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RESEARCH ARTICLE

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Influence of water hardness on zinc toxicity in *Daphnia magna*

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Abstract

Zinc is an essential trace metal required for the maintenance of multiple physiological functions. Due to this, organisms can experience both zinc deficiency and toxicity. Hardness is recognized as one of the main modifying physiochemical factors regulating zinc bioavailability. Therefore, the present study analyzed the effect of hardness on zinc toxicity using *Daphnia magna*. Endpoint parameters were acute-toxicity, development, reproduction, and expression data for genes involved in metal regulation and oxidative stress. In addition, the temporal expression profiles of genes during the initiation of reproduction and molting were investigated. Water hardness influenced the survival in response to exposures to zinc. A zinc concentration of 50 μg/l in soft water (50 mg $CaCO_3$/L) caused 73% mortality after 96 h exposure, whereas the same zinc concentration in the hardest water did not cause any significant mortality. Moreover, increasing water hardness from 100 to 200 mg $CaCO_3$/L resulted in a reduced number of offspring. Fecundity was higher at first brood for groups exposed to higher Zn concentrations. The survival data were used to assess the precision of the bioavailability models (Bio-met) and the geochemical model (Visual MINTEQ). As the Bio-met risk predictions overestimated the Zn toxicity, a competition-based model to describe the effects of hardness on zinc toxicity is proposed. This approach can be used to minimize differences in setting environmental quality standards. Moreover, gene expression data showed that using the toxicogenomic approach was more sensitive than the physiological endpoints. Therefore, data presented in the study can be used to improve risk assessment for zinc toxicity.

KEYWORDS
bioavailability, BLM, gene expression, risk assessment, toxicogenomics

1 | INTRODUCTION

Being an essential trace element, zinc (Zn) is absolutely necessary for the maintenance of life. It is involved in multiple functions in organisms, ranging from gene regulation to protein function (King, 2011). While all living organisms require a minimum level of Zn for proper cell functions, it remains that at high concentrations, Zn can result in toxic effects (DeForest & Van Genderen, 2012; Hogstrand & Wood, 1996; Spear, 1982). Therefore, it is important to determine the boundaries for Zn homeostasis, deficiency, and toxicity. In the present

study, the effects of Zn exposure on *Daphnia magna* were determined. As hardness is a main modifying physiochemical factor regulating Zn bioavailability (Hogstrand, 2011), the effects of hardness on Zn toxicity were evaluated.

Daphnidae is an ecologically important family in freshwater environments as they serve as a food source for fish and other aquatic organisms. Due to their sensitivity to changes in water chemistry and the ease of culturing in a laboratory setting, they are used as standard organisms in aquatic toxicity tests and have been used to establish water quality regulations (Bury et al., 2002). Daphnids, along with most aquatic organisms, are often subjected to a wide array of different heavy metals in their environment, including Zn. In most natural waters, the free divalent Zn ion is the dominant inorganic form, and lethality of waterborne Zn to aquatic organisms is caused by these free and bioavailable Zn ions.

Improving risk assessments for metal species to reduce uncertainty is required to ensure safe environments. Earlier toxicity tests carried out in laboratory settings were practical approaches to link exposures of pollutants and their effects on organisms and populations. This correlation was widely used to develop water quality criteria and subsequently regulations for environmental protection policies by governing bodies. However, over time, it has become apparent that this approach has limitations. Results obtained under strictly controlled laboratory conditions are not always indicative of effects elicited under natural conditions (Pradhan et al., 2017). Moreover, it is clear that the biochemical basis of these exposure-effect relationships needs to be well understood to improve risk assessment. With the advancements in the field, it became evident that the bioavailability of metals was an important factor to assess dose response relationship (de Schamphelaere & Janssen, 2004). Early bioavailability models such as The Windermere Humic Aqueous Model (WHAM) and free-ion activity model (FIAM) were introduced to understand the relationship between bioavailability and the toxicity (Morel, 1983; Tipping, 1994). Improvements on these early models resulted in the development of the biotic ligand model (BLM) to predict metal toxicity as a result of variation in the local physiochemical conditions (Paquin et al., 2002). Although BLM models have shown to be efficient in predicting metal toxicity, several limitations such as effects of salinity have been reported by using environmental water samples (Arnold et al., 2005). Moreover, Zn BLMs have been developed for moderately hard to hard water conditions (Clifford & McGeer, 2009), and their applications to very soft waters are not well defined. These limitations indicate that there is potential for improvement of this widely used tool. Software for geochemical modeling such as Visual MINTEQ (Gustafsson, 2014) stands out as a promising tool used to calculate bioavailability as it provides a more comprehensive toolset. Therefore, comparison and validation of these models was included in the present study.

Environmental quality standards (EQS) for Zn limit show substantial variation between European countries. Most of the countries have several defined limit values by considering hardness when setting limit values for Zn. However, Belgium, Czech Republic, Latvia, Lithuania, Poland, Slovenia, the Netherlands, and Luxembourg have only one set of limit values (Vorkamp & Sanderson, 2016). In most environmental risk assessments, the acceptable exposure levels are based on measured levels of chemical pollutants with an addition of safety factors, also referred to as assessment factors (AFs). Differently interpreted AF values results in even greater diversity for Zn EQS. An EQS variability study conducted in 2016 resulted in Zn limit values from 3 μg/L (Sweden, valid for $CaCO_3$ < 24 mg/L) to 1,300 μg/L (Cyprus, total zinc for lakes) (Table 1) (Vorkamp & Sanderson, 2016). Although some countries updated the EQS values for Zn in the following years (Sweden 5.5 μg/L) (SwAM, 2019), these theoretically calculated limit values represent non-optimal concentrations for essential trace metals such as Zn, due to having a lower limit for functioning metabolism. Therefore, there is a growing need to validate bioavailability models used for Zn to connect water characteristics and bioavailability data in soft natural waters. Data calculated by quantitative computational models based on hard water models need to be verified by biological endpoints in softer waters. Organisation for Economic Co-operation and Development (OECD) guidelines for testing of chemicals offers two test to asses acute toxicology (OECD, 2004) and reproductive success (OECD, 2012) of *D. magna*. The understanding of how metal toxicity occurs can be further improved by analyzing gene expression profiles of the organisms exposed to different metal concentrations. This new toxicogenomic approach has a great potential to give insight to the mode of actions occurring at the cellular level. A toxicogenomic approach can reduce the number of organisms used in test protocols and reduce the analysis time since cellular responses can be observed earlier than they are manifested on an organismal or population level. Moreover, this approach allows researchers to observe physiological responses such as metal regulation and oxidative stress. These responses are usually neglected when traditional toxicity test protocols proposed by regulation bodies are used. Therefore, there is a need to modify the standardized test protocols to include a toxicogenomic approaches. In the present study, different water hardness and Zn concentrations were used to improve the understanding of the variability in Zn toxicity and to add to current toxicity tests and improve bioavailability tools.

