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Combined effects of arsenic, salinity and temperature on *Crassostrea gigas* embryotoxicity

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ABSTRACT

The combined effects of different salinity and temperature levels on the toxicity of Arsenic (As) were studied on the embryonic development of the oyster *Crassostrea gigas*. A standardized embryotoxicity test was performed to assess the interactive effects of these stressors, in a full factorial design experiment including a range of salinities (15, 19, 24, 28 and 32), temperatures (16, 20, 24, 28 and 32 °C) and As concentrations (100, 300, 600, 1200, 2400 μg L^{-1}). The embryotoxicity endpoint was about the determination of normal larvae development rates at various conditions, and median effect concentration (EC_{50}) determination for each As exposure condition. Results showed that toxicity induced by As was characterized by retardation of embryonic development observing toxic effects at lower concentrations than previously reported studies. The presence of As in seawater resulted in a narrower range of tolerance to both salinity and temperature. These findings bring new insights on the impacts of a common contaminant on an important shellfish species having a planktonic early life stage development, with potential implications for population survival and ecosystem functioning in a changing environment.

1. Introduction

Oysters belonging to the *Crassostrea* genus comprise one of the most important group of bivalve molluscs in global fisheries and aquaculture (FAO, 2012). Within this genus, *Crassostrea gigas*, the so-called Pacific oyster, accounts alone for over 90% of the global production (Tidwell, 2012). Oysters have a high socio-economic value, and provide ecosystem services (e.g: water quality improvement; refuge for other species, erosion protection) making this taxonomic group an invaluable keystone species (Coen et al., 2007; Grabowski et al., 2012). Their sessile nature, water filtering capacity and ubiquity make these bivalves very interesting models to assess direct anthropogenic pressures like marine aquatic pollution (e.g., He and Wang, 2013; Ivanina et al., 2008; Zanette et al., 2011), and indirect human impacts such as climate change (e.g., Lannig et al., 2010; Moreira et al., 2016; Tomanek et al., 2012). Recent research on these issues has mostly focused on adults (Ivanina et al., 2008; Lannig et al., 2010; Tomanek et al., 2012; Moreira et al., 2016), but some papers studied also juvenile (e.g., Corsi et al., 2014; Dickinson et al., 2012; Lejart et al., 2011; Minetto et al., 2014; Moreira et al., 2017; Waldbusser et al., 2011) and larval stages (Barton et al., 2012; Libralato et al., 2007, 2009; Miller et al., 2009; Waldbusser et al., 2013; Gamain et al., 2017).

Several groups of marine invertebrates such as oysters, present early free-living benthonic (at a very early stage after fertilization) and planktonic life stages (Pechenik, 1999). During the early life stages, larvae sensitivity to environmental stressors is generally higher than juveniles and adults (Beiras and His, 1994; His et al., 1999; Przeslawski et al., 2008) especially considering salinity and temperature (Gamain et al., 2017; Boukadida et al., 2016). Impacts on these stages can impair the recruitment of adult populations posing at risk the potential survival of the relative stock population (Byrne et al., 2012).

In oyster species within the *Crassostrea* genus, early free-living life stages include gametes, embryos and larvae (Kasyanov et al., 1998). The early development of *C. gigas* embryos into straight hinged larvae (D-shape) is a process of intense cellular activity during which any impairment within a series of biochemical and physiological mechanisms can result in malformed larvae (Leverett and Thain, 2013). Due to the cost effectiveness of *C. gigas in vitro* fertilization, ecological relevance and high sensitivity of embryos to common contaminants (Beiras and His, 1994), some embryotoxicity protocols are available

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like Thain (1991), His et al. (1997), USEPA (1991), ASTM (2004), and ISO (2015). Further applications are available for sediment quality assessment (Geffard et al., 2001; Volpi Ghirardini et al., 2005; Libralato et al., 2008); wastewater toxicity (Libralato et al., 2010; Mamindy-Pajany et al., 2010); emergent pollutant screening (Fabbri et al., 2014) and to climate change stressors (Gamain et al., 2016).

Under the eminent threat of global climate change, the ability of marine species early life stages to cope with a mixture of environmental stressors will condition species survival and ecosystem functioning (Byrne et al., 2011). Seawater temperature rise and increased input of freshwater into the oceans, resulting from atmospheric carbon dioxide build-up, are among the most important climate change related factors affecting costal marine ecosystems (Torresan et al., 2008; Philippart et al., 2011; IPCC, 2013). Shallow water bodies such as estuaries, are especially vulnerable to these stressors (Harley et al., 2006; Hoegh-Guldberg and Bruno, 2010), where organisms are subjected to constant changes in seawater physicochemical parameters, as well as to high levels of anthropogenic pollution (Schropp et al., 1990; Riba et al., 2004). Changes in abiotic factors such as salinity and temperature can directly influence the development of marine invertebrates early life stages (Carballeira et al., 2011; Verween et al., 2007), and can also affect the sensitivity of these stages to pollutants (Coglianese, 1982; His et al., 1989, 1999).

The main driving forces within the climate change scenarios are acidification, salinity and temperature (Ko et al., 2014; Parker et al., 2010; Talmage and Gobler, 2011; Thyiagarajan and Ko, 2012). In most recent studies, the interactive effects on invertebrate species early stages of contaminant exposure and climate change stressors have shown increased metal toxicity to *Mytilus galloprovincialis* embryos under seawater temperature rise (18–24 °C) (Boukadida et al., 2016), increased sensitivity of *Ruditapes philippinarum* larvae to diclofenac under ocean acidification conditions (up to ca. 1100 µatm pCO_2) (Munari et al., 2016), and increased copper and metolachlor toxicity to *C. gigas* larvae under low salinity (18–33) (Gamain et al., 2016). These studies highlight the importance of assessing pollutants toxicity under changing environmental parameters, and are essential to fill the gap in knowledge regarding how climate change may alter organism's sensitivity to pollutants (Schiedek et al., 2007; Noyes et al., 2009).

