Environmental Pollution 228 (2017) 190–200

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Invited paper

# Moderate ocean warming mitigates, but more extreme warming exacerbates the impacts of zinc from engineered nanoparticles on a marine larva☆

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ARTICLE INFO

Article history:
Received 21 December 2016
Received in revised form
10 May 2017
Accepted 11 May 2017
Available online 20 May 2017

Keywords:
Zinc oxide
Climate change
Larvae
*Tripneustes gratilla*
Pollution

ABSTRACT

There is growing concern about the combined effects of multiple human-induced stressors on biodiversity. In particular, there are substantial knowledge gaps about the combined effects of existing stressors (e.g. pollution) and predicted environmental stress from climate change (e.g. ocean warming). We investigated the impacts of ocean warming and engineered nanoparticles (nano-zinc oxide, nZnO) on larvae of a cosmopolitan tropical sea urchin, *Tripneustes gratilla*. Larval *T. gratilla* were exposed to all combinations of three temperatures, 25, 27 and 29 °C (current SST and near-future predicted warming of +2 and + 4 °C) and six concentrations of nZnO (0, 0.001, 0.01, 0.1, 1 and 10 mg nZnO·$L^{-1}$). These stressors had strong interactive effects on fertilization, gastrulation and normal development of 5 day old larvae. High concentrations of nZnO had a negative effect, but this impact was less pronounced for sea urchins reared at their preferred temperature of 27 °C compared to 25 or 29 °C. Larval growth was also impacted by combined stress of elevated temperature and nZnO. Subsequent measurement of the dissolution and aggregation of nZnO particles and the direct effect of $Zn^{2+}$ ions on larvae, suggest the negative effects of nZnO on larval development and growth were most likely due to $Zn^{2+}$ ions. Our results demonstrate that marine larvae may be more resilient to stressors at optimal temperatures and highlight the potential for ocean warming to exacerbate the effects of pollution on marine larvae.



## 1. Introduction

The world's oceans have warmed by 0.6 °C over the past century coinciding with increasing carbon dioxide emissions (Collins et al., 2013). By 2100, the average surface temperature of the oceans is predicted to have increased by up to 2 °C (Collins et al., 2013; Hobday and Lough, 2011). This will be accompanied by greater volatility in sea temperatures resulting in short term marine heatwaves (Wernberg et al., 2013) and localised warming above the global average in climate 'hotspots' (Hobday and Lough, 2011). These changes will influence the distribution, behaviour, morphology, and fitness of marine species that are regulated by environmental temperatures (Lough and Hobday, 2011). Ocean warming is therefore likely to have profound effects on both marine biodiversity and ecosystem function (Byrne et al., 2011; Harley et al., 2006; Przeslawski et al., 2008; Wernberg et al., 2011). Predicting the environmental impacts associated with changing ocean temperatures will be complicated by interactions with other human-induced stressors such as pollution, fishing, coastal development, and ocean acidification (Wernberg et al., 2012). Understanding the environmental consequences of multiple stressors involving climate-driven ocean change will therefore be a critical step in effective marine ecosystem management in the coming decade (Sokolova and Lannig, 2008).

There is increasing concern about the potential for interactive effects of ocean warming and marine pollution on marine species (Wernberg et al., 2012). Marine pollution, such as heavy metals, have deleterious effects on marine organisms (Islam and Tanaka,

☆ This paper has been recommended for acceptance by Dr. Hageman Kimberly Jill.

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http://dx.doi.org/10.1016/j.envpol.2017.05.033
0269-7491/

2004) that are predicted to be exacerbated by increases in temperature in several ways (Sokolova and Lannig, 2008). Elevated temperatures may change the biochemical and physiological processes within an organism and thereby increase susceptibility to pollutants (Bao et al., 2008; Cherkasov et al., 2007; Ivanina et al., 2009), and exposure to toxicants may reduce the thermal tolerance of organisms (Bao et al., 2008; Sokolova and Lannig, 2008). Moreover temperature may change the physical or chemical properties of the pollutant, such as metal solubility.

Engineered nanoparticles are manufactured ultrafine materials (<0.1 μm) that have recently been recognised as a new form of marine pollution (Matranga and Corsi, 2012). Through production processes, general use and mishandling, engineered nanoparticles are entering the environment (Fairbairn et al., 2011; Klaine et al., 2008) and being transported into the ocean (Klaine et al., 2008). Similar to other small pollutants (e.g. microplastics, Kaposi et al., 2013), there is concern over what effects nanoparticles may have on marine biota (Fairbairn et al., 2011; Zhu et al., 2011), particularly given that the level of nanoparticle pollution is predicted to intensify with increasing consumer use (Jarvis et al., 2013).

One such nanoparticle that has been increasing in prevalence in the marine environment is nano-zinc oxide (nZnO) (Jarvis et al., 2013). nZnO enters the marine environment primarily through the use of sunscreen (Ju-Nam and Lead, 2008), in which it is a major active component, but nZnO may also be introduced through wastewater treatment (Lombi et al., 2012). It is estimated that concentrations of nZnO in the marine environment are generally low (~10 ng $L^{-1}$), except for locations where sewage effluents are discharged (0.2–1.4 μg $L^{-1}$) (Gottschalk et al., 2009). However it is likely that nZnO concentrations near other source locations are considerably higher. At a popular swimming beach in Spain, concentrations of another nanoparticle used in sunscreen, titanium dioxide, were up to 0.04 mg $L^{-1}$ (measured as $Ti^{4+}$, Sánchez-Quiles and Tovar-Sánchez, 2014).

