**Metallic nickel nanoparticles and their effect on the embryonic development of the sea urchin *Paracentrotus lividus***

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**Abstract**

The presence of nanoparticles in many industrial applications and daily products is making it nowadays crucial to assess their impact when exposed to the environment. Metallic nickel nanoparticles (Ni NPs) are of high industrial interest due to their ability to catalyze the reversible hydration of $CO_2$ to carbonic acid at ambient conditions. We characterized metallic Ni NPs by XRD, HRTEM and EDS and determined the solubility of free nickel ions from 3 mg/L metallic Ni NPs in seawater by ICP-MS over 96 h, which was below 3%. Further, embryonic development of the sea urchin *Paracentrotus lividus* was investigated for 48 h in the presence of metallic Ni NPs (0.03 mg/L to 3 mg/L), but no lethal effects were observed. However, 3 mg/L metallic Ni NPs caused a size reduction similar to 1.2 mg/L $NiCl_2$*6 $H_2O$. The obtained results contribute to current studies on metallic Ni NPs and point to their consequences for the marine ecosystem.

**Capsule**

Metallic nickel nanoparticles display weak dissolution rates in seawater, but higher concentrations resulted in similar effects on sea urchin embryonic development as nickel salt.

**Keywords**

Metallic nickel nanoparticle; Solubility; Seawater; Sea urchin; Embryonic development

## 1. Introduction

Today, there is a great variety of engineered nanoparticles (NPs) available that find use in numerous applications due to their favorable properties (Ju-Nam and Lead, 2008). Some of the most commonly found NPs are $TiO_2$, Ag, CuO, ZnO and NiO, which are present in various products such as cosmetics, health care products, clothing, and electronic devices or find use as catalysts. As a consequence of their application they can reach surrounding ecosystems, including estuarine, freshwater and marine ecosystems, for example by wastewater output or aerial deposition (Wiesner et al., 2006; Baker et al., 2014). However, their potential effect on the environment and therein living organisms is still investigated insufficiently (Nowack and Bucheli, 2007; Ju-Nam and Lead, 2008) and suitable tools to identify interactions of NP with organic material are still deficient in order to achieve a better understanding and determine guidelines for their safe application (Love et al., 2012; Smita et al., 2012). So far, most studies focused on the effects of NPs exposure to freshwater organisms, but progressively more information on marine organisms is gathered (reviewed by Matranga and Corsi, 2012; Baker et al., 2014), which is important since oceans fulfill principal functions for a stabile ecosystem and as food source.

Nickel is the 24$^{th}$ most abundant element in the Earth's crust. The International Nickel Study group (INSG) reported that the global primary nickel production in 2014 was 1.93 Mt and projected production for 2015 to ~ 1.95 Mt (INSG Insight, 2014). Nickel is used in many industrial and commercial applications including electroplating, catalysis, battery manufacture, forging, metal finishing and mining; all of which lead to environmental pollution by nickel. Nickel is a transition metal that exists in five oxidation states and can be divided into four categories – soluble, sulphidic, oxidic and metallic – and toxicity can depend on the state of nickel (Muñoz and Costa, 2012). Exposure to highly nickel-polluted environments has the potential to produce various pathological effects in humans, such as contact dermatitis, lung fibrosis, cardiovascular and kidney diseases and cancer (e.g. Huang et al., 2011; Coman et al., 2013). With the common industrial use of nickel, the application of metallic nickel nanoparticles (Ni NPs) has progressed. The most recent potential application of metallic Ni NPs is to catalyze the reversible hydration of $CO_2$ to carbonic acid at room temperature and atmospheric pressure, which is of high importance for $CO_2$ capture technologies and mineralization processes, and has been suggested to be applied primarily to point flue sources such as power plants or air-condition outlets on the top of buildings (Bhaduri and Šiller, 2012; Bhaduri and Šiller, 2013a). Those NPs are capable of accelerating the mineral carbonation process when alkaline ions ($Ca^{2+}$, $Mg^{2+}$) are readily available in solution (Bhaduri and Šiller, 2013b; Bodor et al., 2014). Ni NPs can further be immobilized on silica which solves the issue of recovering Ni NPs from slurry containing carbonate precipitates (Han et al., 2015). Nevertheless, the effect of metallic Ni NPs when exposed to the environment, including freshwater and marine ecosystems, remains to be investigated in order to ensure their safe use.

