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Aquatic Toxicology

Influence of environmentally relevant concentrations of Zn, Cd and Ni and their binary mixtures on metal uptake, bioaccumulation and development in larvae of the purple sea urchin *Strongylocentrotus purpuratus*

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ABSTRACT

Metal accumulation, disturbance of Ca^{2+} homeostasis, and occurrence of abnormalities are well-established consequences of single metal exposure during early development stages of sea urchins. However, the effects caused by low concentrations of metals and metal mixtures need to be better understood in marine invertebrates. Therefore, the present study investigated the effects of environmentally relevant concentrations of Zn (9 µg/L), Cd (30 µg/L) and Ni (5 µg/L) in single and binary exposures (Zn + Cd, Cd + Ni and Ni + Zn) to the early life stages of the purple sea urchin *Strongylocentrotus purpuratus*. Endpoints checked in all treatments after 48-h exposure were unidirectional metal influx rates, bioaccumulation, and Ca^{2+} influx rates. Additionally, the presence of abnormal larvae and developmental delay was evaluated at 24 h, 48 h and 72 h of exposure. Unidirectional influx rates of all three metals were significantly higher than control background rates in all single exposures and binary mixtures, and were generally not different between them. Net accumulation (body burden) of both Zn and Cd increased significantly as a result of their respective single exposures, while Ni accumulation decreased considerably. When Zn or Cd were presented in binary exposures with other metals, the net accumulations of Zn or Cd were reduced relative to single exposures to these metals, whereas this did not occur for Ni accumulation. Thus, bioaccumulation proved to be a better metric than influx rate measurements to analyze metal competition at a whole organism level at these low metal concentrations. Short-term Ca^{2+} influx also did not appear to be a sensitive metric, as the measured rates did not vary among all single and binary exposures, with the exception of a lower rate in Ni + Zn binary exposure. A critical aspect observed was the relationship between bioaccumulation *versus* influx measurements, which proved positive for Cd, but negative for Zn and Ni, demonstrating possible capacities for both Zn and Ni regulation by sea urchin larvae. Increases in larval abnormalities relative to controls occurred only after binary mixtures, starting at 48 h exposure and maintained until 72 h. However, delay of the sea urchin development by the presence of gastrula stage at 72 h exposure occurred in Zn and Ni single exposures and all metal mixtures, with very high abnormal development when Ni was present.

1. Introduction

Complex mixtures of trace metals occur naturally in the aquatic environment, and concentrations may rise through anthropogenic input.

Bioavailability-based models on metal bioaccumulation (Meyer et al., 2015; Van Genderen et al., 2015) have been proposed to predict the toxicity of metal mixtures to freshwater animals, and some direct bioaccumulation measurements in mixture exposures have been made in

sensitive model organisms such as rainbow trout (Brix et al., 2016; Niyogi et al., 2015) and great pond snails (Crémazy et al., 2019, 2018). In seawater, only a few studies have examined the effects of metal mixtures in sensitive model species (Phillips et al., 2003; Rouchon and Phillips, 2017), and to our knowledge, only one has tried to link bioaccumulation to the biochemical impacts of metal exposures (Klein et al., 2019).

The early life stage of sea urchins is one of the most sensitive models for acute bioassays of marine pollution (Blewett et al., 2016; Kobayashi, 1990, 1985, 1974; Tellis et al., 2014a, 2014b). Aquatic pollutants such as metals can induce abnormalities at different development stages (Nadella et al., 2013; Phillips et al., 2003; Tellis et al., 2014b, 2014a), at least in part due to competitive interactions with essential ions for binding sites ("biotic ligands") in surface cell membranes. This competition occurs because of the chemical similarity, known as "ionic mimicry", of some metals to essential ions (Bury et al., 2003; Niyogi and Wood, 2004). Cd and Zn are examples of metals that act as Ca^{2+} analogues, directly affecting Ca^{2+} homeostasis. This mechanism is still controversial for Ni since it has been previously reported to disturb Mg^{2+}, Na^+, and K^+ as well as Ca^{2+} homeostasis (Blewett et al., 2016; Blewett and Leonard, 2017; Leonard and Wood, 2013).

Recently, we reported that simultaneous exposure to the essential metal Zn reduced the bioaccumulation of non-essential Cd (but not vice versa) in larval sea urchins (*Strongylocentrotus purpuratus*), whereas each metal tended to protect against the oxidative stress effects of the other metal (Klein et al., 2019). The present study was performed in parallel, focusing on metal influx rates and bioaccumulation, Ca^{2+} influx rates, and developmental aspects in response to environmentally relevant concentrations of Zn (9 µg/L), Cd (30 µg/L) and Ni (5 µg/L) in single and binary exposures (Zn + Cd, Cd + Ni and Ni + Zn) in the same model system.

Unidirectional influx rates of Zn, Cd and Ni were measured using radiotracer techniques in the early life stages of sea urchins under control conditions (no addition of metals) and after 48-h exposures. Ca^{2+} uptake rates were also measured after 48-h exposure. Lastly, the effects of metals and their binary combinations in inducing possible developmental effects were evaluated at 24 h, 48 h and 72 h of exposure, in light of evidence that Ca homeostasis is critical to calcification of the internal skeleton ("spicule") and therefore normal sea urchin development (Raz et al., 2003; Tellis et al., 2013; Wilt, 2002, 1999). Our overall hypothesis was that cationic competition would occur during the metal mixture exposures, affecting the uptake and bioaccumulation of all metals and Ca compared to single exposures. We further predicted that the disturbance of Ca homeostasis would occur, resulting in adverse developmental effects.

2. Material and methods

2.1. Sea urchins

Adults of the purple sea urchin *S. purpuratus* were obtained from WestWind SeaLab Supplies located in Victoria (BC, Canada). Animals were transported to Bamfield Marine Sciences Centre (BMSC) and held in 200-L tanks at $10-15\,°C$ in aerated seawater (32 ppt). Males and females were induced to spawn using the 0.5 mM KCl haemocoel injection method (Hinegardner, 1975). Sperm from spawning males was collected in 50 mL of filtered seawater, then 500 µL of diluted sperm was added to the pooled sea urchin eggs (collected in 250 mL of filtered seawater). Once fertilization of 90 % of the eggs was achieved, the stock was diluted as necessary for the experiments. All exposure experiments were conducted in an incubator at $15 \pm 1\,°C$ under 16:8 h light/dark cycle.

2.2. Exposure solutions

Sea urchin larvae were exposed to Ni, Cd and Zn, their binary

combinations (Zn + Cd, Cd + Ni, Ni + Zn) and natural seawater (background levels; control). Concentrated stock solutions were made in deionized water with analytical grade $ZnSO_4$, $Cd(NO_3)_2.4H_2O$ and $NiCl_2.6H_2O$ (Sigma-Aldrich, St. Louis, MO, USA). The exposure solutions were made by adding the required volumes (<1%) of metal stock solutions to filtered (0.2 µm) seawater (32 ppt) to obtain the desired final metal concentrations. They were allowed to equilibrate for 24 h before the beginning of the experiments.

Nominal metal concentrations in the exposures were 9.7 µg/L (0.15 µmol/L) for Zn, 34.0 µg/L (0.30 µmol/L) for Cd, and 5.0 µg/L (0.08 µmol/L) for Ni. The Zn and Cd levels represent approximately 10 % of the 96-h EC50 values for this species whereas the Ni concentration is only about 2 % (Blewett et al., 2018; Phillips et al., 2003). A relatively lower Ni concentration was studied in light of recent reports of much greater Ni sensitivity in other sea urchin species (Bielmyer et al., 2005; Blewett et al., 2016; Rosen et al., 2015). To our knowledge, there is an absence of regulatory guidelines for multiple metal exposures, but in marine waters, guidelines for Zn range from 15 µg/L (chronic, Australia/New Zealand) to 86 µg/L (acute, USA), for Cd 0.12 µg/L (Canada, chronic) to 33 µg/L (USA, acute), and Ni from 8.2 (chronic, USA) to 70 µg/L (chronic, Australia) (USEPA, 2005; ANZECC, 2000; CCME, 2007).

Measured concentrations are reported in Table 1 and were close to nominal levels; note that the Zn, Cd, and Cd + Zn concentrations were previously reported by Klein et al. (2019). For analyses, water samples were acidified (1% final concentration) with 65 % HNO_3 (Suprapur, Merck, Darmstadt, Germany) and kept refrigerated (4 °C). Samples were desalted following the procedures described by Nadella et al. (2009), and analyzed by graphite furnace atomic absorption spectrophotometry. Full analytical details and quality controls have been reported by Klein and co-authors (2019).