TABLE 1 Comparison of Zn EQS values within EU countries

Country	Value (μg/L)	Country	Value (μg/L)
Austria	7.8	Belgium	20
Croatia	7.8 (35, 52)	Czech Republic	92
Denmark	7.8 (3.1)	Cyprus	1100/1300
France	7.8 (3.1)	Estonia	10
Netherlands	7.8	Ireland	8 (50,100)
Portugal	7.8	Latvia	120
Slovakia	7.8 (35.1, 52)	Lithuania	100
Slovenia	7.8	Luxemburg	7.2
Sweden	8 (3)	Romania	11.8 (73)
Used EU (2010)[a]		Not used EU (2010)[a]	

Note: Values in brackets (presumably) refer to other hardness categories.
[a]EU (2010): European Risk Assessment Report for Zinc.

2 | MATERIALS AND METHODS

2.1 | Water composition and metal bioavailability calculation

Test waters used in the survival, reproduction, and qRT-PCR (quantitative real-time polymerase chain reaction) assays were prepared by adjusting the hardness of the water obtained from the pristine Kimativajärvi Lake (67°44′33.2″N, 22°10′49.5″E), Sweden. $CaCl_2$ and $MgSO_4$ were used in a 2:1 ratio to achieve total hardness equivalent to 50, 100, and 200 mg $CaCO_3$/L for soft, medium, and hard water, respectively. Potassium concentrations for all test waters were set to 3 mg/L using $KHCO_3$. The natural Zn concentration of the lake water was 3.1 μg/L (Table 2). Additional Zn was added as $ZnCl_2$ dissolved in distilled water to achieve concentrations of 5, 10, 25, 50, 100, and 500 μg/L of total Zn. Water chemistry analyses were conducted before and after the exposures to take into account any deviations from the nominal concentrations. Anions were quantified in filtered solutions (0.20 μm polypropylene syringe filters) with ion chromatography using a Dionex AS12A column with a Dionex AG12A guard column (Thermo Fisher Scientific, USA). Metals and metalloids were quantified using ICP-QMS with Agilent 7500 cx equipped with a MicroMist nebulizer (Agilent, USA) using external calibration solutions (Merck 10580 multi-element standard VI). Isotopes and their concentration range are listed in Data S1. Test waters were aerated before the exposure and stored in translucent HDPE containers at 22 ± 1°C.

TABLE 2 Composition of the water obtained from Lake Kimativajärvi

Element	LOQ[a] (μg/L)	Measured Concentration (μg/L)	SD	RSD (%)
Ca	5.00	302	7.85	2.60
K[b]	18.40	125	6.61	5.29
Mg	0.05	115	0.24	0.21
Na	8.50	359	9.69	2.70
Ag	0.05	<0.05		N/A
Al	0.10	23.40	0.26	1.13
As[b]	0.10	0.17	0.04	23.62
Ba	0.05	3.58	0.29	8.20
Be	0.05	<0.05		N/A
Bi	0.05	0.12	0.00	1.92
Cd	0.05	<0.05		N/A
Co	0.05	<0.05		N/A
Cr[b]	0.20	0.60	0.28	46.61
Cu[b]	0.05	0.76	0.10	13.09
Fe[b]	0.10	36.80	3.60	9.77
Ga	0.05	0.18	0.01	3.91
Li	0.10	<0.10		N/A
Mn	0.10	0.84	0.06	7.26
Mo	0.05	<0.05		N/A
Ni	0.20	<0.20		N/A
Pb	0.05	0.07	0.01	12.46
Rb	0.05	0.51	0.00	0.90
Se[b]	1.30	5.7	0.45	7.94
Sr	0.05	3.03	0.07	2.33
Te	0.30	<0.30		N/A
Tl	0.05	<0.05		N/A
U	0.05	<0.05		N/A
V[b]	0.05	0.13	0.02	17.06
Zn	0.10	3.11	0.50	16.10
pH		6.7		
DOC		2.9 mg/L		

Abbreviations: RSD, relative standard deviation (SD/mean*100); SD, standard deviation.

[a]Limit of quantification.

[b]Analysis using collision cell to avoid impact from di-and polyatomic interferences.

Bioavailability calculations were performed using Bio-Met Bioavailability Tool version 5.0 (www.bio-met.net) and Visual MINTEQ 3.1 software by inputting the measured concentrations of each element that was above detection limit as well as data for pH, hardness, and content of organic material in test media. The bio-met bioavailability tool is a "user-friendly" excel-based software validated towards full Biotic Ligand Models (BLMs) for Zn, nickel, copper, and lead. Bio-met calculates Bioavailability Factors (BioF) and Local HC5 (hazardous concentration at 5% assuming a lognormal Species Sensitivity Distribution) values for metals based on information on three local water quality parameters (pH, dissolved organic carbon [DOC], and calcium [Ca^{2+}]). The Bio-met tool contains a large database of more than 20,000 different combinations of key input parameters (pH, DOC, and Ca^{2+} concentrations) and corresponding HC_5 calculations for various metals, using their respective BLM. The database then functions as a lookup table. The physio-chemistry of the assessed local site is compared to the physio-chemistry of existing simulations in the lookup table. The lowest HC_5 of the two "best-matching" lookup table entries is selected as local HC_5.

2.2 | *D. magna* culture and feeding

Ephippia from the Daphtoxkit (MicroBioTests Inc., Belgium) were activated by rinsing with tap water and incubated for 72 h in standard freshwater (prepared using 67.75 mg/L $NaHCO_3$, 294 mg/L $CaCl_2$, 123.25 mg/L $MgSO_4$, and 5.75 mg/L KCl), with a hardness of 250 $CaCO_3$/L under continuous illumination at a temperature of 22 ± 1°C as this results in optimal hatching. Newly hatched juveniles (<24 h) were transferred to the test waters directly after hatching and kept in 16-h light and 8-h dark cycle. *D. magna* (strain MBP996) were fed with a mixture of microalgae *Spirulina* and yeast with adjusted feeding ratios during the exposures to ensure proper feeding.

2.3 | Zn exposure protocol

For survival assays, newly hatched *D. magna* juveniles (<24 h) were transferred to 8 ml soft, medium, or hard water with different Zn concentrations in 6 well plates (BD Falcon, Germany). Each well contained 20 animals, and four replicates were used for each exposure (total 80 animals for each hardness and Zn combination). Half of the exposure water was changed at 48 h after initial exposure. Mortality was screened using a light microscope and recorded at 24, 48, 72, and 96 h. For qRT-PCR analysis, newly hatched juveniles were transferred to soft, medium, or hard water with no added Zn and were kept until 72 h post hatch. They were then exposed to different Zn concentrations for 24 h in six well plates containing 8 ml exposure water. Each well contained 14 animals to ensure adequate amounts of RNA following extraction, and eight replicates were used for each exposure. For temporal gene expression analysis, newly hatched juveniles (<24 h) were transferred to hardness adjusted Kimativajärvi water. Each well contained 20 animals due to small animals at first sampling points and sampling was carried out at 24, 48, 72, 76, 80, 84, 88, 96, 100, 104, 108, and 112 h post-hatching. Daphnids were collected and snap frozen using liquid nitrogen and stored at −80°C until RNA extraction.