The physio-chemical characteristics (e.g. size, chemical composition, dissolution, agglomeration) of NPs are often depending on the environmental conditions (Griffitt et al., 2008; Keller et al., 2010; Odzak et al., 2014) and can account for their impact on organisms. The main characteristics of NPs in seawater were reviewed by Baker et al. (2014): first, the high ionic strength in this medium is supposed to increase agglomeration of NPs and thereby reduces the dissolution potential, but these effects are still in correlation with the concentration; second, dissolved metal cations will be complexed by free anions in salt water (e.g. chlorine anions) but uptake of agglomerates and/or ions depends on the organism.

The early embryonic development of sea urchins is a commonly used, highly sensitive and suitable marine *in vivo* model system for toxicological and eco-toxicological studies (reviewed by Baker et al., 2014; Matranga and Corsi, 2012). Several studies used this model to examine the effects of NPs. ZnO NPs were highly toxic in low concentrations ($EC_{50}$ 99.5 μg/L) to embryonic development of *Lytechinus pictus* due to ion dissolution, whereas insoluble $CeO_2$ NPs and $TiO_2$ NPs induced no abnormalities up to 10 mg/L (Fairbairn et al., 2011). ZnO NPs also induced toxicity in *Paracentrotus lividus* sperm and embryos which was not exclusively due to dissolution but also affected by interactions of NPs with the seawater and/or the organism (Manzo et al., 2013). Further, Ag NPs were reported to be toxic to *P. lividus* embryonic development rather than the dissolved Ag ions (Šiller et al. 2013). Offspring from NPs (Ag, $TiO_2$ and Co) exposed *P. lividus* sperm displayed morphological abnormalities and spicule deformations (Gambardella et al., 2015). A comparative study on three Mediterranean sea urchins further reported that the effects of Ag NPs were species-specific and that the developmental stage, when embryos were first exposed to the NPs, was important (Burić et al., 2015). For adult sea urchins that were forced to ingest NPs ($10^{-2}$ g/L), agglomerates of nanoparticles ($SnO_2$, $CeO_2$ and $Fe_3O_4$) were found in the digestive, immune and reproductive system and caused mortality within few days (Falugi et al., 2012).

The effects of nickel-containing NPs were investigated mainly on freshwater organisms. A comparative study on metallic NPs, including nickel, used three different freshwater organisms showing that effects on daphnia were mainly due to ion dissolution (1% after 48 h) and tested alga were most vulnerable to nano-nickel (Griffitt et al., 2008). Highlighting the fact that toxicity differs with chemical composition of the NPs and the exposed organism. In zebrafish, the configuration of metallic Ni NPs affected toxicity stronger than shape and size, and the NPs acted differently than soluble nickel salt (Ispas et al., 2009), whereas NiO NPs caused cumulative mortality in adults and embryos (Kovriznych et al., 2013). NiO NPs were also tested on aquatic macrophytes and caused cellular oxidative stress (Oukarroum et al., 2015). In addition several investigations with metallic Ni NPs and nickel-based NPs were performed on mammalian cells *in vitro* and *in vivo* (reviewed by Magaye and Zhao, 2012) and showed that cytotoxicity was a function of surface charge, available surface sites and ion dissolution (Chusuei et al., 2013). However, investigations on nickel-based NPs are rather limited for marine organisms. Low ion dissolution from NiO NPs (21% after 28 days) was reported in seawater and up to 1000 µg/g nickel was accumulated in marine amphipods, but no significant mortality was observed for up to 2000 µg/g NiO NPs (Hanna et al., 2013). Another study reported $EC_{50}$ with 32.28 mg/L NiO NPs after 72 h for marine microalga and ion dissolution was stated with 0.14%, which was contributing in addition to aggregate attachment to the cell surface (Gong et al., 2011). Similar studies for nickel-based NPs on marine organisms have not been reported so far, leaving possible effects on the marine ecosystem largely unanswered.