2.3. Total metal accumulation after 48 h in single and binary exposures

Once fertilization reached 90 %, embryos were transferred to polyethene beakers containing 180 mL of single metals (Ni, Zn and Cd), their binary mixtures (Zn + Cd, Cd + Ni, Ni + Zn), or natural seawater (control), totaling 3600 larvae per replicate (20 animals/mL), with five replicates per treatment. At the end of 48-h exposure, samples were vacuum-filtered (~10 s) using polycarbonate membrane filters (8.0 µm, Whatman Nucleopore®Track-Etched Membranes) and rinsed using a seawater solution with 10 mM EDTA (5 mL) to remove surface-bound metal. Each filter was dried and weighed before sample processing and reweighed after drying with the larvae at 65 °C for 24 h. As a result, we had the dry weight of the larvae, which was used to normalize the data. The filters plus larvae were digested in full-strength HNO_3 (Suprapur, Merk, Darmstadt, Germany) at 65 °C for 24 h. Metal concentrations were analyzed by graphite furnace atomic absorption spectrophotometry as for water samples; again, full details are provided by Klein et al. (2019). Digestion of the filter alone showed the background level of metals to be negligible. Data were expressed as µg/g dry larvae.

Table 1
Measured metal concentrations in control, single metal, and binary metal exposures. Means ± SEM (n = 5). Nominal concentrations were Ni = 5.0 µg/L, Cd = 34.0 µg/L, and Zn = 9.7 µg/L.

Treatment	Ni (µg/L)	Cd (µg/L)	Zn (µg/L)
Control*	0.17 ± 0.07	0.18 ± 0.04	0.19 ± 0.11
Zn*	–	–	8.99 ± 0.33
Ni	4.94 ± 0.76	–	–
Cd*	–	29.66 ± 4.91	–
Ni + Zn	4.06 ± 0.25	–	7.92 ± 0.35
Zn + Cd*	–	28.49 ± 4.97	8.69 ± 1.46
Cd + Ni	4.88 ± 0.82	28.70 ± 4.34	–

* Previously reported by Klein et al. (2019).

2.4. Time course of unidirectional influxes

Initially, separate time-series experiments were performed to determine the time course of unidirectional Zn, Cd and Ni uptake in *S. purpuratus* embryos, according to Nogueira et al. (2015). After 90 % egg fertilization, sea urchin embryos were pooled in a concentration of 20 animals/mL and transferred to "cold" single metal solutions (60 mL = 1200 organisms) in polyethene beakers at nominal dissolved concentrations of 9 µg Zn/L, 34 µg Cd/L or 5 µg Ni/L, the same as used in the experimental exposures. Each batch of 1200 animals represented one replicate. The time course of unidirectional influxes was determined by adding 0.2 µCi/mL ^{65}Zn (as ZnCl$_2$, PerkinElmer, Guelph, ON, Canada) or ^{63}Ni (as NiCl$_2$, IsoSolutions™, Vancouver, BC, Canada) or ^{109}Cd (as CdCl$_2$, Eckert and Ziegler, Valencia, CA, USA), per replicate. Radioactivity of 3 replicates per metal was measured every 15 min until the maximum-saturation values were achieved (180 min for Zn and Cd, and 120 min for Ni). At the end of each experimental time, samples were vacuum filtered (~10 s) using polycarbonate membrane filters (8.0 µm, Whatman Nucleopore®Track-Etched Membranes) and then rinsed with 5 mL of a cold metal solution. This solution contained a ten-fold higher "cold" metal concentration (ZnSO$_4$, Cd(NO$_3$)$_2$.4H$_2$O, or NiCl$_2$.6H$_2$O) than the corresponding exposure solution to displace any radioisotope loosely bound to the external surface of the embryos. The polycarbonate membrane was then removed from the vacuum filter and placed in a vial for subsequent measurement.

In order to correct for any radioisotope that remained bound to the filter paper after rinsing, a correction factor was determined for each exposure concentration using blank samples (experimental media with no sea urchin embryos, but containing the respective radioisotope). These samples were filtered and rinsed as described above. For each exposure solution, the radioactivity measured in the filter paper without the larvae was subtracted from that measured in the filter paper with larvae samples. This correction typically amounted to less than 5% of the radioactivity in a sample.

^{63}Ni radioactivities in water (2 mL) and filters + larvae were measured by adding 4 mL of scintillation fluid (Ultima Gold AB, Packard Bioscience, Groningen, The Netherlands) to each sample. Samples were then held in the dark for 3 h prior to counting using a scintillation counter (Tri-carb liquid scintillation analyzer, Perkin Elmer, Illinois, USA). Quench correction was performed through internal standardization. A portable gamma counter (Triathler, Hidex, Helsinki, Finland) was used to measure ^{65}Zn and ^{109}Cd radioactivities, for which quench correction was unnecessary. Data were expressed as ng/g of embryos following the equation:

$$J_{in} = CPM \times (1/SA) \times (1/g \text{ of embryos})$$

where J_{in} is the unidirectional influx rate of the metal, CPM is counts per minute in the larvae, and SA is the specific activity of the experimental medium. The specific activity was calculated using the following equation:

$$SA = [\text{radioactivity concentration (CPM/L)}]/[(\text{metal concentration (µg/L)}]$$

2.5. Unidirectional influx rates after 48 h in single and binary exposures

Unidirectional Ni, Zn, Cd and Ca^{2+} influx rates (Jin) were determined after sea urchin larvae had been exposed for 48 h in polyethene beakers, each containing 60 mL of single metals (Ni, Zn and Cd), their binary mixtures (Zn + Cd, Cd + Ni, Ni + Zn), or natural seawater (control), totaling 1200 larvae per replicate (20 animals/mL). Metal influx rates were measured in the respective single and binary exposure treatments by adding 0.2 µCi/mL ^{65}Zn or ^{63}Ni or ^{109}Cd per sample (7 replicates for each treatment), as described above. A flux period of 60 min was chosen for all measurements of unidirectional influx rates

based on the results of the time-series experiment. Sample filtration, washing, and radioactivity measurement procedures were performed as described previously. Data were expressed as ng/g dry embryos/h.

Additionally, the Ca^{2+} influx rate was measured in sea urchin embryos exposed to all single metals and binary mixtures by adding 0.2 µCi/L ^{45}Ca per sample (as CaCl$_2$, Perkin Elmer, Guelph, ON, Canada) for a flux period of 20 min (7 replicates for each treatment). No additional Ca^{2+} was added in the natural seawater that contained 10 mmol/L Ca. ^{45}Ca radioactivity measurements (4 mL of scintillation fluid added to each 2 mL water sample or filter + larvae sample) were performed by scintillation counting with internal standardization for quench correction, as described above for ^{63}Ni. Ca^{2+} influx data were expressed as pmol/embryos/h.

2.6. Abnormalities

Experiments were conducted in 25-mL polyethene vials, and three replicates were performed per treatment. Each treatment vial contained 20 mL seawater with 20 larvae/mL, totaling 400 larvae per sample. At the end of 24 h, 48 h and 72 h of single and binary metal exposures, samples were vacuum filtered (~10 s) using polycarbonate membrane filters (GE Polycarbonate, 8.0 µm, GE Water and Process Technologies, Trevose, Pa, USA), then rinsed with 5 mL of EDTA solution (10 mM) and fixed with formalin (5%) in 5 mL seawater. First, a screening of larvae with normal and abnormal morphology was evaluated at 400x magnification. Subsequently, all larvae from each sample were evaluated and counted under a microscope with 100x magnification. Embryos that did not display typical blastula (24 h), gastrula (48 h) or pluteus (72 h) morphology, relative to their respective controls were considered as larvae with abnormal development.

2.7. Data presentation and statistical analysis

Data were expressed as means ± standard errors (SEM) where n represents the number of samples per treatment. Influxes in the time series experiments (Ni, Zn and Cd) were analyzed by non-linear regression analyses ("exponential rise to a maximum"), yielding the constants of maximum uptake (i.e. B$_{sat}$ = saturation at this concentration) and the time to half-maximum (K$t_{1/2}$; SigmaPlot11, Systat Software, USA). Comparison of abnormalities in development, unidirectional influx rates, and metal accumulation were made using one-way analysis of variance (ANOVA) followed by Tukey's test. Data were log-transformed when necessary to satisfy ANOVA assumptions (data normality and homogeneity of variances). The significance level adopted was 95 % ($\alpha < 0.05$).