2.4 | RNA isolation and qRT-PCR

Following sampling, *D. magna* were lysed in 350 µl of TRIzol reagent (Sigma, USA) using tissue homogenizer (Precellys Evolution, Bertin Technologies, USA), and RNA extraction was performed using Direct-zol Kit (Zymo Research, USA) according to the manufacturer's instructions. DeNovix DS-11 spectrophotometer (Wilmington DE, USA) was used to measure the RNA concentration. cDNA was synthesized from 300 ng of RNA using the qScript cDNA synthesis kit (Quanta Biosciences, USA) according to the manufacturer's instructions. qRT-PCR was performed to quantify the expression of the genes using qPCRBIO SyGreenMix Lo-ROX (PCR Biosystems, USA) on CFX384 Real time PCR detection system (Bio-Rad, USA) with thermo cycling profiles of initial denaturation step at 95°C for 2 min followed by 35 cycles of 95°C for 5 s and 60°C for 30 s. Expression ratios were calculated based on ΔΔCt method (Schmittgen & Livak, 2008), further modified by the Pfaffl method (Pfaffl et al., 2002) using primer efficiencies (Data S2) with *actin* as the housekeeping gene.

2.5 | Reproduction and development

Newly hatched *D. magna* were transferred to 50 ml beakers filled with 40 ml soft, medium, or hard water with Zn concentrations ranging from 3.1 to 100 µg/L. Each beaker contained a single animal, and six replicates were used for each exposure. The number of progenies was recorded for 21 days, and the development of body length, tail length, and body width was measured at the end of exposure using stereo microscope Lumar V12 (ZEISS, Germany).

2.6 | Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.0.2 software (GraphPad Software, USA) using one-way ANOVA for multiple group comparison. Dunnett's post-test was used to compare the mean of each column with the mean of a control column, whereas Tukey's post-test was used to compare the mean of each column with the mean of every other column. Statistically significant differences were considered when p values were <0.05 ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). A total of 25 µg/L Zn was selected as a control group for gene expression analysis since the lowest mean mortality rate was observed with 25 µg/L Zn exposure across all three water hardness at the time of sampling. Principal component analysis (PCA) was used to analyze multivariate data using the SIMCA software, v13.0.3 (Umetrics, Sweden). A number of significant components were validated using cross validation rules. Expression profiles of 10 genes

were used as variables to analyze correlations between exposure groups. Tolerance ellipse based on Hotelling's T2 was used to check outliers (95%).

3 | RESULTS

3.1 | Zinc bioavailability and *D. magna* survival

Bioavailabilty calculations were carried out using two different software. The Bio-Met bioavailability tool indicated BioF values of 0.69, 0.69, and 0.52 for soft, medium, and hard water, respectively (Table 3). Risk characterization ratio (RCR) was also calculated for each exposure condition. In all three hardness groups, RCR values exceeded 1 at concentrations at or above 25 μg/L Zn. Calculations using the Visual MINTEQ 3.1 were also carried out to determine free divalent Zn, calcium (Ca), and magnesium (Mg) concentrations. BioF values were calculated as decimal fraction of free divalent Zn over total measured Zn concentrations. BioF values according to Visual MINTEQ 3.1 were 0.83, 0.77, and 0.71 for soft, medium, and hard water, respectively. The theoretically calculated bioavailable Zn concentrations varied between different software models (Table 3). Although both models predicted lower bioavailable Zn in the hard water, the Bio-met model delivered equal BioF values for soft and medium water whereas Visual MINTEQ predicted soft water to have more bioavailable Zn.

A 96 h survival assay was conducted to determine the acute toxicity of Zn with selected concentrations (3.1, 5, 10, 25, 50, 100, 250, and 500 μg/L) at different water hardness using *D. magna*. Significant mortality was observed as early as 24 h with 500 μg/L Zn in soft water, reaching to 100% mortality at 72 h (Figure 1A). However, the mortality rates for 500 μg/L Zn in medium and hard water was 85% and 31%, respectively, at the same time point (Figure 1B,C). Moreover, 100 μg/L Zn at 96 h resulted in higher mortality in soft water,

TABLE 3 Composition of the water used for experiments

	Zinc[a]					RCR[b]	Calcium[c]		Magnesium[d]	
	Measured (μg/L)	Free Zn (VM)[e] (μg/L)	Bioavailable Zn (BLM)[f] (μg/L)	Measured to Free Zinc (%)	Measured to Bioavailable Zinc (%)	(BLM)	Measured (μg/L)	Free Ca (VM) (μg/L)	Measured (μg/L)	Free Mg (VM) (μg/L)
Soft	3	2.49	2.06	82.78	68.56	0.19	11,078	9,169	5,218	4,319
	6.48	5.35	4.44			0.41				
	9.31	7.7	6.38			0.59				
	23	19.1	15.8			1.45				
	54.3	45	37.3			3.42				
	108	89.5	74			6.79				
	508	419	348			31.93				
Medium	3.41	2.64	2.34	77.36	68.56	0.21	20,996	16,248	11,960	9,255
	5.21	4.03	3.57			0.33				
	12.7	9.84	8.7			0.8				
	28.2	21.8	19.3			1.77				
	52.9	40.9	36.3			3.33				
	91.7	70.7	62.9			5.77				
	465	359	319			29.23				
Hard	3.14	2.23	1.64	70.99	52.26	0.15	40,491	28,733	21,930	15,562
	4.8	3.4	2.51			0.23				
	12.5	8.85	6.52			0.6				
	27.4	19.5	14.3			1.32				
	50.9	36.3	26.6			2.44				
	93.6	66.1	48.9			4.49				
	459	326	240			22				

[a]Zinc concentrations were reported for each test water.
[b]Risk characterization ratio (RCR) calculated using BLM is also reported.
[c]Calcium concentrations were reported as a mean of each hardness group.
[d]Magnesium concentrations were reported as a mean of each hardness group.
[e]Free ion concentrations were calculated using Visual MINTEQ 3.1 (VM).
[f]Free ion concentrations were calculated using biotic ligand model (BLM).