We characterized metallic Ni NPs by different methods, including high resolution transmission electron microscopy, Energy dispersive X-ray spectroscopy and X-ray diffraction. The solubility of free nickel ions from metallic Ni NPs in seawater was determined by inductively coupled plasma mass spectroscopy. Additionally, the impact of different metallic Ni NPs and nickel salt concentrations was investigated in a comparative manner on the embryonic development of the sea urchin *P. lividus*.

## 2. Materials and methods

### *2.1. Characterization of metallic nickel nanoparticles*

Metallic Ni NPs were purchased from Nano Technologies (Korea). The size distribution of metallic Ni NPs was analyzed by high resolution transmission electron microscopy (HRTEM) using a JEOL 2100F field emission gun instrument operating at 200 keV located in Durham University, UK, as reported previously (Bhaduri and Šiller, 2013a). Samples for HRTEM measurements were prepared on Cu grids with lacey carbon films (300 mesh, Agar Scientific). The presence of metallic nickel was confirmed using energy dispersive X-ray spectroscopy (EDS). X-ray diffraction (XRD) was carried out using PANalytical X'pert Pro diffractometer with a X'Celerator area detector and Cu Kα X-rays were used.

### *2.2. Solubility of nickel ions from metallic nickel nanoparticles*

As published previously (Šiller et al., 2013), a dialysis membrane (MWCO=3,500, diameter=11.5 mm; Spectrum Laboratories) was used to determine dissolution of free nickel ions from metallic Ni NPs in artificial seawater (ASW, see 2.3.). For suitable dispersion of the particles, metallic Ni NPs (final concentration: 3 mg/L) were treated in 10 mL ASW for 3 min with ultra-sonication (Hilsonic, UK) , filled in a dialysis membrane and kept in 1 L of deionized water.  Samples of 10 mL were collected after 24, 48, 72, 96 h and 46 days from the beaker. Free nickel ions in solution were determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500 ICP-MS; detection limit: ±0.001 mg/L) at Environmental Scientific Group (ESG), UK (http://www.esg.co.uk) with standard solutions as reference. Dynamic light scattering (DLS) analysis of metallic Ni NPs were performed in deionized water and ASW. Detailed information on ICP-MS and DLS measurements is provided in supplementary material.

*2.3. Sea urchin larval culture*

Adult *Paracentrotus lividus* were collected in the area of Rovinj (Croatia) and maintained in seawater tanks at 18°C at the Dept. Zoology (University of Stuttgart, Germany). Artificial seawater (ASW) with pH 8.0 and salinity 35‰ was prepared with nanopore-filtered deionized water. ASW was prepared freshly for each experiment and supplemented either with metallic Ni NPs (final concentrations: 3 mg/L, 0.3 mg/L and 0.03 mg/L) followed by 3 min ultra-sonication treatment (Sonopuls and UW 3100, Bandelin electronic, Berlin, Germany) or with nickel salt ($NiCl_2$*6 $H_2O$; final concentrations: 0.03 mg/L. 0.3 mg/L, 1.2 mg/L, 3 mg/L, 12 mg/L and 24 mg/L). Spawning of gametes was induced by injection of 0.5 M KCl. Gametes from different individuals were pooled and fertilized in ASW at 18-19°C. At the 4- to 8-cell stage (2 h post-fertilization (hpf)) embryos were washed and transferred to culture dishes containing ASW or ASW supplemented with metallic Ni NP or nickel salt. Embryonic development was monitored using an Olympus SZH 10 binocular (Olympus, Hamburg, Germany). Separate experiments were carried out with at least duplicate samples for each condition. Detailed information is provided in supplementary methods.

*2.4. Morphological characterization*

Three separate experiments, 100 embryos per concentration (triplicate samples) were classified for normal or delayed development 24 and 48 hpf using a light microscope (Axioskop, Zeiss, Germany). Embryos were either fixed in 0.1% formaldehyde (final concentration) or arrested in movement by 5% $MgCl_2$. Fixed embryos (48 hpf, 50 individuals per condition) were measured in length (Scheitel to the postoral rod) in triplicates (NikonDsFi1, NIS-ElementsD, Nikon Instruments Europe, Amstelveen, Netherlands). Statistical analyses for significance were performed with one-way ANOVA (α=0.05). Light microscopic images were obtained using an Axiovert 200M microscope (software Axio Vision, Zeiss, Germany). Detailed information is provided in supplementary methods.