3. Results

3.1. Time course of unidirectional influxes

The time course of metal influxes radiolabeled with ^{65}Zn, ^{109}Cd and ^{63}Ni were well described by "exponential rise to maximum" curves. The B*sat* levels ranged from 58 ng/g for Zn (Fig. 1A), to 28 ng/g for Cd (Fig. 1B), to 17.5 ng/g for Ni (Fig. 1C) at these concentrations (Table 1), which were all significantly different from one another. The corresponding K$t_{1/2}$ times were 45 min for Zn (Fig. 1 A), 54 min for Cd (Fig. 1B), and 11 min for Ni; the Ni K$t_{1/2}$ was significantly different from those of the other two metals (Fig. 1C). Based on these results, the 60-min influx period was chosen for all further influx tests.

3.2. Metal influx rates and accumulation

Unidirectional metal influx rates (Jin) in larvae were measured with radiotracers, over a 60-min period, after 48 h of continuous exposure to elevated metal concentrations (Table 1). For all experimental treatments, influx rates were measured in the presence of the elevated metal
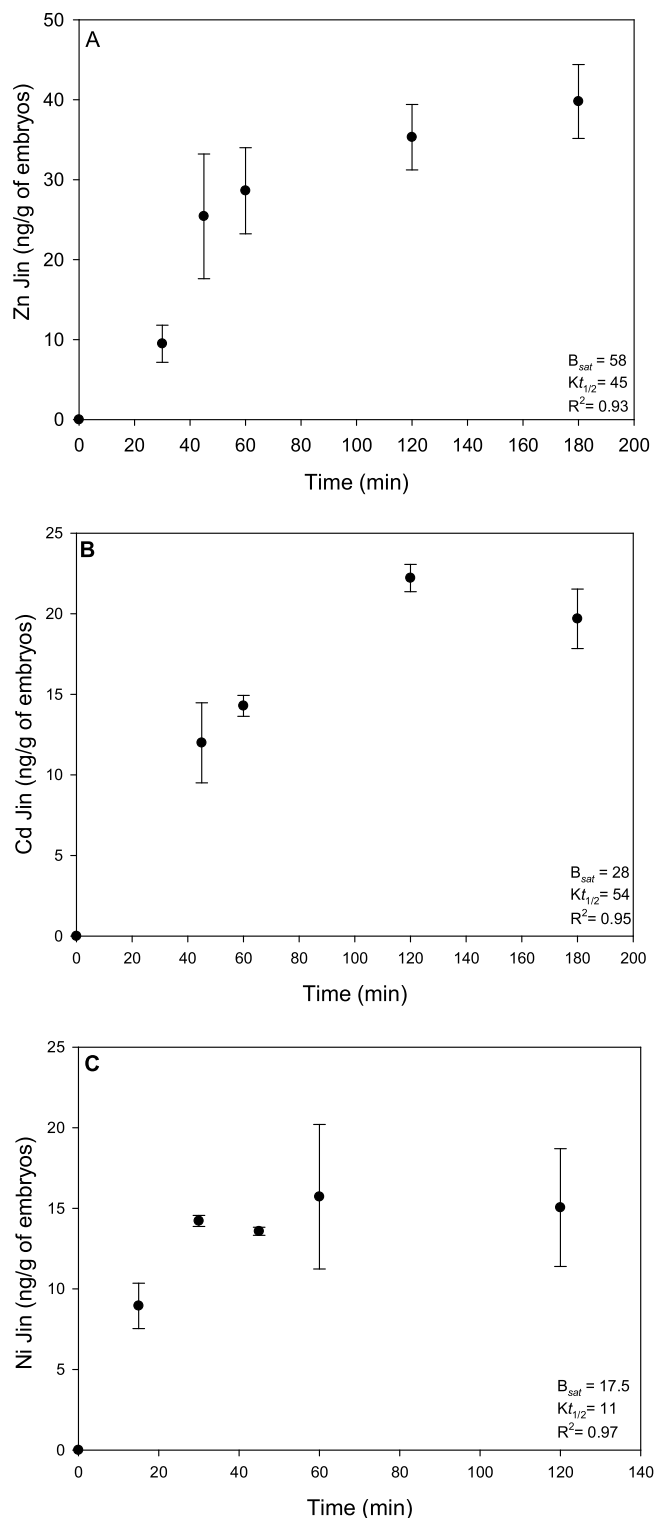
were many-fold greater than in the controls. Absolute rates were generally highest for Zn (Fig. 2A), intermediate for Cd (Fig. 2B), and lowest for Ni (Fig. 2C), in accord with the patterns established in the time series pilot experiment (Fig. 1). Whole body accumulations were similarly greatest for the essential metal Zn (Fig. 2A), but much lower for non-essential Cd (Fig. 2B) and Ni (Fig. 2C).

Influx rates of Zn, Cd and Ni in the early life stages of sea urchin exposed to single and mixtures metals exhibited the same pattern of response (Fig. 2). Metal influxes significantly increased in the single
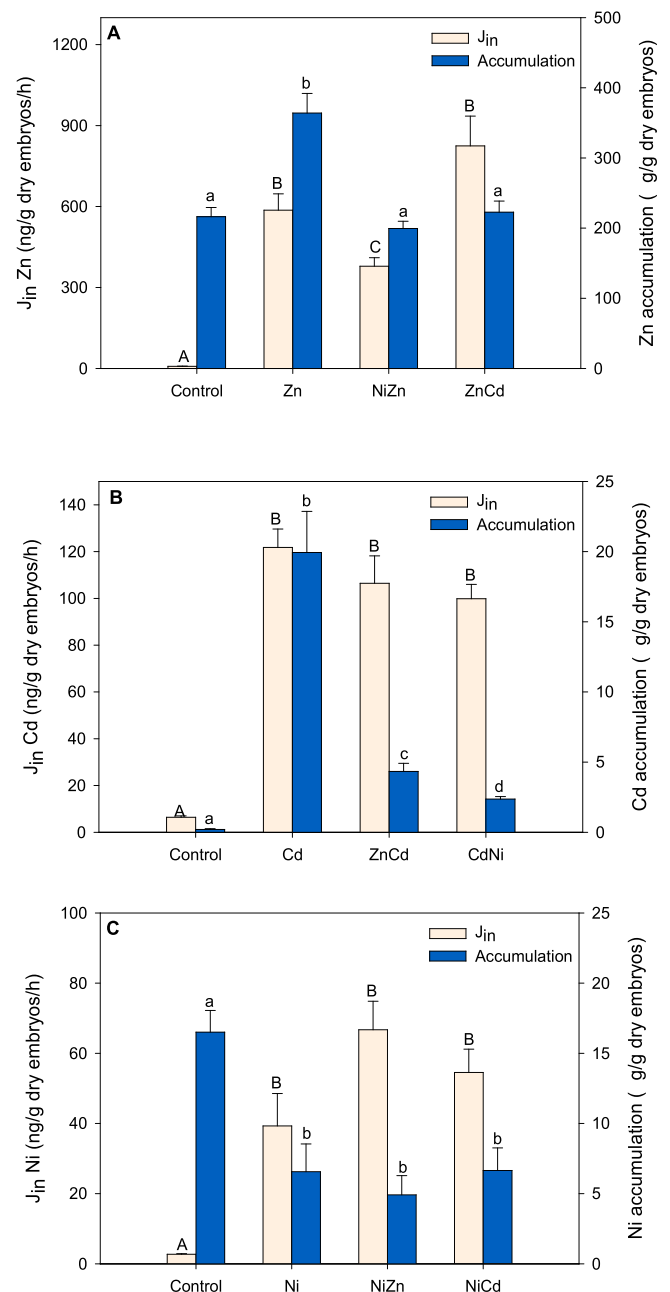

Fig. 1. Time series for unidirectional uptake of (A) Zn, (B) Cd, and (C) Ni influx in sea urchin larvae incubated with ^{65}Zn, ^{109}Cd, and ^{63}Ni respectively. Larvae were sampled after 15, 30, 45, 60, 120 and 180 min. Data are expressed as mean ± SEM (n = 3). The maximum influx (B_{sat}) values were all significantly different among the three metals, while the time to half-maximum influx ($Kt_{1/2}$) values for Ni was significantly different from those for Zn and Cd (p < 0.05).