Arsenic (As) is one of the most common aquatic contaminant worldwide (Mandal and Suzuki, 2002; Ng, 2005) originating from both natural and anthropogenic sources (Bissen and Frimmel, 2003) with sediment concentrations ranging up to 489 mg/kg (Patel et al., 2005). Estuaries are a major sink of As (Bone et al., 2006) posing a real threat to inhabiting biota and surrounding coastal waters also due to sediment resuspension phenomena and washout events thus increasing the natural background of As in water column up to several thousands of microgram per litre (Mamindy-Pajany et al., 2013).

Arsenic embryotoxicity in *C. gigas* has been investigated by Martin et al. (1981) and Mamindy-Pajany et al. (2013) (48 h EC_{50} of 326 µg L^{-1}; and 24 h EC_{50} of 920 µg L^{-1}, respectively), but the role of salinity and temperature remains under-explored.

The aim of this research is to evaluate the potential embryotoxicity on *C. gigas* after 24 h of contact time considering various As-spiked scenarios (100, 300, 600, 1200, 2400 µg L^{-1}), changing salinities (15, 19, 24, 28 and 32) and temperatures (16, 20, 24, 28, 32 °C) in the perspective of climate change events.

2. Material and methods

2.1. Testing solutions

Natural seawater, sampled in Venice lagoon (Italy) (45°26′15.97″N and 12°22′43.76″E), was filtered at 0.2 µm (stored at 4 °C in the dark) and used for the entire experiment. Reagent grade sodium arsenate (Na_3AsO_4, As^{5+}) (CAS#10048-95-0) was used to prepare a concentrated As stock solution with ultra-pure water. The stock solution was further spiked into previously prepared seawater to obtain the following interval of As nominal concentrations: 100, 300, 600, 1200 and 2400 µg L^{-1} for each testing salinity. Exposure concentrations were chosen referring to Mamindy-Pajany et al. (2013).

Five different salinities were used for As exposures (15, 19, 24, 28 and 32 ± 1). Control seawater (32), was diluted in ultra-pure water to obtain the lower salinity levels. Salinity levels were selected according *C. gigas* larvae tolerance range (Nell and Holliday,1988), and measured with a calibrated refractometer (Atago, Japan). Seawater pH and conductivity of all salinity batches were measured before exposures with a multiparametric probe (HQ40d Portable, Hach Lange) (Table S2 and Table S3).

Prior to embryotoxicity test, treatments (various salinities and As concentrations) were prepared and transferred just 24 h before the bioassay began in test vessels. The same experimental design was replicated 5 times, one per each exposure temperature, in separate test vessels. Five independent climatic chambers were simultaneously used to keep constant the relative temperature (16, 20, 24, 28 and 32 °C). Exposure temperatures were selected considering *C. gigas* natural habitat temperatures for warmer months (between 14 and 29 °C) (Carrasco and Barón, 2009), up to 32 °C (within the expected increase in seawater surface temperature for the end of the 21st century of +4 °C (Solomon, 2007)).

For the embryotoxicity positive control, a stock solution of Cu $(NO_3)_2$ was prepared in ultra-pure water and was used as reference toxicant according to Libralato et al. (2009) (3, 6, 12, 18 and 24 µg L^{-1}).

Testing media were analysed for effective As concentration by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) equipped with a collision/reaction cell (ICP-ORS-MS) (Agilent 7500 ORS).

2.2. Embryotoxicity assay

Sexually mature *C. gigas* were purchased from Guernsey Sea Farm (UK) and induced to spawn immediately after arrival. Male and female oysters were kept in separate containers and gamete emission induced by thermal stimulation according to Libralato et al. (2007, 2013). Sperm and egg gametes were qualitatively assessed for fertilization success via a microscope. Zygotes per unit volume were determined as well and transferred to 3 mL 24 wells microplates with lids (Iwaki, Japan) in order to reach approximately 200 embryos per replicated treatment (3 replicates). Microplates were transferred to the goal temperature and kept in the dark for 24 h. Larvae development ended by the addition of buffered formalin (4%, approximately 10 µL) to each well. One hundred larvae were counted per well distinguishing between normal (D-shape) and abnormal (kidney shape, indented shell, protruded mantle and pre-D stages) embryos/larvae according to His et al. (1997) using an inverted stereomicroscope (Leica DMIL), coupled to a digital camera (Nikon DS-Fi1).

Toxicity data were determined as percentages of abnormal larvae (retarded and malformed larvae). EC50 on the exposed populations have been provided as well as the relative confidence limit values at 95% after parametric data fitting. The responses for each treatment were corrected for effects in the negative control by applying Abbott's formula (ASTM, 2004). The hypothesis test was verified using Analysis of Variance (ANOVA) and Tukey's test to check any difference among the groups after lognormal transformation of concentration data. When ANOVA revealed significant differences among treatments, *post-hoc* tests were carried on with Dunnett's method testing the pairwise difference between each treatment and the control. Parametric or non-parametric methods were considered for points' estimation. Statistical analyses were performed using Microsoft® Excel 2013/XLSTAT©-Pro (Version 7.2, Addinsoft, Inc., Brooklyn, NY, USA).

Contour plots were used to depict at the same time salinity, temperature and As toxicity effects using SigmaPlot (Version 11.0, Systat Software, Inc., San Jose California USA).