## 3. Results and discussion

The crystal structure of metallic Ni NPs was characterized displaying the XRD pattern and a FCC (Fm3m) type of single phase structure with the cell parameters of 3.52 Å (Fig. S1). The observed peaks in the pattern correspond to the lattice planes of [111], [200] and [220], [311] and [222], respectively (Dellis et al., 2013). HRTEM was used to characterize the particle size (Fig. 1A) as well as the size distribution (Fig. 1B) of metallic Ni NPs. In total 279 particles were measured and the diameter was shown to be below 100 nm, with an average size of approximately 48 nm. This nanometer size is in the range that typically describes NPs (Nowack and Bucheli, 2007).

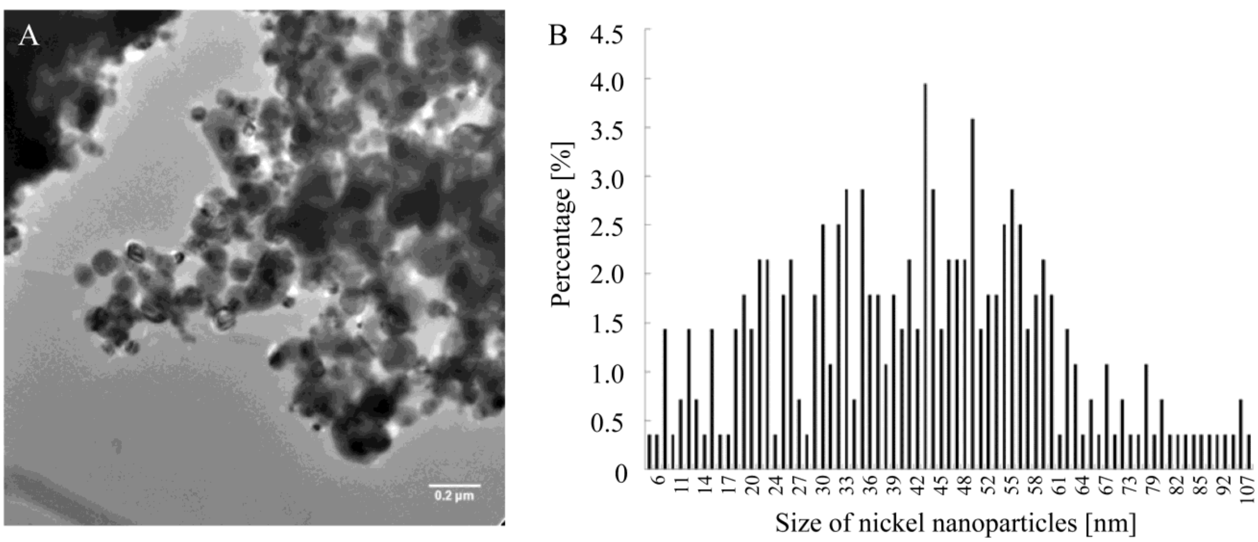


Fig. 1. HRTEM image of metallic Ni NPs (A) and size distribution of the particles (B).

The presence of metallic nickel was confirmed using EDS analysis (Fig. S2A) and Selected Area Electron Diffraction (SEAD) (Fig. S2B) pattern confirmed the crystal planes of metallic Ni NPs with the corresponding lattice planes at [220], [222], [311], [400], [422] and [531]. The [220], [311] and [222] agree with XRD results, confirming the metallic nature of metallic Ni NPs.