Fig. 2. Unidirectional influx rates (beige bars) and total metal accumulation (blue bars) after 48 h exposure of sea urchin larvae to (A) Zn, (B) Cd, and (C) Ni, alone and in binary mixtures. For influx measurements, the exposure solutions were radiolabeled with ^{65}Zn, ^{109}Cd, and ^{63}Ni, respectively, and fluxes were measured over a 60-min period. Data are expressed as mean ± SEM (n = 7 for influx rates, n = 5 for total accumulation). Within a specific metal, different letters indicate significantly different mean values among treatments with upper-case letters for influx rates, and lower-case letters for accumulation. Means sharing the same letter are not significantly different at p < 0.05.

concentrations. For the time-matched controls, the larvae had been kept in natural seawater with background metal levels throughout, with influx rate measurements made in these same background concentrations. For all three metals, influx rates in the experimental exposures

exposures and binary mixtures (Cd + Zn, Ni + Zn and Ni + Cd) when compared to their respective control treatment (no metal added), with no differences between them, except for a slightly lower influx rate of Zn when presented in binary combination with Ni (i.e. Ni + Zn) (Fig. 2A).

The high total Zn accumulation seen in the controls was further elevated by Zn single exposure, but levels decreased back to control in the binary exposures (Cd + Zn, Ni + Zn; Fig. 2A). Total Cd accumulation was also greatly increased by a single exposure but decreased significantly in the Zn + Cd exposure, and even further in the Ni + Cd exposure treatment (Fig. 2B). The basal level of Ni accumulation was quite high in the control treatment, and surprisingly it was depressed by more than 50 % after single Ni exposure and both binary mixture treatments (Zn + Ni, Cd + Ni; Fig. 2C). Thus the binary exposures did not reduce Ni bioaccumulation relative to the Ni alone exposure.

3.3. Ca²⁺ influx rate

The unidirectional Ca^{2+} influx rate determined with ^{45}Ca at 48 h was not affected by any of the single metal exposures (Zn, Cd and Ni) or the binary exposures to Zn + Cd. The decrease in the Cd + Ni treatment was not significant. However embryos exposed to Ni + Zn exhibited a significant decrease of Ca^{2+} influx rate when compared to the control and their respective single metal exposures (Cd and Ni; Fig. 3).

3.4. Abnormalities

The types of abnormalities observed in *S. purpuratus* were degenerated, atrophied and deformed larvae. These were low in most treatments up to 24 h, but increased in some treatments thereafter. Sea urchin larvae exposed to single metals (Zn, Cd and Ni) presented similar levels of abnormalities along the times of exposure of 24, 48 and 72 h. However, binary combinations of metals increased the incidence of abnormalities in exposed larvae significantly. Interesting, larvae exposed to the Zn + Cd mixture presented a peak of abnormalities after 48 h then returned to intermediate levels after 72 h of exposure. However, a significant increase of larval abnormalities occurred after Cd + Ni and Ni + Zn exposures at 48 h, and this remained elevated at 72 h (Fig. 4).

3.5. Development

The expected occurrence of the pluteus stage at 72 h of the development was significantly lower in larvae treated with single Zn, Ni, and all binary mixtures (Zn + Cd, Cd + Ni, Ni + Zn) compared to the control
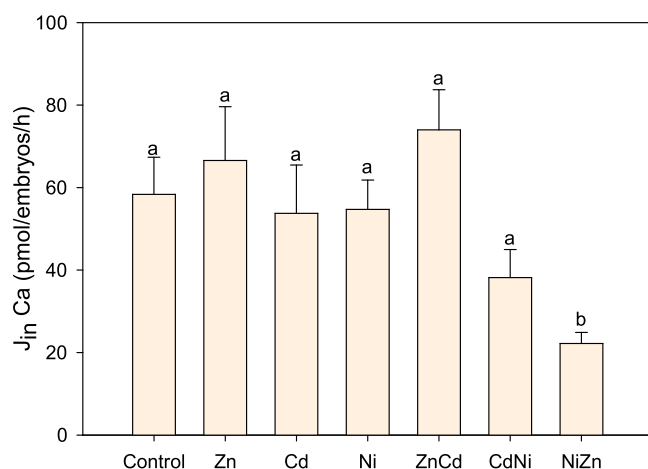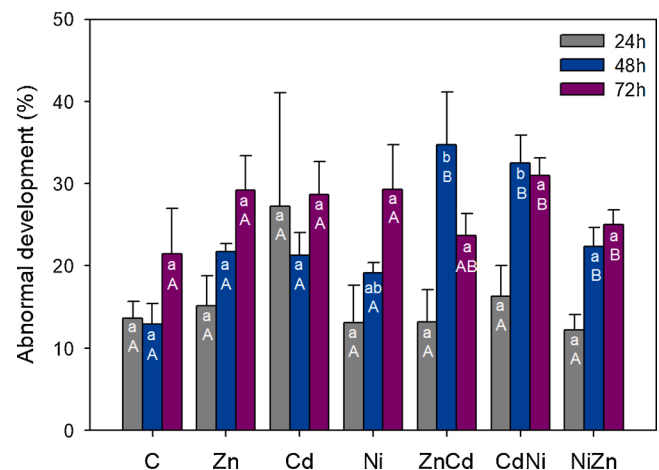

Fig. 4. Percentage occurrence of abnormal larvae of *S. purpuratus* at 24 h. (grey bars), 48 h (blue bars) and 72 h (purple bars) exposure to Zn, Cd, and Ni alone and in binary mixtures. Data are expressed as mean ± SEM (n = 3). Different lower-case letters indicate significantly different mean values among treatments within the same exposure time at p < 0.05. Different upper-case letters indicate significantly different mean values among exposure times within the same treatment at p < 0.05.

group. A remarkably low occurrence of pluteus larvae was observed in larvae exposed to Ni and Ni mixtures, corresponding to 7.8 ± 1.8 % in Ni and 5.5 ± 1.6 % in Cd + Ni and 1.3 ± 0.6 % in Ni + Zn. The treatments that presented low occurrence of the pluteus stage were marked by a concomitant high occurrence of the gastrula stage after 72 h of development, above 90 % (Fig. 5).

4. Discussion

4.1. Overview

The responses of sea urchin embryos exposed to Zn, Cd, Ni and their binary mixtures did not fully correspond with our initial hypotheses. Firstly, we had hypothesized that in combined metal exposures, the metal cations in the water would interfere due to their competition for binding sites in the cellular membranes, and this would be detectable by unidirectional metal influx measurements. However, this was not supported. Bioaccumulation measurements provided a better metric,
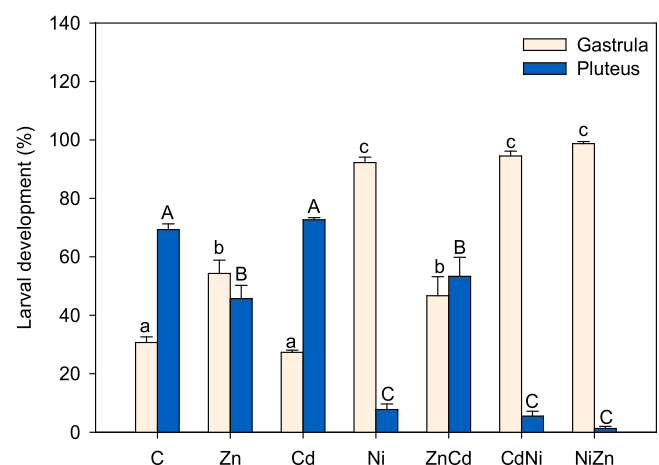

Fig. 3. Unidirectional calcium influx rate (measured with ^{45}Ca) in sea urchin larvae after 48 h exposure to Zn, Cd, and Ni alone and in binary mixtures. Data are expressed as mean ± SEM (n = 7). Means sharing the same letter are not significantly different at p < 0.05.

Fig. 5. Detailed analysis of the development of *S. purpuratus* larvae at 72 h of exposure to Zn, Cd and Ni and their binary mixtures. Data are expressed as mean ± SEM (n = 3). Different letters indicate significant differences among treatments (lower-case letters for gastrula and upper-case letters for pluteus). Means sharing the same letter are not significantly different at p < 0.05.

showing that at least for Zn and Cd, the presence of any of the other metals in binary exposures reduced their body burdens relative to single exposures. One possible explanation is that influx rates represent, in our study, 1-h measurements at the end of 48-h exposure, while the bioaccumulation reflects the total period of 48-h exposure, and integrates not only the influx over this whole period, but also the efflux, and both parameters may be subject to physiological regulation.