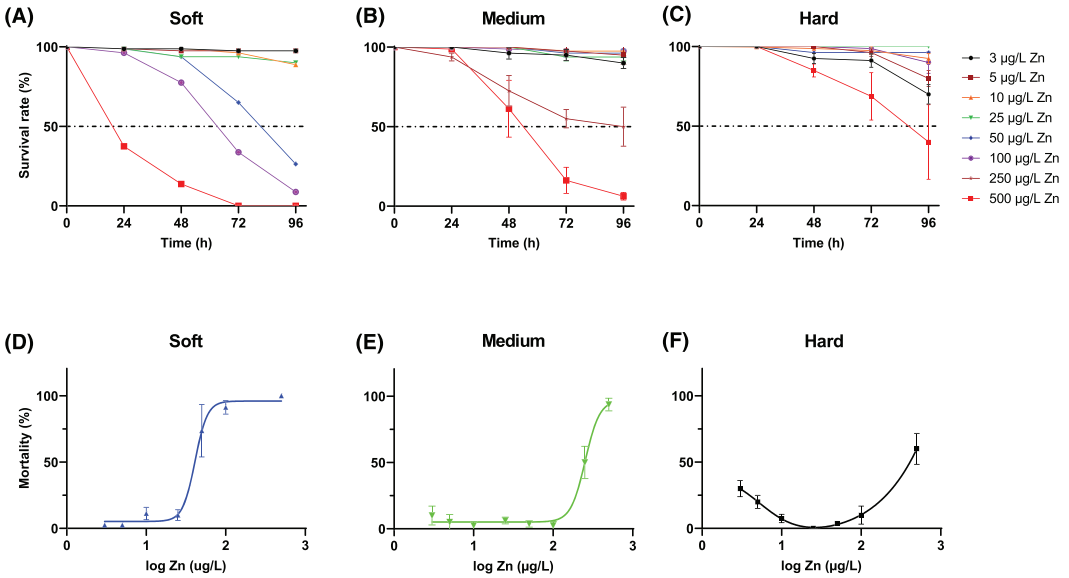

FIGURE 1 Twenty *Daphnia magna* neonates (<24 h old) per well were exposed to varying Zn concentrations for 96 h, and the survival rate was recorded for soft (A), medium (B), and hard water (C). Dose–response curves were generated for each water hardness by plotting mortality rates at the end of 96 h exposure (D–F). Mean ± SEM. $n = 4$

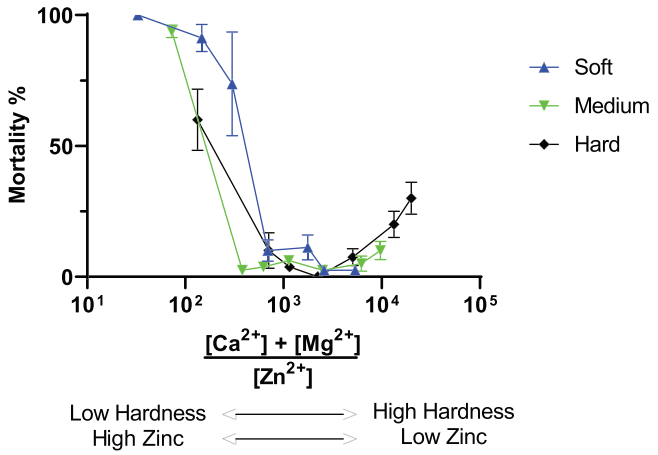

FIGURE 2 Free ion concentrations were calculated using Visual MINTEQ. The sum of Ca^{2+} and Mg^{2+} concentrations was divided by the Zn concentrations and plotted against the mortality rates of daphnids at the end of 96 h exposure. Mean ± SEM. $n = 4$

compared to medium and hard water. In contrast, the lowest Zn concentration (3.1 µg/L) resulted in lower mortality rates in soft water compared to medium and hard water. The dose response curves for the 96 h time point are shown in Figure 1D–F for each water hardness.

Survival data for *D. magna* agreed with the predictions made by Visual MINTEQ since lower LC_{50} values were observed in soft water compared to medium water. Additionally, in the Bio-met model, RCR values equal to or greater than 1 are considered as a potential risk to organisms. However, the calculated RCR values for 25 µg/L Zn in soft, medium, and hard water (1.45, 1.77, and 1.32, respectively) clearly overestimated the effects since there was no significant mortality in soft and medium water with Zn concentrations up to 25 µg/L, and 25 µg/L Zn also resulted in the lowest mortality in hard water.

Based on the present results, a hardness-based toxicity approach for Zn exposure was developed to better demonstrate the effect of water hardness on Zn toxicity by using free ion concentrations calculated by Visual MINTEQ. In this approach, the sum of Ca^{2+} and Mg^{2+} concentrations divided by the Zn concentrations were plotted against the mortality rates (Figure 2). The lower ratios (lower values on the *x* axis) represented the high Zn and low Ca and Mg concentrations, whereas the higher ratios represented the low Zn and high Ca and Mg concentrations. Mortality rates were highest when there was abundant free Zn, and the addition of Mg and Ca reduced the mortality rate until an optimal balance is achieved between Mg, Ca, and Zn that provides best survival conditions. After this point, further increase in Ca and Mg led to increased mortality in a dose dependent manner.

3.2 | *D. magna* reproduction and development

Cumulative number of offspring per individual daphnid during the 21 days reproduction assay ranged from 14 to 40 offspring for soft, medium, and hard water (Figure 3A–C). First offspring were observed between 9 and 10 dph. Since the daphnids were kept individually, it was possible to track brood cycles for each daphnid. For each treatment group, four brood releases were observed between the 9dph and 21dph. The number of progenies produced in the first brood was shown to corelate with Zn concentration in soft and medium water (Figure 3C–E).

Body length and width were measured at the end of the 21 days exposure. Body length ranged from 2.21 to 2.98 mm, and body width