In order to differentiate between the effects of metallic Ni NPs and nickel ions, the dissolution in seawater was determined. For ASW without metallic Ni NPs, a nickel ion concentration of 0.005 mg/L was measured by ICP-MS. For nanopore-filtered deionized water, which was used for ASW preparation, a nickel ion concentration of <0.001 mg/L was detected. The low amount of nickel ions present in control ASW is presumably due to minor impurities in salts that were used. Solubility of free nickel ions from 3 mg/L metallic Ni NPs in ASW was determined after 24 h, 48 h, 72 h and 96 h of dialysis (Fig. 2). After 48 h, approximately 0.088 mg/L nickel ions were detected, corresponding to about 2.93% dissolution. This concentration is above the recommended chronic dose by EPA and SEPA in salt water with 8.2 µg/L in the US and 30 µg/L in the UK, respectively (EPA, 2009; SEPA, 2005). For the subsequent time points (72 h and 96 h), there was no further detectable increase in dissolution of nickel ions. Measurements after 46 days resulted in a dissolution of 0.244 mg/L free nickel ions from 3 mg/L metallic Ni NPs in ASW, corresponding to approximately 8.13% (data not shown). In contrast, dissolution of NiO from 10 mg/L NiO NPs in seawater was reported to be about 21% of NiO after 28 days (Hanna et al., 2013), which is more than twice the dissolution of metallic Ni NPs after a shorter period. Interestingly, dissolution of 3 mg/L metallic Ni NPs in nanopore-filtered deionized water was 0.257 mg/L after 46 days, corresponding to 8.57% (data not shown). Results and explanations of DLS analysis are presented in the supplementary section (Fig. S3). To summarize, for 3 mg/L significant agglomeration of metallic Ni NPs has been observed in both deionized water and ASW, which is expected to hinder metallic Ni NPs ion dissolution due to smaller surface area (Baker et al., 2014).

These results show that nickel ion dissolution occurs for metallic Ni NPs over time and highlights that ion dissolution from metallic Ni NPs in seawater is a crucial property and a critical factor for potential toxicity, as it was already evidenced for other NPs (Misra et al., 2012; Maurer et al., 2014).

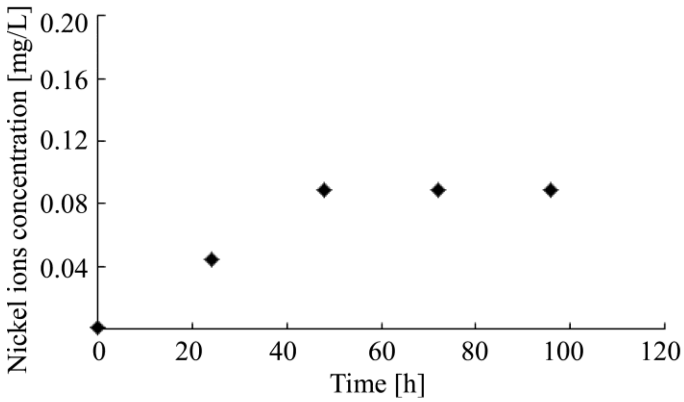


Fig. 2. Dissolution of free nickel ions from 3 mg/L metallic Ni NPs in artificial seawater after dialysis for 24 h, 48 h, 72 h and 96 h, determined by ICP-MS.

A study using photoemission spectroscopy showed that in deionized water mainly OH-groups are attached to the surface of metallic Ni NPs (Bhaduri and Šiller, 2013a). Solubility studies of NiO compared to $Ni(OH)_2$ in deionized water have shown that solubility depends on pH and temperature of the solution (Palmer et al., 2011). The $Ni(OH)_2$ phase transition to NiO in deionized water is predicted to occur at 77°C or 89°C (Palmer et al., 2010, 2011), therefore it is expected that metallic Ni NPs in seawater at pH 8 are still predominantly metallic at 25°C. The solubility constant at room temperature (T=25°C) is several orders of magnitude higher for NiO in deionized water than for $Ni(OH)_2$. It was predict by modeling the solubility constant to be $\log_{10}K^{\circ}$= -6.8 and -10.7 for NiO and $Ni(OH)_2$, respectively (Palmer et al., 2011). This qualitatively explains in addition, why dissolution of metallic Ni NPs is much smaller than of NiO NPs in seawater. A study on zebrafish embryos with Ag NPs highlights further the fact that high $Cl^-$ levels attenuated the toxic effect of Ag NPs (Groh, et al., 2015), indicating that the composition of the medium can have a pivotal influence on potential toxicity.