Another surprising result was the apparent toxic effect of Ni at very low concentrations, especially in binary mixtures with Zn and Cd. This toxicity was reflected in elevated abnormalities and delayed development. We had also predicted that disturbance of Ca homeostasis would occur, resulting in adverse developmental effects. Tellis et al. (2014a) and Blewett et al. (2016) had demonstrated that one mechanism of toxicity in the early life stages of sea urchins is the disruption of Ca^{2+} homeostasis, but in our experiments, the Ca^{2+} influx rate measured at the end of 48-h exposure was not a useful metric to predict such disruption, though it did tend to be lower in binary exposures in which Ni was present. However, we discuss below the influence of metals (especially Zn and Cd) on other physiological mechanisms, such as the induction of oxidative stress.

4.2. Time course of unidirectional influxes versus bioaccumulation

The time course experiments demonstrated a shorter $Kt_{1/2}$ for Ni (i.e. a faster turnover rate) than for Zn or Cd, in accord with the very rapid Ni uptake rate (complete within 10 min) reported for early life stages of the sea urchin *Lytechinus pictus* by Timourian and Watchmaker (1972). These data suggest that the body pool of Ni is more labile than that of Zn or Cd where $Kt_{1/2}$ values were 4 to 5-fold longer. This may explain why the body burden of Ni seen in the control situation could be reduced in all three of the metal exposure treatments, though it does not explain how the presence of elevated external Ni could actually induce a reduction in total Ni body burden (Fig. 2C). Nevertheless, it is interesting that exactly the same phenomenon [lower whole body Ni concentration in Ni-exposed organisms (40 μg /L) than in control organisms] was reported by Tellis et al. (2014b) in *S. purpuratus* larvae at all time points from 12 h to 60 h. Presumably, increased efflux of Ni is activated early in exposure and stays activated in *S. purpuratus*, as clearly shown in *Lytechinus pictus* (Timourian and Watchmaker, 1972).

The time course experiments, as they were performed immediately post-fertilization, provided useful additional information. The 60-min values in Fig. 1 represent the one-hour unidirectional influx rates during the first hour of metal exposure in the very early life of the sea urchin, while the influx rates in Fig. 2 shows the one-hour influx rates at the end of 48-h metal exposure. Thus, when we compare those values, Ni has a fairly similar influx rate at 1-h (15.72 ± 4.48 ng/g of embryos) and 48-h exposure (39.31 ± 9.22 ng/g of embryos), increasing only 2.5 × . In contrast, Zn influx at 1-h exposure was 28.62 ± 5.38 ng/g of embryo and by 48 h had increased more than 20-fold to 586.34 ± 69.84 ng/g. Similarly, Cd influx rate at 1 h was 14.28 ± 0.64 ng/g of embryos while the 48-h exposure value was 121.79 ± 7.84 ng/g of embryo, an 8.5-fold elevation. Embryo mass does not change appreciably over the first 48 h of development (Tellis et al., 2013, 2014b), so these increases are real. It is known that Zn and Cd are taken up via Ca^{2+} pathways, reflecting their chemical similarities (see Introduction), and according to Tellis et al. (2013, 2014a,b), the unidirectional influx rate of Ca^{2+} in *S. purpuratus* early life stages increases at least 7-fold over the same period. Interestingly, if the unidirectional influx rates of Zn and Cd at 48 h had been maintained over the entire 0–48 h period, they would have accounted for only about 30 % of the net bioaccumulation (above control) of either Zn or Cd measured at 48 h (Figs. 2A,B). The clear implication is that unidirectional influx rates of Zn and Cd must have been considerably higher in the intervening period. Of course, since the net bioaccumulation of Ni actually declined over the same period (Fig. 2C), Ni influx rate was not a limiting factor. Thus, the non-similarity of the Ni pattern compared to the Zn and Cd patterns may implicate an uptake

pathway for Ni different from that for Ca^{2+} in *S. purpuratus*.

The present measurements suggest that the influx rate for Ni is lower than for Cd or Zn (Figs. 1,2) but it must be taken into account that the exposure concentration was also lower – on a molar basis Ni 0.08 μmol/L, Zn 0.15 μmol/L, Cd 0.30 μmol/L – so the comparison is not parallel. At present we know very little about the transport physiology of Ni in seawater or freshwater animals. Suggested mechanisms include facilitated diffusion through channels, transport by divalent metal-specific transporters such as DMT1, or transport by ionic mimicry on nutrient-metal transporters such as those for Mg^{2+} or Ca^{2+} (Pane et al. 2003; Deleebeeck et al. 2009; Niyogi et al. 2014; Blewett et al., 2016; Brix et al., 2017b; Blewett and Leonard, 2017) but there is no definitive evidence.

4.3. Bioaccumulation

The whole body burdens of all three metals at 48 h (Fig. 2) were generally comparable to those previously reported in the same species at this time (Zn: Nadella et al., 2013; Tellis et al., 2014a; Klein et al., 2019; Cd: Klein et al., 2019; Ni: Tellis et al., 2014b), even though in some of these previous Zn and Ni studies, the exposure concentrations were considerably higher. Indeed, for Zn and Ni at 48 h, the bioaccumulation levels usually remained in the same general range as those in unexposed animals, providing strong evidence that these metals are subject to physiological regulation. This was not the case for the non-essential Cd where whole body burden increased dramatically in response to exposure (Fig. 2b; see also Klein et al., 2019). However, regulation of body burden is not unexpected for an essential element such as Zn, and has also been seen for another essential element Cu (Tellis et al., 2014b), but the occurrence of the same phenomenon for Ni, as seen in the present study as well as by Tellis et al. (2014b) supports the controversial contention that it too may be an essential element in aquatic animals (Muyssen et al., 2004; Chowdhury et al., 2008; Blewett and Leonard, 2016; Brix et al., 2017a). One difference from earlier studies (Nadella et al., 2013; Tellis et al., 2014a; Klein et al., 2019) was the significant 60 % increase in Zn body burden at 48 h relative to the control (Fig. 2A). The explanation is unknown; presumably, it reflects imperfect regulation. In the exactly parallel experiments of Klein et al. (2019), there was a non-significant increase of 15 % at this time.

4.4. Metal interactions in binary mixtures

An antagonistic effect was observed in the Zn + Cd treatment, where both metals significantly decreased their accumulation when compared to the respective single metal exposures. In our parallel study (Klein et al., 2019), a significantly decreased bioaccumulation of Cd was observed in the presence of Zn, but no changes in Zn bioaccumulation in the presence of Cd. However, in that study, the presence of Zn protected against oxidative stress caused by Cd, and to a lesser extent, the presence of Cd protected against the oxidative stress caused by Zn (i.e. less than additive effects). In classical toxicity tests with the same species, Phillips et al. (2003) demonstrated antagonism between Cd and Zn. The chemical similarities between Zn and Cd facilitates their competition for binding sites on proteins (Reid and McDonald, 1988; Tellis et al., 2014a). In freshwater organisms, the affinity constant (log K) for Cd (8.6) is higher than that for Zn (5.4; Niyogi and Wood, 2004), so we would anticipate that Cd would compete more effectively against Zn, rather than *vice versa*, though this did not appear to be the case with sea urchin early life stages, suggesting that other mechanisms are also at play (e.g. excretion, physiological regulation).

The log K for Ni (4.0) is even lower in freshwater organisms, but it probably targets different binding sites on proteins than Cd or Zn (Niyogi and Wood, 2004). Therefore it is surprising that presence of a very low concentration of Ni in binary mixtures of Ni + Zn and Ni + Cd generated a decrease in the bioaccumulation of both Zn and Cd (Figs. 2A,B), but there were no accompanying changes of Ni

bioaccumulation (Fig. 2C). However, the presence of Ni alone in the exposure water decreased Ni body burden relative to non-exposed animals, again suggesting physiological regulation (Fig. 2C). According to the toxicity tests performed by Phillips and co-authors (2003) on *S. purpuratus* embryos, Ni + Zn exerted synergistic toxicity, while Ni + Cd exerted additive toxicity. These effects on toxicity differ from the present antagonistic effects on bioaccumulation, though they do concur with effects on development, as discussed below. However, it is important to point out that concentrations used by Phillips et al. (2003) were many-fold higher, with EC50 concentrations of Zn = 97 μg/L, Ni = 341 μg/L and Cd = 342 μg/L.

Interestingly, the whole body Ni levels in *S. purpuratus* embryos were approximately 5 μg/g dry embryos in the present study as well as in Tellis et al. (2014b), but Tellis et al. (2014b) exposed the embryos to 40 μg Ni/L while our study was performed using only 4.9 μg Ni/L. To explore this issue of possible Ni homeostasis further, we carried out an analysis of the relationship between metal bioaccumulation and the influx measurements at 48 h for Ni, as well as for the other two metals (Fig. 6). Unexpectedly, Zn and Ni exhibited similar relationships, and both were very different from the Cd relationship.