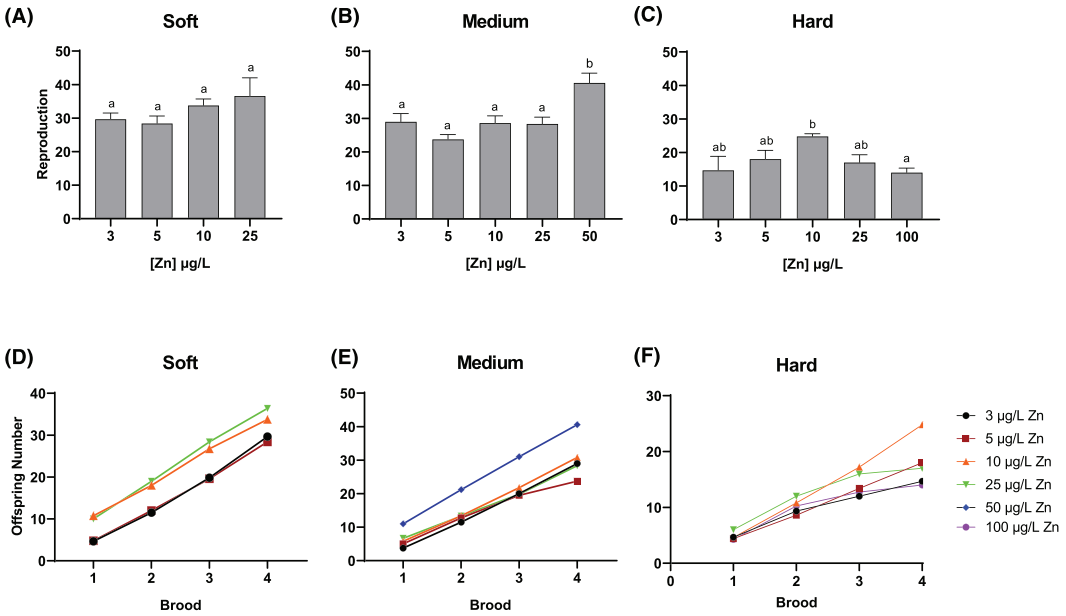

FIGURE 3 *Daphnia magna* neonates (<24 h old) were kept individually in 40 ml exposure water for 21 days, and progeny numbers were recorded during the exposure. Total number of offspring at the end of the exposure period is shown (A–C). Number of cumulative progenies produced during first four brood release is shown (D–F). Mean ± SEM. $n = 6$

TABLE 4 Body measurements of *Daphnia magna* at the end of 21 days exposure to varying zinc concentrations in different water hardness

	Zinc (µg/L)	Body Length (µM)	Body Width (µM)	Tail length (µM)
Soft	3	2,682 ± 286^{abc}	1866 ± 198^{abcd}	499 ± 209^{abcd}
	5	2,496 ± 229^{cd}	1,627 ± 116de	442 ± 206^{bcd}
	10	2,581 ± 214^{bc}	1,725 ± 127^{bcd}	536 ± 169^{abcd}
	25	2,969 ± 236^{a}	1,939 ± 168^{abc}	738 ± 273^{a}
Medium	3	2,543 ± 81^{bcd}	1,714 ± 73^{bcd}	375 ± 129^{bcd}
	5	2,524 ± 138^{cd}	1,688 ± 59^{bcde}	310 ± 71^{d}
	10	2,964 ± 219^{a}	1,998 ± 152^{a}	486 ± 72^{abcd}
	25	2,903 ± 112^{ab}	1,943 ± 47^{ab}	546 ± 153^{abcd}
	50	2,954 ± 45^{a}	1,954 ± 57^{ab}	600 ± 70^{abc}
Hard	3	2,894 ± 164^{ab}	1,912 ± 204^{abc}	364 ± 35^{bcd}
	5	2,979 ± 92^{a}	1,896 ± 179^{abc}	564 ± 192^{abcd}
	10	2,755 ± 123^{abc}	1,743 ± 139^{abcd}	332 ± 48^{bcd}
	25	2,804 ± 52^{abc}	1,673 ± 35^{cde}	619 ± 10^{ab}
	100	2,211 ± 282^{d}	1,432 ± 146^{e}	312 ± 69^{cd}

Note: Significant differences were determined using ANOVA followed by Tukey posttest. Different letters within the same column represent significant differences ($p < 0.05$, $n = 6$).

ranged from 1.43 to 2.00 mm (Table 4). Zn concentrations of 10 µg/L and higher in medium water resulted in increases in both body length compared to lower concentrations in same hardness. On the other hand, with the highest Zn concentration in hard water (100 µg/L Zn), body length was shorter, and body width was narrower compared to other concentrations at the same water hardness. Shortened tails were observed in some of the daphnids, but no correlation to the exposure conditions was observed. Due to this shortening in the tail length for some individuals, tail length measurements were excluded from further analysis.

3.3 | Gene expression

qRT-PCR analysis was performed to determine the effects of different physiochemical water properties on gene expression profiles in *D. magna*. Test concentrations were chosen based on the survival assays.

The expression of metallothionein (*mt*) genes was analyzed to gain insight to metal regulation in different water hardness. The *mt-a* gene was upregulated at 25 µg/L Zn in soft water compared to the lower concentrations (Figure 4A). However, same Zn concentration

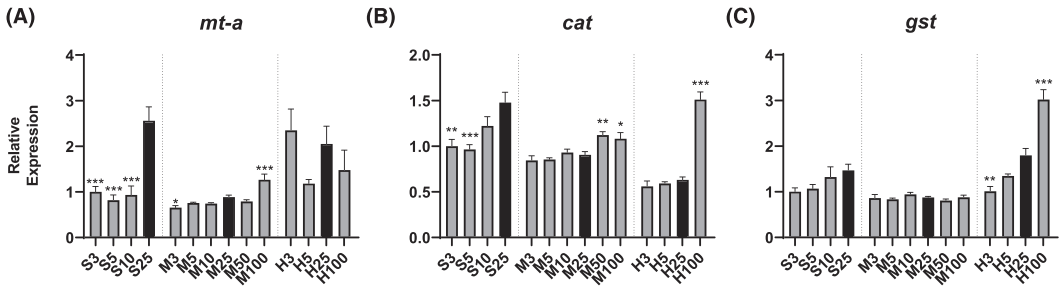

FIGURE 4 Fourteen 72 h old daphnids were exposed to varying zinc concentrations for 24 h in different water hardness for 24 h. qRT-PCR was performed to analyze the gene expression profiles for (A) *mt-a*, (B) *cat*, and (C) *gst*. The 25 μg/L Zn concentration at all three water hardness was selected as a control for one-way ANOVA followed by Dunnett posttest to determine statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). Mean ± SEM. $n = 8$

did not show any significant changes in medium water where 100 μg/L Zn was required for upregulation of *mt-a*.

Expression of genes related to oxidative stress such as *cat* and *gst* was analyzed. A Zn concentration dependent expression profile was observed for *cat*; that is, the higher Zn concentration resulted in higher transcript levels at each hardness. Expression levels of *gst* was upregulated only in hard water with 100 μg/L Zn. While *cat* expression levels were slightly reduced with increasing hardness, the *gst* levels remained similar between different water hardness groups at low Zn concentrations (Figure 4B,C).