The effect of metallic Ni NPs on embryonic development of *P. lividus* was investigated within this study for the first time and the developmental stages were characterized morphologically 24 and 48 hpf. After 24 h, control embryos had completed gastrulation and were in the early prism stage, with the archenteron reaching the roof of the blastocoel, vital swimming behavior and spicule formation. Similar observations were made for embryos that were cultured in the presence of metallic Ni NPs or low nickel salt concentrations (Fig. S4A-D). However, high nickel salt concentrations (12 and 24 mg/L) resulted in delayed development and rotating swimming behavior (Fig. S4E and F, respectively). Similar observations were made 48 hpf, normal morphological development with species-specific spicule formation was observed for all tested concentrations of metallic Ni NPs and low nickel salt concentrations (Fig. 3, exemplary images; Fig. S5). However, larvae cultured in 3 mg/L metallic Ni NPs and 3 mg/L nickel salt appeared considerably smaller compared to the control (Fig. 3B and C, respectively). For the two highest concentrations of nickel salt, strong morphological deformations were observed (Fig. 3D exemplary image; Fig. S5). These observations were supported by classifying embryos in normal or delayed/arrested development (Fig. 3E). In conclusion, no lethal impact of metallic Ni NPs with concentrations between 0.03 mg/L and 3 mg/L was detected after a period of 48 h, but 12 and 24 mg/L nickel salt were lethal. Previous studies using nickel chloride with concentrations ranging between 1 µM and 10 mM showed delayed development or morphological deformations in sea urchin embryos (*L. pictus*) (Timourian and Watchmaker, 1972) due to the impact of nickel ions on ectodermal cells and their positioning (Hardin et al., 1992), indicating that low concentrations of nickel ions effect sea urchin embryonic development considerably.

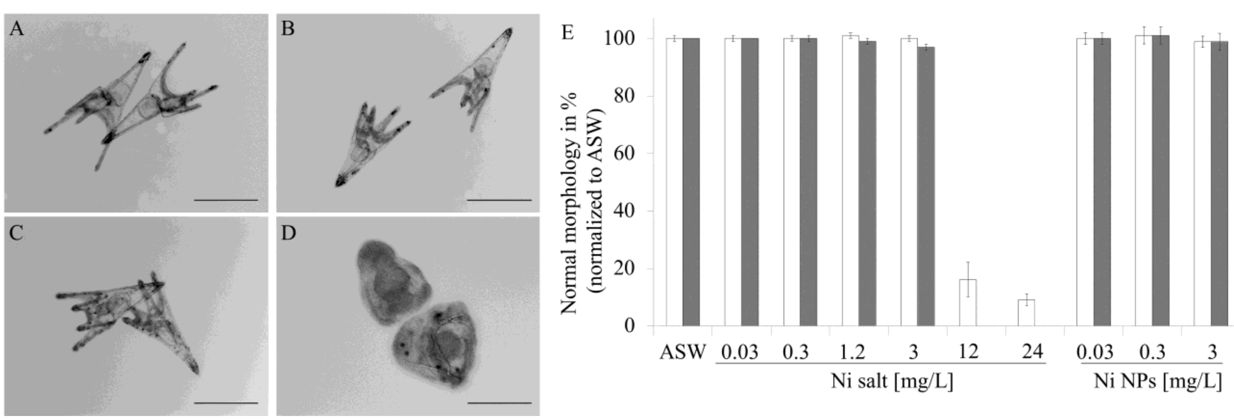


Fig. 3. *P. lividus* embryos 48 hpf (scale bar A-C: 200 µm, D: 100 µm). A: ASW; B: 3 mg/L metallic Ni NPs; C: 3 mg/L $NiCl_2*6\ H_2O$; D: 24 mg/L $NiCl_2*6\ H_2O$. Percentage of embryos 24 hpf (white bars) and 48 hpf (grey bars) with normal morphology, normalized to ASW as 100% (E).

Interestingly, 0.3 mg/L Ag NPs resulted in much stronger effects on embryonic development of *P. lividus* but the effect was rather correlated to the NPs than ion dissolution (0.03mg/L after 51 h) (Šiller et al., 2013). It was also reported that embryonic development of *L. pictus* in the presence of $CeO_2$ and $TiO_2$ NPs, which are insoluble and aggregate in freshwater as well as seawater (Keller et al., 2010), did not result in developmental abnormalities for concentrations up to 10 mg/L over 96 h (Fairbairn et al., 2011). ZnO NPs were toxic in much lower concentrations, presumably caused by the high dissolution of zinc ions (Fairbairn et al., 2011; Manzo et al., 2013). Highlighting, that ion dissolution can be an important factor for toxicity, but is not the case for all NPs.