Bioaccumulation (*y-axis*) was plotted as a function of unidirectional influx rate for the same metal (*x-axis*), regardless of the presence or absence of other metals (Fig. 6). Not surprisingly, an exponentially rising curve was observed for the non-essential metal Cd (R^2 = 0.99; Fig. 6B) where an increase of Cd influx was associated with increased Cd bioaccumulation. However, both Zn (Fig. 6A) and Ni (Fig. 6C) presented robust negative relationships between bioaccumulation and influx and with R^2 of 0.99 and 0.97, respectively. In both cases, higher rates of metal influx had no effect on bioaccumulation, which was greatest at lowest rates of metal influx, suggesting physiological regulation of both metals in the early life stages of *S. purpuratus* as would be expected for an essential metal such as Zn, and by inference suggesting the possible essentiality of Ni, as discussed earlier.

4.5. Ca^{2+} influx

The absence of changes in the Ca^{2+} influx rates after 48-h exposures of the early life stages of *S. purpuratus* to single Zn, Cd and Ni environments, as well to binary Zn + Cd and Cd + Ni mixtures (Fig. 4) is not in general accord with previous studies, where Ca^{2+} influx rates were inhibited by continuous prior exposure to each of these three metals, albeit at much higher concentrations (Blewett et al., 2016; Tellis et al., 2014b, 2014a). A significant decrease of Ca^{2+} influx rate occurred only in the Zn + Ni binary mixture (Fig. 4). However, it must be remembered that our measurements captured only one 20- min period at the end of 48-h exposure, and that in these previous studies, there were individual times during that 48-h period where significant inhibition did not occur. Furthermore, Brix et al. (2017b) have suggested that the capacity of Ca^{2+} to inhibit Ni uptake or the absence of a Ca^{2+} effect on Ni uptake are related to the metal concentration used in the experiments, as well as the existence of Ca-sensitive and Ca-insensitive uptake pathways.

4.6. Influence of metals on sea urchin development

Zn, Cd and Ni at higher concentrations have been reported to induce patho-physiological changes in various species of sea urchin embryos that result in larval abnormalities and delayed development (Filosto et al., 2008; Kobayashi, 1990; Morroni et al., 2018). Here we have demonstrated that environmentally relevant concentrations of Zn, Ni and binary mixtures of these metals are also able to negatively affect the early development of *S. purpuratus*

Most previous developmental studies on the early life stages of sea urchins have examined only the effects of single metals, at higher concentrations. In the present investigation, despite the anti-oxidant effects of 9 μg/L Zn (Klein et al., 2019), it still caused greater developmental delay than Cd (30 μg/L), and did not protect against the delay caused by
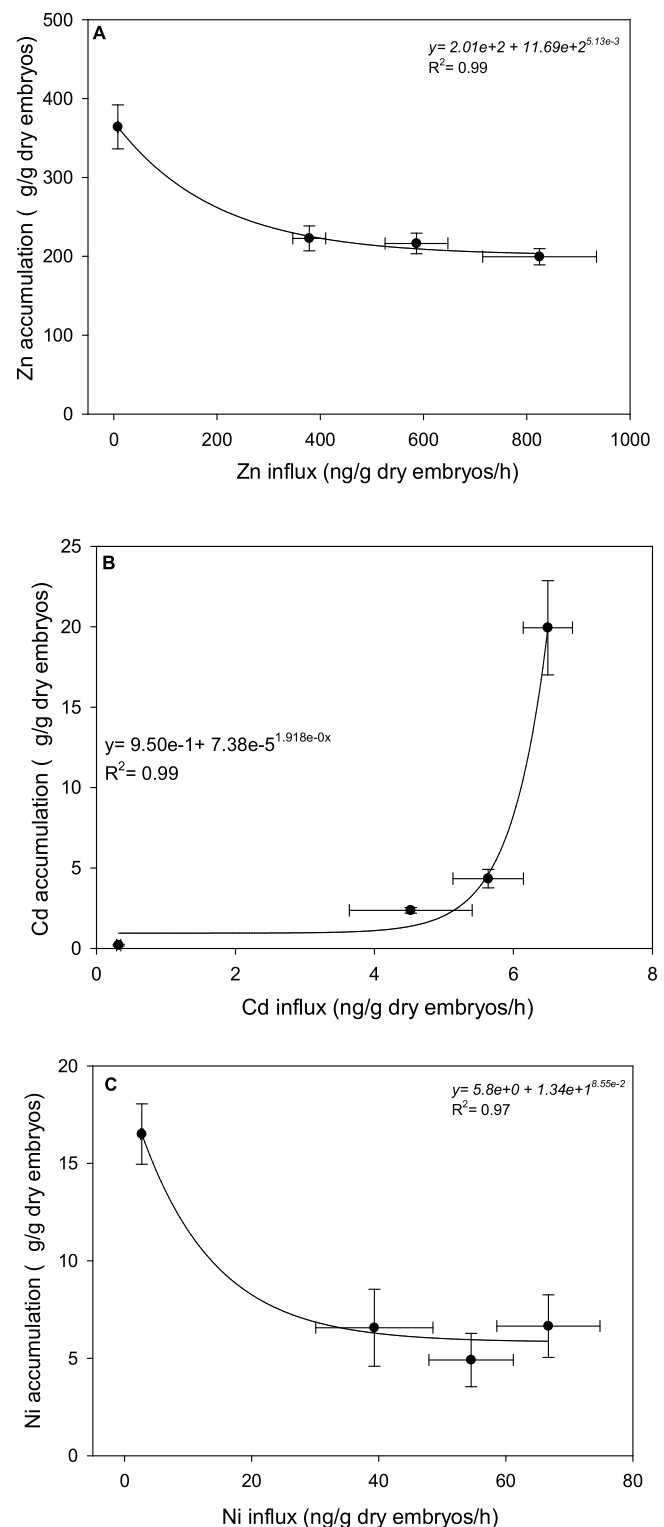
$$y = 2.01e+2 + 11.69e+2^{5.13e-3}$$
$$R^2 = 0.99$$

$$y = 9.50e-1 + 7.38e-5^{1.918e-0x}$$
$$R^2 = 0.99$$

$$y = 5.8e+0 + 1.34e+1^{8.55e-2}$$
$$R^2 = 0.97$$

Fig. 6. The relationships between total accumulation *versus* unidirectional influx rate at 48 h of exposure for (A) Zn, (B) Cd, and (C) Ni in *S. purpuratus* larvae. Data are expressed as means ± 1 SEM ((n = 7 for influx rates, n = 5 for total accumulation).

Ni (4.9 μg/L; Fig. 6). *Anthocidaris crassipina* exhibited abnormal development after 24-h exposure to 50–500 μg/L of Zn, but 98 % of larvae exposed to 20 μg/L of Zn presented healthy development at the same time (Kobayashi, 1990). This same species exposed to a range of Zn concentrations (14–480 μg /L) for 48 h exhibited arrested pluteus

formation, but 3.8 and 7.2 µg Zn/L did not cause the same effects on development (Kobayshi and Okamura, 2004). Skeletal abnormalities were observed in the embryos of the sea urchin *Evechinus chloroticus* exposed to nominal concentrations of 5 and 10 µg Zn/L of Zn (Rouchon and Phillips, 2017) for 72 h. Not only the metal concentration but also the time of exposure seems to be critical in determining the toxic effects of Zn on the normal development of the sea urchin embryos.

The situation appears similar for the non-essential metal, Cd. The induction of abnormalities and delay in development both seem to be related to higher exposure concentrations of this metal (Chiarelli et al., 2014; Filosto et al., 2008; Roccheri et al., 2004) and/or prolonged exposure times in sea urchin early life stages (Filosto et al., 2008). In the present study, exposure to 30 µg/L Cd alone, despite the greater oxidative stress response (Klein et al., 2019), did not increase the occurrence of abnormalities (Fig. 4) or delay development (Fig. 5) relative to the control treatment. Lister and co-authors (2017) demonstrated that abnormal embryonic development in sea urchin is mostly independent of oxidative damage after exposure to polycyclic aromatic hydrocarbons.

