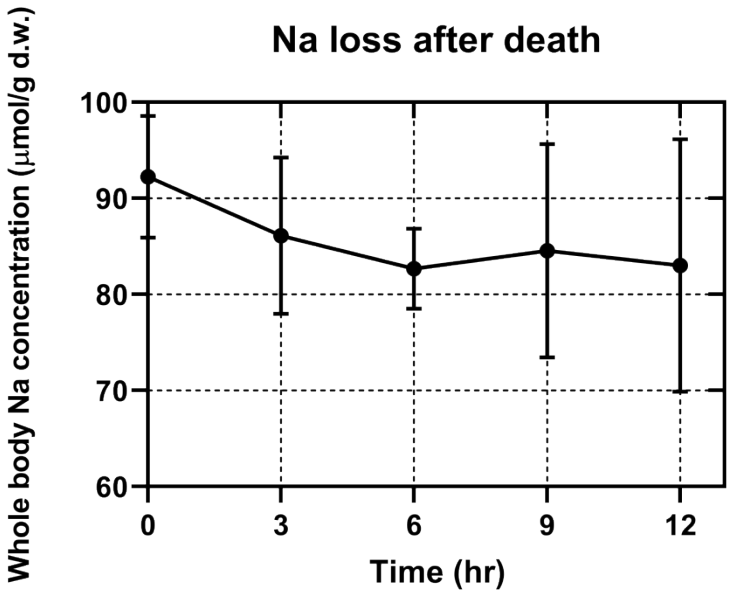


Fig. S4. The whole body sodium concentration in zebrafish following death at time = 0 hr. Values are means ± SD. Complementary experiment 1.

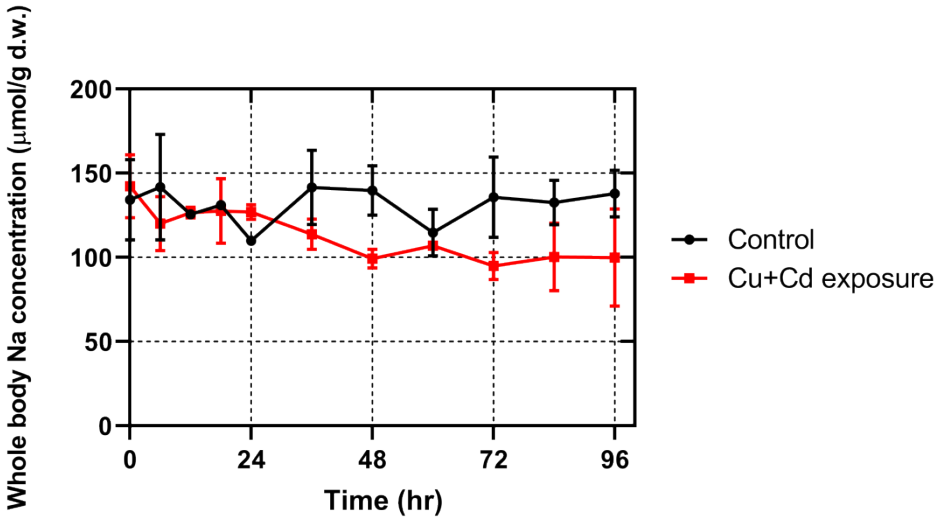


Fig. S5. The whole body sodium concentration of live zebrafish during 96 hours of a control exposure (no added metals) and exposure to Cu (1.2 µM) + Cd (0.2 µM) at 28 °C. Values are means ± SD. There is a significant (Two Way ANOVA, $p<0.05$) reduction in the Na^+ level of the fish exposed to Cu+Cd. Complementary experiment 2.