Length measurements of pluteus larvae 48 hpf (Fig. 4) cultured in the presence of nickel salt concentrations (0.3, 1.2 and 3 mg/L) resulted in a significant ($p < 0.05$) reduction in length (2.9%, 15.2% and 25.7%, respectively). Similarly, for embryos cultured in the presence metallic Ni NPs (0.3 and 3 mg/L) a significant ($p < 0.05$) reduction was observed (3.6% and 13.2%, respectively). Apparently, the size reduction is comparable between nickel salt and metallic Ni NPs for those concentrations. In respect to the measured dissolution rate of metallic Ni NPs in seawater after 48 h it is proposed that the size reduction is caused by nickel ion dissolution. However, additional effects of metallic Ni NPs cannot be ruled at this point and higher dissolution rates after longer incubation of metallic Ni NPs in seawater could also result in stronger deformations. Therefore subsequent investigations and studies with environmental relevant concentrations are required.

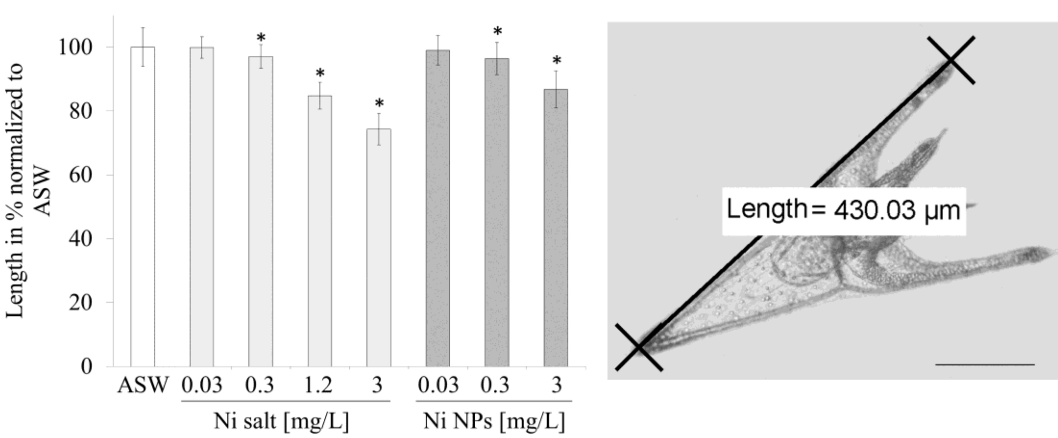

Fig. 4. Length measurement of *P. lividus* embryos 48 hpf presented in percentage normalized to ASW as 100 % and a representative measurement of a pluteus larva from ASW (scale bar: 100 µm). Stars indicate significant difference to larvae from ASW (one-way ANOVA).

However, Ni-containing NPs showed toxic effects when tested on freshwater organisms or in cell culture (e.g. Ispas et al., 2009; Griffitt et al., 2008; Magaye and Zhao, 2012). Only a limited number of studies on Ni-containing NPs were performed on marine organisms. Marine amphipods showed no increased mortality after 10 days in the presence of NiO NPs with similar concentrations as in our study (Hanna et al., 2013). Therefore, the present study contributes to the so far limited understanding of Ni-containing NPs in the marine environment and their effects on marine organism. Further, more attention should be payed to marine environments when assessing the effects of NPs, since NPs properties strongly depend on the environmental condition and cannot be compared implicitly with results from freshwater or cell culture.

## 4. Conclusion

This study demonstrates for the first time the effects of metallic Ni NPs on the embryonic development of the sea urchin *P. lividus* and supports the sea urchin as a highly sensitive *in vivo* marine test system. Exposure to 3 mg/L metallic Ni NPs did not cause lethality, but a size reduction that was comparable with the one for 1.2 mg/L $NiCl_2$* 6 $H_2O$, indicating that the dissolution of nickel ions from metallic Ni NPs is interfering with the developmental process.

This study is of high ecological interest due to the use of metallic Ni NPs in industrial applications and contributes to the overall understanding of metallic Ni NPs in the marine environment. However, results on one class of NPs should not be generalized since they can have versatile properties and effects on different organisms and environments. Therefore, full characterization of NPs is required to ensure their save application in technologies.

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