The hormetic effects of Cd may contribute to these observations. Cd is one of the well-documented xenobiotics that promotes the hormesis phenomenon in a variety biological models (Gaddipati et al., 2003; Jia et al., 2013; Liu et al., 2015; Zhang et al., 2009). In toxicology, hormesis is a biphasic dose-response relationship characterized by improvement of biological fitness at low doses and inhibitory or toxic effects at a high doses (Mattson, 2008). According to Pagano et al. (1982, 1986), the Cd concentration used here ($30 \mu g/L = 0.27 \times 10^{-6}$ M) belongs to a concentration interval below toxic Cd levels (10^{-5} M) and close to hormetic Cd levels (10^{-7} M) for sea urchin early life stages. The hormesis phenomenon may be reflected in the Zn + Cd abnormalities. In this mixture exposure, the abnormalities in the early life of sea urchin increased significantly between 24 h and 48 h but decreased after 72 h exposure back to values similar to those at 24 h. Pagano et al. (1982) also observed the inversion tendency of toxic effect in the fertilization of the gametes of sea urchins exposed to a Zn + Cd mixture by the reduction of the depression of fertilization success when compared to the effects caused by isolated Zn and Cd exposures. However, in the present study the presence of Cd did not avoid abnormalities in the Ni + Cd mixture, and also developmental delay in the mixtures remained similar to the delays in Ni and Zn isolated exposures.

The adverse effects of the low concentration of Ni (4.9 µg/L) on the *S. purpuratus* development surprised us. The delay in development was above 90 % in both single and binary mixtures. To our knowledge, this is the first report of delay on development caused by such a low concentration of Ni (approximately 0.8×10^{-7} M). Timourian and Watchmaker (1972) found that 10^{-6} M Ni delayed the development of dorsoventral symmetry in *Lytechinus pictus*, while Blewett et al. (2016) reported abnormal development in *Evechinus chloroticus* exposed to 30 µg/L (5×10^{-7} M) Ni for 96 h, and Bielmyer et al. (2005) demonstrated abnormal pluteus development in Diadema antillarum exposed to 15 µg/L (2.5×10^{-7} M), the lowest concentration tested but still three times higher than the concentration used here.

Ni appears to be particularly potent in causing developmental delay, as the phenomenon was much less marked in single exposures to Zn or Cd, but was always prominent in mixtures whenever Ni was present (Fig. 5). Skeletal mineralization is critical to development in sea urchin larvae (Raz et al., 2003; Tellis et al., 2013; Wilt, 2002, 1999) and the presence of Ni is frequently associated with decreased Ca^{2+} uptake (Blewett et al., 2016; Tellis et al., 2014b). However, we cannot confirm this inhibition from our Ca^{2+} influx measurements, which were recorded only at 48 h of exposure (Fig. 4). At higher Ni concentrations (10^{-7} M and upwards), several authors have reported delayed and/or abnormal development manifested as decreased skeletal formation, disrupted dorsoventral differentiation of ectodermal cells, and lack of dorsoventral symmetry (Timourian and Watchmaker, 1972; (Hardin et al., 1992).

An increased occurrence of abnormal larvae was observed after sea urchin embryos were exposed for 48 h to all three binary mixtures (Zn + Cd, Cd + Ni and Ni + Zn; Fig. 4), despite generally antagonistic effects on bioaccumulation (Fig. 2). There was greater developmental delay associated with the latter two (Fig. 5), in accord with the toxicity data of Phillips et al. (2003). Our results reinforce that the first 48 h of the sea urchin development are especially sensitive to the presence of low concentrations of metal mixtures, resulting in abnormal development, and that by 72 h, developmental delay becomes very prominent, especially when Ni is present. It remains unknown whether recovery from these abnormalities and delays ever occurs, or whether these larvae ultimately die.

Author contributions

The study was conceived, the experiments were performed, and the data were analyzed by all authors. CMW and AB provided the funding. LSN, FXVDM, and CMW wrote the first draft, and all authors revised the manuscript.

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Lygia S. Nogueira: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Fabíola Xochilt Valdez Domingos-Moreira:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Roberta Daniele Klein:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Adalto Bianchini:** Conceptualization, Methodology, Resources, Writing - review & editing. **Chris M. Wood:** Conceptualization, Methodology, Formal analysis, Resources, Writing - review & editing.

Declaration of Competing 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.

References

ANZECC, 2000. Australian and New Zealand Guidelines for Fresh and Marine Water Quality.

Bielmyer, G.K., Brix, K.V., Capo, T.R., Grosell, M., 2005. The effects of metals on embryo-larval and adult life stages of the sea urchin, *Diadema antillarum*. Aquat. Toxicol. 74, 254–263. https://doi.org/10.1016/j.aquatox.2005.05.016.

Blewett, T.A., Leonard, E.M., 2017. Mechanisms of nickel toxicity to fish and invertebrates in marine and estuarine waters. Environ. Pollut. 223, 311–322. https://doi.org/10.1016/j.envpol.2017.01.028.

Blewett, T.A., Smith, D.S., Wood, C.M., Glover, C.N., 2016. Mechanisms of nickel toxicity in the highly sensitive embryos of the sea urchin *Evechinus chloroticus*, and the modifying effects of natural organic matter. Environ. Sci. Technol. 50, 1595–1603. https://doi.org/10.1021/acs.est.5b05626.

Blewett, T.A., Dow, E.M., Wood, C.M., McGeer, J.C., Smith, D.S., 2018. The role of dissolved organic carbon concentration and composition on nickel toxicity to early life-stages of the blue mussel Mytilus edulis and purple sea urchin *Strongylocentrotus purpuratus*. Ecotoxicol. Environ. Saf. 160, 162–170. https://doi.org/10.1016/j. ecoenv.2018.05.029.

Brix, K.V., Tellis, M.S., Crémazy, A., Wood, C.M., 2016. Characterization of the effects of binary metal mixtures on short-term uptake of Ag, Cu, and Ni by rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 180, 236–246. https://doi.org/10.1016/j.aquatox.2016.10.008.

Bury, N.R., Walker, P.A., Glover, C.N., 2003. Nutritive metal uptake in teleost fish. J. Exp. Biol. 206 (11), LP–23. https://doi.org/10.1242/jeb.00068.

CCME, 2007. Canadian Environmental Quality Guidelines: Canadian Water Quality Guidelines for the Protection of Aquatic Life.

Chiarelli, R., Agnello, M., Bosco, L., Roccheri, M.C., 2014. Sea urchin embryos exposed to cadmium as an experimental model for studying the relationship between autophagy and apoptosis. Mar. Environ. Res. 93, 47–55. https://doi.org/10.1016/j.marenvres.2013.06.001.

Crémazy, A., Brix, K.V., Wood, C.M., 2018. Chronic toxicity of binary mixtures of six metals (Ag, Cd, Cu, Ni, Pb, and Zn) to the great pond snail *Lymnaea stagnalis*. Environ. Sci. Technol. 52, 5979–5988. https://doi.org/10.1021/acs.est.7b06554.

Crémazy, A., Brix, K.V., Wood, C.M., 2019. Using the Biotic Ligand Model framework to investigate binary metal interactions on the uptake of Ag, Cd, Cu, Ni, Pb and Zn in the freshwater snail *Lymnaea stagnalis*. Sci. Total Environ. 647, 1611–1625. https://doi.org/10.1016/j.scitotenv.2018.07.455.

Filosto, S., Roccheri, M.C., Bonaventura, R., Matranga, V., 2008. Environmentally relevant cadmium concentrations affect development and induce apoptosis of *Paracentrotus lividus* larvae cultured in vitro. Cell Biol. Toxicol. 24, 603–610. https://doi.org/10.1007/s10565-008-9066-x.

Gaddipati, J.P., Rajeshkumar, N.V., Grove, J.C., Maharaj, S.V.M., Centeno, J.A., Maheshwari, R.K., Jonas, W.B., 2003. Low-dose cadmium exposure reduces human prostate cell transformation in culture and up-regulates metallothionein and MT-1G mRNA. Nonlinearity Biol. Toxicol. Med. 1, 199–212.

Hardin, J., Coffman, J.A., Black, S.D., McClay, D.R., 1992. Commitment along the dorsoventral axis of the sea urchin embryo is altered in response to NiCl2. Development 116, 671.

Hinegardner, R.T., 1975. Morphology and genetics of sea-urchin development. Am. Zool. 15, 679–689.

Jia, L., He, X., Chen, W., Liu, Z., Huang, Y., Yu, S., 2013. Hormesis phenomena under Cd stress in a hyperaccumulator–*Lonicera japonica* Thunb. Ecotoxicology 22, 476–485. https://doi.org/10.1007/s10646-013-1041-5.