3.4 | Multivariate data analysis and temporal gene expression

Multivariate data analysis was used to analyze the effects of hardness and zinc on *D. magna*. A total of 10 stress related genes were used as variables to identify independent components that explains the variation between exposure groups (Figure 4 and Data S3). The PCA analysis resulted in a goodness of fit value of 0.67 (R^2X) and a goodness of prediction value of 0.49 (Q^2X). First principal component, PC1 explained 29% of the variation and PC2 explained 26% of the variation observed in the dataset. In order to simultaneously display and interpret score and loading, a biplot was generated (Figure 5A). Exposure groups were also clustered hierarchically using PCA (Figure 5B). The biplot displays similarities and dissimilarities between exposure groups and allows for correlation of these groups with gene expression profiles. Exposure groups located near a gene variable showed high expression levels whereas distantly located pairs explained lower expression. Exposure groups close to plot origin showed average properties. Metallothionein genes have relatively higher *x* values whereas heat shock protein genes had relatively higher *y* values. Thus, the PCA biplot indicated that metallothionein genes were the main variables to explain variations seen in the exposure groups since they were correlated with the first component. Daphnids exposed to hard water was paired up with the stress related genes such as *gst* and *cat* located on *y* axis. PCA plot demonstrated that hardness had a strong effect on differences observed between exposure groups. Even though the exposure groups were sampled at the same time point, the changes in timing of molting-reproduction cycles makes it challenging to compare gene expression profiles in waters of different composition since changing the water chemistry may speed up or slow down the reproduction-molting cycles. To test this hypothesis, a temporal gene expression analysis was performed. Most gene expression studies conducted with *D. magna* use 24 h post-hatching as a sampling point. However, we observed that the vitellogenins had very low transcript levels prior to 96 hours post hatching (hph). After 96 hph, they start their cyclic expression profiles which usually lasts around 3 to 4 days at 21°C. To analyze reproductive development, daphnids were sampled at 12 different time points during their early life stages. First hatchings were observed at 8 am, and this time point was used as a start of the16:8 light dark cycle. Daily samplings were carried out until 3 dph (72hph) with 24 h intervals. After 72 hph, sampling intervals was set to four hours until the end of the exposure to obtain a better resolution on gene expression profiles at the start of vitellogenin accumulation. There was no sampling at 92 hph to not disturb the dark cycle.

Juvenile hormone esterase (*jhe*) showed significant temporal differences in gene expression between the hardness exposures (Figure 5C). On the other hand, both *vtg1* and *vtg2* transcripts remained stable at basal levels for all three hardness groups until significant upregulation was observed at 96 hph for soft and medium water. However, this upregulation was delayed until 104 hph in hard water (Figure 5D,E). At 100 hph *vtg* levels continued to increase in medium water, whereas there was a decline in *vtg* transcripts in soft water compared to 96 hph. This downregulation in *vtg* levels was also observed in medium water with 4 h delay, falling between 100 and 104 hph. Delayed onset of *vtg* production in hard water and contrasting expression profiles observed in soft and medium water with delays was in accordance with the hypothesis that water hardness changed the expression profiles of *vtg* transcripts by affecting the developmental pace of the organism. This also suggests that daphnids should be sampled no earlier than 96 hph to study late vitellogenesis.

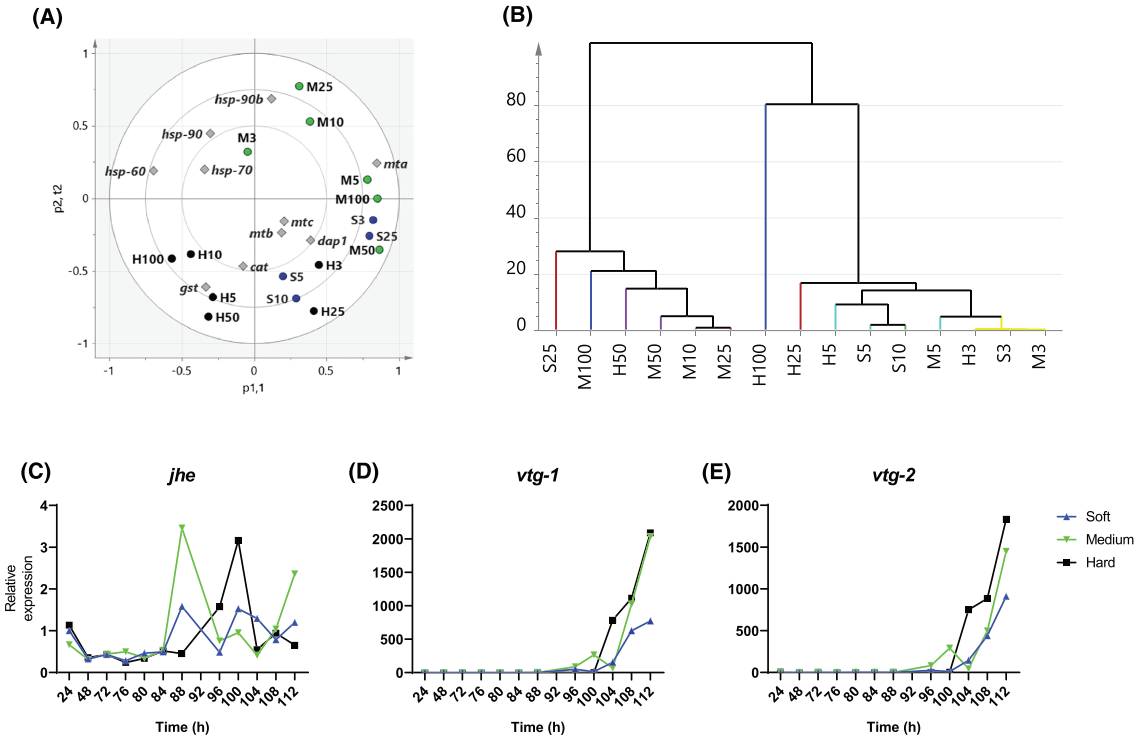

FIGURE 5 PCA bipolt (A) and hierarcichal clustering of exposure groups (B) were generated using principal component analysis. In addition, temporal expreesion level of genes related to reproduction was analyzed using qRT-PCR. Twenty newly hatched daphnids were exposed to soft, medium, and hard water for 112 h. Expression levels are shown relative to 24 h in soft water. (C) *jhe*, (D) *vtg-1*, and (E) *vtg-2*

4 | DISCUSSION

Exposure with increasing Zn concentrations for 96 h in different hardness resulted in significantly altered survival rates in *D. magna*. Survival assay results were similar to previous studies conducted with *D. magna* exposed to varying Zn concentrations (Muyssen et al., 2006; Muyssen & Janssen, 2005; Paulauskis & Winner, 1988; Winner & Gauss, 1986). Barata et al. (1998) reported that the LC_{50} values of Zn exposure, for the least and most sensitive strains of *D. magna*, ranged from 131 to 259 μg/L for soft water (46 mg/L $CaCO_3$), 457 to 1,060 μg/L for moderate-hard water (90 mg/L $CaCO_3$) and 601 to 962 μg/L for hard water (179 mg/L $CaCO_3$). In the present study, calculated LC_{50} values at 48 h were similar to the previously reported values (Barata et al., 1998) with 191.3 μg/L for soft water and 638.9 μg/L for medium water, although the observed LC_{50} value for hard water (1828 μg/L) was higher than for the reported most tolerant strain. In another study conducted with four daphnid species in water with hardness of 41 mg/L $CaCO_3$, LC_{50} value for *D. magna* was 819.9 μg/L Zn after 48 h exposure, whereas LC_{50} values ranged from 260.9 to 304.7 μg/L Zn for other daphnid species (Shaw et al., 2006). This suggested that *D. magna* was less sensitive to zinc exposure compared to other daphnid species investigated in the study. However, in our study, soft water LC_{50} value for the same time point for *D. magna* was observed to be similar to those observed with *Daphnia ambigua*, *Daphnia pulex*, and *Ceriodaphnia dubia*. Therefore, our data for *D. magna* did not show any significant differences in survival compared to previously reported survival data for other daphnid species (Data S4).