Zinc oxide nanoparticles are highly toxic to marine organisms and can cause abnormal development (Fairbairn et al., 2011; Manzo et al., 2013), reduced growth (Jarvis et al., 2013; Peng et al., 2011), altered protein levels (Wong et al., 2010), mechanical injuries (Wong et al., 2010), cytotoxic and genotoxic effects (Schiavo et al., 2016), and mortality (Hanna et al., 2013; Jarvis et al., 2013). Photo-excitation of nZnO in seawater can form $H_2O_2$, a chemical that negatively affects the growth of phytoplankton (Sánchez-Quiles and Tovar-Sánchez, 2014). However, little is known about how the impacts of nZnO may interact with climate-driven ocean change. In one of the only studies examining such impacts, Wong and Leung (2014) found the effects of nZnO on photosynthesis of the diatom *Skeletonema castatum* and protein expression of the fish *Oryzias melastigma* was dependent on temperature, but there were no interactive effects of these factors on growth and mortality.

The aim of this study was to examine the combined effects of nZnO and predicted near-future ocean warming on the early life stages of the tropical sea urchin *Tripneustes gratilla*, an ecologically important species distributed throughout the Indo-Pacific region (Lawrence and Agatsuma, 2013). *T. gratilla* larvae appear in the water column during summer (Juinio-Menez et al., 1998), which is also when peak concentrations of nZnO may occur (Tashiro and Kameda, 2013). This study was done in context of ocean warming along the subtropical east coast of Australia, a climate change hotspot (Hobday and Lough, 2011; Lough and Hobday, 2011). Sea surface temperatures (SST) along the coast of SE Australia have risen by 2.3 °C since 1940 (Lough and Hobday, 2011) and are currently warming four times faster than the global average (Lough and Hobday, 2011; Wernberg et al., 2011). It is predicted that in the near future (ca. 2100), ocean temperatures along SE Australia will rise by a further 4 °C (Hobday and Lough, 2011). To understand if toxicity could be attributed to the presence of zinc oxide as nanoparticles or ions resulting from the dissolution of nanoparticles, dissolution and aggregation of nZnO were measured, and the toxicity of nZnO was compared to dissolved $Zn^{2+}$ ions.

## 2. Methods and materials

### 2.1. Study organisms and gamete collection

Larvae were obtained from *Tripneustes gratilla* collected from Coffs Harbour, NSW, Australia (30°12.5′S, 153°16.1′E). Intracoelomic injections of 1–2 mL of 1 *M* KCl were used to induce spawning in six males and three females. Eggs were collected in 500 mL beakers containing natural seawater (pH ~8.1, salinity ~35, total alkalinity ~2310 μmol $L^{-1}$, <6.4 μg $L^{-1}$ $Zn^{2+}$) filtered to 1.0 μm and UV sterilized (hereafter FSW). Prior to fertilization, eggs from each female were microscopically examined to ensure uniform sphericity and then pooled. Sperm was collected dry and activated in FSW prior to use. Sperm motility was confirmed microscopically and an equal amount of sperm from each male was pooled to create a concentration of $10^{-6}$ sperm·$mL^{-1}$ in FSW.

### 2.2. Chemicals and test solution preparation

To examine the interactive effects of zinc oxide nanoparticles (nZnO) and near-future ocean warming on the early life stages of *T. gratilla,* larvae were reared in eighteen treatments determined by the factorial cross of six nZnO concentrations (0 = control, 0.001, 0.01, 0.1, 1 and 10 mg nZnO·$L^{-1}$) and three temperatures (25, 27 and 29 °C), chosen to represent current ambient SST and warming of +2 and + 4 °C respectively. Zinc oxide nano-powder (<50 nm, 6% Al; Sigma-Aldrich, 677 450, characterised by Miao et al., 2013, mean size ~30 nm) was used as a source of nZnO. Experimental concentrations were made in 1 L Schott bottles by serial dilution of an 800 mg $L^{-1}$ stock solution of nZnO in FSW. 5 mL of the stock solutions and 35 mL of FSW were added to 50 mL specimen containers (Techno Plas); total 40 mL of each experimental solution at a final concentration of 0.001, 0.01, 0.1, 1.0 or 10.0 mg $L^{-1}$ respectively. Controls had FSW only. Experimental temperatures (25, 27 and 29 °C) were maintained using a climate-controlled room and water baths. Each nZnO and temperature treatment in all combinations was replicated in five independent 50 mL containers (n = 5).

### 2.3. Effects of nZnO and temperature on fertilization success and embryogenesis

Approximately 2000 eggs were added to each replicate container (50 eggs·$mL^{-1}$) and exposed to treatments for 10 min before sperm was introduced (100 μL sperm solution, sperm to egg ratio 500:1). After 3 h development time, the fertilization success of *T. gratilla* was determined by assessing 30 eggs/zygotes from each replicate and scoring the number of fertilized and unfertilized eggs.

To determine if nZnO and temperature impacted gastrulation of *T. gratilla* larvae, at 24 h, ~20 randomly selected larvae from each replicate were examined microscopically and the percentage of embryos that reached the early gastrula stage was determined.

### 2.4. Effects of nZnO and temperature on larval development and growth

At the onset of feeding at day 5 (Mos et al., 2011), the remaining larvae in each replicate were fixed using 50–100 μL of 10% formalin in 1.5 mL Eppendorf tubes and examined within five days. To assess the percentage of normal development, all larvae in a haphazardly chosen 1 mL sample of the fixed larvae were scored as normal or