Klein, R.D., Nogueira, L.S., Domingos-Moreira, F.X.V., Costa, P.G., Bianchini, A., Wood, C.M., 2019. Effects of sublethal Cd, Zn, and mixture exposures on antioxidant defense and oxidative stress parameters in early life stages of the purple sea urchin *Strongylocentrotus purpuratus*. Aquat. Toxicol. 217, 105338.

Kobayashi, N., 1974. Marine pollution bioassay by sea urchin eggs, an attempt to enhance accuracy, I. Publ. Seto Mar. Biol. Lab. XXI (5/6), 377–391.

Kobayashi, N., 1985. Marine pollution bioassay by sea urchin eggs, an attempt to enhance accuracy, II. Publ. Seto Mar. Biol. Lab. 34, 225–237.

Kobayashi, N., 1990. Marine pollution bioassay by sea urchin eggs, an attempt to enhance sensitivity. Publ. Seto Mar. Biol. Lab. 34, 225–237.

Leonard, E.M., Wood, C.M., 2013. Acute toxicity, critical body residues, Michaelis–menten analysis of bioaccumulation, and ionoregulatory disturbance in response to waterborne nickel in four invertebrates: <i>Chironomus riparius, Lymnaea stagnalis, Lumbriculus variegatus and Daphnia pule. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 158, 10–21. https://doi.org/10.1016/j.cbpc.2013.03.008.

Liu, Z., Chen, W., He, X., Jia, L., Yu, S., Zhao, M., 2015. Hormetic responses of *Lonicera japonica* Thunb. To cadmium stress. DoseResponse 13 (1). https://doi.org/10.2203/dose-response.14-033.He.

Mattson, M.P., 2008. Hormesis defined. Ageing Res. Rev. 7, 1–7. https://doi.org/10.1016/j.arr.2007.08.007.

Meyer, J.S., Farley, K.J., Garman, E.R., 2015. Metal mixtures modeling evaluation project: 1. Background. Environmental Toxicology and Chemistry 34, 726–740. https://doi.org/10.1002/etc.2792.

Morroni, L., Pinsino, A., Pellegrini, D., Regoli, F., 2018. Reversibility of trace metals effects on sea urchin embryonic development. Ecotoxicol. Environ. Saf. 148, 923–929. https://doi.org/10.1016/j.ecoenv.2017.11.013.

Nadella, S.R., Fitzpatrick, J.L., Franklin, N., Bucking, C., Smith, S., Wood, C.M., 2009. Toxicity of dissolved Cu, Zn, Ni and Cd to developing embryos of the blue mussel (*Mytilus trossolus*) and the protective effect of dissolved organic carbon. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 149, 340–348. https://doi.org/10.1016/j.cbpc.2008.09.001.

Nadella, S.R., Tellis, M., Diamond, R., Smith, S., Bianchini, A., Wood, C.M., 2013. Toxicity of lead and zinc to developing mussel and sea urchin embryos: critical tissue residues and effects of dissolved organic matter and salinity. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 158, 72–83. https://doi.org/10.1016/j.cbpc.2013.04.004.

Niyogi, S., Wood, C.M., 2004. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. Environ. Sci. Technol. 38, 6177–6192. https://doi.org/10.1021/es0496524.

Niyogi, S., Nadella, S.R., Wood, C.M., 2015. Interactive effects of waterborne metals in binary mixtures on short-term gill–metal binding and ion uptake in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 165, 109–119. https://doi.org/10.1016/j.aquatox.2015.05.016.

Pagano, G., Esposito, A., Giordano, G.G., 1982. Fertilization and larval development in sea urchins following exposure of gametes and embryos to cadmium. Arch. Environ. Contam. Toxicol. 11, 47–55.

Pagano, G., Cipollaro, M., Corsale, G., Esposito, A., Ragucci, E., Giordano, G.G., Trieff, N.M., 1986. The sea urchin: bioassay for the assessment of damage from environmental contaminants. In: Cairns Jr., J. (Ed.), Community Toxicity Testing. Association for Standard Testing and Materials, Philadelphia, pp. 67–92.

Phillips, B.M., Nicely, P.A., Hunt, J.W., Anderson, B.S., Tjeerdema, R.S., Palmer, S.E., Palmer, F.H., Puckett, H.M., 2003. Toxicity of cadmium-copper-nickel-zinc mixtures to larval purple sea urchins (*Strongylocentrotus purpuratus*). Bull. Environ. Contam. Toxicol. 70, 592–599. https://doi.org/10.1007/s00128-003-0026-0.

Raz, S., Hamilton, P.C., Wilt, F.H., Weiner, S., Addadi, L., 2003. The transient phase of amorphous calcium carbonate in sea urchin larval spicules: the involvement of proteins and magnesium ions in its formation and stabilization. Adv. Funct. Mater. 13, 480–486. https://doi.org/10.1002/adfm.200304285.

Reid, S.D., McDonald, D.G., 1988. Effects of cadmium, copper, and low pH on ion influxes in the rainbow trout, *Salmo gairdneri*. Can. J. Fish. Aquat. Sci. 45, 244–253. https://doi.org/10.1139/f88-029.

Roccheri, M.C., Agnello, M., Bonaventura, R., Matranga, V., 2004. Cadmium induces the expression of specific stress proteins in sea urchin embryos. Biochem. Biophys. Res. Commun. 321, 80–87. https://doi.org/10.1016/j.bbrc.2004.06.108.

Rosen, G., Rivera-Duarte, I., Colvin, M.A., Dolecal, R.E., Raymundo, L.J., Earley, P.J., 2015. Nickel and copper toxicity to embryos of the long-spined sea urchin, *Diadema savignyi*. Bull. Environ. Contam. Toxicol. 95, 6–11. https://doi.org/10.1007/s00128-015-1457-0.

Rouchon, A.M., Phillips, N.E., 2017. Acute toxicity of copper, lead, zinc and their mixtures on the sea urchin *Evechinus chloroticus*. N. Z. J. Mar. Freshwater Res. 51, 333–355. https://doi.org/10.1080/00288330.2016.1239643.

Tellis, M.S., Lauer, M.M., Nadella, S., Bianchini, A., Wood, C.M., 2013. Ionic status, calcium uptake, and Ca2+ -ATPase activity during early development in the purple sea urchin (*Strongylocentrotus purpuratus*). Comp. Biochem. Physiol., Part A Mol. Integr. Physiol. 166, 272–277. https://doi.org/10.1016/j.cbpa.2013.05.028.

Tellis, M.S., Lauer, M.M., Nadella, S., Bianchini, A., Wood, C.M., 2014a. Sublethal mechanisms of Pb and Zn toxicity to the purple sea urchin (*Strongylocentrotus purpuratus*) during early development. Aquat. Toxicol. 146, 220–229. https://doi.org/10.1016/j.aquatox.2013.11.004.

Tellis, M.S., Lauer, M.M., Nadella, S., Bianchini, A., Wood, C.M., 2014b. The effects of copper and nickel on the embryonic life stages of the purple sea urchin (*Strongylocentrotus purpuratus*). Arch. Environ. Contam. Toxicol. 67, 453–464. https://doi.org/10.1007/s00244-014-0069-8.

Timourian, H., Watchmaker, G., 1972. Nickel uptake by sea urchin embryos and their subsequent development. J. Exp. Zool. 182, 379–387. https://doi.org/10.1002/jez.1401820309.

USEPA, 2005. Updates: Water Quality Criteria Documents for the Protection of Aquatic Life in Ambient Water (EPA-820-B-96-001).

Van Genderen, E., Adams, W., Dwyer, R., Garman, E., Gorsuch, J., 2015. Modeling and interpreting biological effects of mixtures in the environment: introduction to the metal mixture modeling evaluation project. Environ. Toxicol. Chem. 34, 721–725. https://doi.org/10.1002/etc.2750.

Wilt, F.H., 1999. Matrix and mineral in the sea urchin larval skeleton. J. Struct. Biol. 126, 216–226. https://doi.org/10.1006/jsbi.1999.4105.

Wilt, F.H., 2002. Biomineralization of the spicules of sea urchin embryos. Zool. Sci. 19, 253–261. https://doi.org/10.2108/zsj.19.253.

Zhang, Y., Shen, G., Yu, Y., Zhu, H., 2009. The hormetic effect of cadmium on the activity of antioxidant enzymes in the earthworm *Eisenia fetida*. Environ. Pollut. 157, 3064–3068. https://doi.org/10.1016/j.envpol.2009.05.039.