Constructed dose–response curves after 96 h exposure showed that the optimal Zn concentration for survival was inversely proportional for soft and medium water; that is, lower Zn amounts resulted in better survival. However, increased hardness combined with low Zn concentrations led to reduced survival rates compared to soft and medium water. Improved survival rates by the addition of Zn in hard water is likely to be associated with Zn deficiency in organisms cultured without added Zn (Lam & Wang, 2008; Muyssen et al., 2006; Muyssen & Janssen, 2005). As Zn is an important cofactor to numerous enzymes, in the body, including both the antioxidant and immune systems, a deficiency in Zn can lead to both increased oxidative stress and immune dysfunctions (Kling et al., 2013; Olechnowicz et al., 2018). Thus, the increased mortality at low Zn concentrations may be associated with these dysfunctions.

Daphnids were able to tolerate Zn concentrations up to 25 μg/L in soft water and 100 μg/L in medium water. On the other hand, Zn concentrations lower than 10 μg/L resulted Zn deficiency in hard water. Thus, optimal Zn concentration for hard water ranged from 10 to 100 μg/L Zn. Acute lethality of Zn also reduces with increasing hardness for fish species (Hogstrand & Wood, 1996). Spear reported the LC_{50} values of 1 to 10 mg/L in soft water and 3 to 20 mg/L Zn for different fish species (Spear, 1982). Similar results were observed for

phytoplankton where the no-observed effect concentration (NOEC) values for phytoplankton kept in 22–35 mg/L calcium was 75 μg/L Zn, whereas 12–22 mg/L calcium resulted NOEC value of 21 μg/L Zn (Hoang et al., 2021; Van de Perre et al., 2016). These results demonstrated that increased hardness result in decreased acute toxicity across greatly distant species.

Variations in physiochemical conditions of the water also affect the mortality rates. Therefore, bioavailability models have been developed to predict metal toxicity (Paquin et al., 2002). Similar approaches have been investigated to demonstrate the correlation between bioavailability of Zn and its toxic effects on *D. magna* (Heijerick et al., 2002, 2003, 2005; Komjarova & Blust, 2009; Santore et al., 2002). In the present study, two computational models, Bio-met and Visual Minteq, were compared. Visual Minteq can handle more input parameters in its speciation modeling than does Bio-met; hence, Visual Minteq is more complex in that regard. Bio-met is a simplified model compared to the full BLM for Zn; however, it is a pragmatic compromise for making it user-friendly for broad use in compliance assessment.

The overestimated RCR values observed with the Bio-met model are in line with the model's aim to avoid underestimating the effect when used in compliance assessments under the water framework directive. Bio-met indicates the boundaries regarding pH (6–8.5) and hardness (Ca^{2+} between 5 and 160 mg/L) within which it has been validated. Correspondingly, DOC content has no defined boundaries in the model. Therefore, using data outside the defined boundaries for pH and hardness results in higher uncertainties, although it still yields valuable bioavailability estimations (WCA, 2015). In addition to overestimations of Zn toxicity at all three-water hardness levels, Bio-met was not able to predict any Zn deficiency in hard water. The Bio-met tool database does not contain data indicating where deficiency occurs. RCR values increased steadily with the addition of Zn in hard water, whereas better survival rates were observed with addition of Zn up to a concentration of 25 μg/L. These observations indicate that the limitations of Bio-met are a result of limited input options that are important to include in order to determine water hardness. In addition, it is not able to fully consider the possibility of bell-shaped mortality curves for essential metals. Moreover, exposures with environmental waters could yield more relevant data since laboratory studies often overestimate the observed responses (Pradhan et al., 2017). The UK Technical Advisory Group for the implementation of the WFD (WFD-UKTAG) set 10.9 μg/L bioavailable Zn as the generic EQS (WFD, 2012). This value was accepted by European Union Scientific Committee on Health and Environmental Risks and generally implemented by the member states of EU since the value already considered a reasonable worst-case scenario regarding bioavailability, such as low pH, low DOC, and soft waters. However, each member state further modified the recommended EQS value for Zn by introducing additional AFs by considering national physio-chemical water properties which led to Zn values that possess greater risk in deficiency compared to toxicity.

The observed correlation between Ca and Mg over Zn concentrations suggests competition between Zn against Ca and Mg. The molecular basis for this competitive correlation has previously been investigated for Zn and other metals (Ballatori, 2002; Bridges & Zalups, 2005). In both freshwater and marine fish, Zn is absorbed from the water primarily through gills and high Ca concentrations have shown to greatly reduce the uptake rate of Zn (Bentley, 1992; Bradley et al., 1985). Although less is known about the kinetics of Zn uptake in *D. magna*, hardness-based toxicity approach presented in this study accounts for Zn deficiency and should be considered as a complementary tool to improve risk assessment.

Understanding the relationship between metals and their regulation at the molecular level is important to gain insights on how toxicity emerges. Metallothionein (MT) plays an important role on the metal regulation process because of their ability to bind and sequester excess metals (George & Olsson, 1994; Olsson, 1993). Analysis of the *mt* gene regulatory region in *D. magna* indicates that only *mt-a* contains multiple metal response elements (MREs) as well as antioxidant response elements (AREs) (Asselman et al., 2013). While MREs have been shown to be regulated by Zn, AREs have been shown to be regulated by oxidative stress (Kling et al., 2013; Olechnowicz et al., 2018). Thus, *mt-a* may be a good indicator of how *D. magna* responds to increased Zn concentrations as well as to reduced antioxidant protection caused by reduced Zn concentrations.

The MT protein concentrations increase progressively with positive correlations between MT and Zn concentrations in *D. magna* (Fan et al., 2009). In this study, the same positive correlation for *mt-a* in soft and medium water was observed. This indicates that water hardness plays a key role in gene regulation of *mt* and that varying water characteristics might alter the expression patterns for each homolog resulting in distinct response profiles that can be used to analyze mode of action at the subcellular level.

Next, analysis of transcription of genes related to oxidative stress was performed. Formed reactive oxygen species can damage lipids, carbohydrates, proteins, and DNA in the absence of antioxidants (Fan et al., 2012). Genes involved in cellular anti-oxidative defense and stress response (heat-shock proteins) have been recognized as suggestive biomarkers in response to environmental stress (Kim et al., 2015). Upregulation of genes coding heat shock proteins has been proposed as a stress response for daphnids exposed to zinc (Vandegehuchte et al., 2010). Therefore, expression levels of genes involved in antioxidation pathways such as catalase (*cat*) and glutathione s-transferase (*gst)* provide valuable insights on how organisms cope with oxidative stress. Both *cat* and *gst* respond to exposures of *D. magna* with nanomaterials containing metals (Klaper et al., 2009). Another study showed that changes caused by waterborne Zn on CAT and GST at the protein levels differed depending on salinity for Atlantic killifish (Loro et al., 2012). The result demonstrated that while *cat* levels were positively correlated with bioavailable Zn and that increasing water hardness resulted in lower gene expressions, *gst* expression was significantly upregulated in only hard water with 500 μg/L Zn and remained similar between hardness groups.

Reproduction and development were altered by both hardness and Zn. Variations in water characteristics affect the reproductive

performance of *D. magna* and are used as an endpoint for toxicity tests. Previous studies have shown that hardness plays an important role for reproduction in *D. magna* (Lewis & Maki, 1981). Paulauskis and Winner (1988) reported higher number of progenies in water with the total hardness of 197 mg/L as $CaCO_3$ compared to medium (101.8 mg/L as $CaCO_3$) and soft (51.9 mg/L as $CaCO_3$) water at the end of day 50. However, Terra and Feiden (2003) did not observe any significant differences in progeny numbers from 10 mg/L $CaCO_3$ up to a hardness of 200 mg/L $CaCO_3$. In contrast to this, Cowgill and Milazzo (1991) reported the decreased number of progeny when the hardness increased from 129 to 213 mg/L as $CaCO_3$. In our study, water hardness was shown to affect reproductive success of *D. magna* more effectively than changing the Zn concentrations. Zn concentrations up to 25 μg/L in soft water did not show any significant changes in total offspring number at the end of 21 days reproduction assay. However, in medium water hardness, 50 μg/L Zn produced around 50% more offspring then the lower Zn concentrations at the same water hardness. Although the best reproductive performance was observed with 10 μg/L Zn in the hard water, the number of progenies was significantly lower compared to soft and medium water at concentrations of 3.1 μg/L Zn. These results emphasize the requirement of bioavailable Zn for successful reproduction in *D. magna*.

To understand the variations in reproductive success, the fecundity for each brood was analyzed. During the test period of 21 day, four sets of brood releases were observed for all groups with no significant difference at the first day of reproduction (data not shown). However, in soft and medium water, higher Zn concentrations resulted in a higher number of offspring in first brood. Offspring releases from subsequent broods were similar among the exposure groups. A similar result was observed in *D. magna* exposed to different dietary Zn levels (de Schamphelaere et al., 2004). Although this effect was less pronounced in the hard water, there was still a significant decrease in the number of progenies at first brood with less Zn. This reduction in progeny number was also observed to be a result of increased water hardness in another study (Cowgill et al., 1991). Martins et al. (2017) suggested that the growth characteristics at different water compositions might also affect reproductive success of daphnids. However, body length and body width measurements at the end of the 21 days test period did not show any significant differences in soft water. There was an increase in body size in medium water for Zn concentrations of 10 μg/L and higher. Although no correlation between the size of the daphnids and their reproductive performance was observed, improved reproduction success for the first brood with the addition of Zn was evident.

Reproduction is a commonly used endpoint in toxicity studies performed with *D. magna*. However, most studies carried out according to commonly used test protocols such as OECD *D. magna* reproduction test (OECD, 2012) with focus on changes in fecundity while overlooking the biological processes taking place at the molecular level. Including the changes in expression of genes involved in reproductive processes has the potential to be used as an alternative reproductive endpoint. Temporal gene expression analysis showed that water hardness altered the onset of *vtg* and *jhe* expression. Vitellogenin genes showed a distinct expression level for different water hardness prior to initial expression. Juvenile hormone antagonists have shown to affect crustacean vitellogenesis differently among species. JH analog Altosid ZR-515 and fenoxycarb resulted in reduced growth and reproduction in *D. magna* (Hosmer et al., 1998; Templeton & Laufer, 1983). Juvenoids also elicit anti-ecdysteroid activity by potentially stimulating receptor dimerization between USP and HR38 (Mu & Leblanc, 2004). Thus, JHE plays an important role in reproduction by degrading JH, which blocks ecdysone dependent downstream pathways leading to vitellogenesis involving the HR3:E75 dimer and *FTZ-f1* (Sumiya et al., 2014). Temporal expression levels of *hr3* were similar in each water hardness. However, initial *jhe* upregulation was observed at 88 hph in medium and soft water followed by a downregulation after four hours, whereas initial accumulation in hard water started at 96 hph and reached its peak level at 100 hph. JH degradation by *jhe* resulted in increased *vtg* levels with a delay of four hours. The correlation between *vtg* and *jhe* transcript levels with four hours of delay was in accordance with the anti-ecdysteroid roles of JH. This correlation between *vtg* and *jhe* was not evident until the temporal gene expression analyses were conducted. Thus, carrying out a temporal gene expression analyses improved the understanding of the mode of action governing biological pathways that are dynamic at the gene expression level.

5 | CONCLUSIONS

The exposure of *D. magna* to varying Zn concentrations at different water hardness showed that hardness plays an important role for Zn toxicity by effectively changing the bioavailability of Zn. Risk for Zn deficiency is shown to be higher than its toxic effects in hard water. This differences in bioavailable Zn, combined with the competition between Ca, Mg, and Zn, resulted in altered survival and reproduction. Tools used for Zn bioavailability calculations can be improved by approach presented in this study by taking above-mentioned competition into account as well as considering mortality that can occur due to lack of Zn. This consideration can help to reduce overestimations and overly conservative setting of water quality criteria for Zn and thus better consider Zn essentiality and risk for deficiency scenarios. Using environmental water samples should also be considered to achieve more relevant results. The gene expression data indicates that the toxicogenomic approach is more sensitive compared to mortality and reproduction tests. However, careful consideration is needed to interpret the gene expression data, as it may be caused by the different treatments leading to unsynchronized development. Thus, temporal gene expression analysis can provide valuable insights into the mode of action and help researchers to design sampling strategies. Gene expression assays can also reduce the number of animals used for testing, as well as shortening the test period, especially for reproduction and should be considered as a supplementary method to improve environmental risk assessments.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

Berkay Paylar was involved in conceptualization, carried out investigation and formal analysis, drafted original manuscript. Solomon Asnake was involved in conceptualization. Viktor Sjöberg carried out investigation. Daniel Ragnvaldsson carried out formal analysis. Jana Jass was involved in resources and funding acquisition. Per-Erik Olsson was involved in supervision, Conceptualization, Resources and Funding acquisition. All authors contributed to the interpretation of data and the revision of the manuscript.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material and on request from the authors

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