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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).

Table 1
Analytical arsenic (As) concentrations (μg L^{-1}) prepared considering five salinity exposure scenarios (15, 19, 24, 29, and 32).

As[a] (μg L^{-1})	Salinity				
	15	**19**	**24**	**29**	**32**
0[b]	20.6 ± 0.5	26.1 ± 0.5	33.0 ± 0.5	39.9 ± 0.5	44.0 ± 0.5
100	103.4 ± 0.8	109.4 ± 3.0	121.5 ± 21.2	102.9 ± 5.0	103.0 ± 13.9
300	245.0 ± 3.6	291.1 ± 8.6	301.2 ± 4.5	288.8 ± 6.4	310.2 ± 38.2
600	620.0 ± 115.0	553.6 ± 1.2	576.5 ± 24.7	700.5 ± 161.1	559.2 ± 9.5
1200	1221.9 ± 59.0	1114.8 ± 25.5	1033.7 ± 135.5	1051.2 ± 124.0	1163.0 ± 34.2
2400	2407.3 ± 127.1	2140.1 ± 174.6	2204.6 ± 60.4	2248.2 ± 54.4	2141.2 ± 3.9

[a] Nominal concentration.
[b] Natural As concentration.

3. Results and discussion

3.1. Controls and exposure scenarios

Negative controls were verified at standard assay conditions (24 °C and at 32 salinity). Normally developed D-shape larvae average percentage was > 70%, according to ASTM (2004). Positive controls showed an EC50 value of 8.39 μg L^{-1} (6.78–9.79 μg L^{-1}), within the acceptable range proposed by His et al. (1999) (5–13 μg/L).

Exposure scenarios were investigated considering the potential effects of dissolved organic carbon (DOC) into filtered seawater as well as its main constituents (Table S1) according to the Visual MINTEQ software (version 3.1) (Gustafsson, 2014). Results (Table S2) indicated that most As (97%) is present in solution as $HAsO_4^{2-}$ (97%) and as $H_2AsO_4^-$ (3%) with no substantial changes according to the range of the investigated temperatures and salinities (data not reported here). The analysed concentrations of As were summarised in Table 1 according to each salinity scenario.

3.2. Multiple stressor effects on embryonic development

The combined effects of salinity and temperature on the embryotoxicity of As are represented in Fig. 1. Each contour plot represents the average percentage of abnormal larvae at different salinity and temperature levels at a fixed As exposure concentration.

At 16 and 20 °C and salinity 15, 100% abnormal developed larvae were observed regardless of As exposure concentration.

The lowest percentage of abnormally developed larvae (maximum of 30%) were observed at 0 μg L^{-1} of As (Fig. 1A), at salinities ranging from 20 to 32, and temperature ranging from 24 to 28 °C. In embryos exposed to 100 μg As L^{-1} (Fig. 1B) the lower percentage of abnormal larvae (ca. 40%) were observed at salinities ranging between 24 and 32, and a temperature of 28 °C. At 300 μg As L^{-1} (Fig. 1C), abnormality percentage was over 90% for most of the testing conditions, and the lowest percentage of abnormal larvae of approximately 85%, was observed at salinity 32 and 28 °C. In embryos exposed to 600 μg As L^{-1} (Fig. 1D), as well as for those exposed to 1200 and 2400 μg As L^{-1} (not represented), approximately 100% abnormal larvae were observed regardless of salinity and temperature.

3.3. Types of abnormalities observed

The relative frequency of different abnormalities recorded at 0, 100 and 300 μg As. L^{-1} are depicted in Fig. 2 (A, B and C respectively). The most prevalent type of abnormality was the pre-D larval stage. This category of abnormal developed embryo-larvae includes all developmental stages from unfertilized eggs to trochophore larvae (see Fig. 3). In oyster embryos exposed to the 15 salinity at 16 and 20 °C, pre-D stages accounted for 100% of abnormal percentage observed, regardless of As exposure concentration (Fig. 2). At higher salinities and temperature levels, the exposure to As generally led to an increased frequency of pre-D larvae, with the exception of oyster embryos exposed at 28 °C and high salinities (28 and 32) (Fig. 2B).

Other types of abnormal developed larvae were observed, such as those presenting protruded mantle, indented shell, kidney shape or dead (see Fig. 3). Generally, at 0 μg As. L^{-1} larvae presenting protruded mantle were the second most prevalent abnormality (after pre-D), followed by larvae presenting indented shell and kidney shape, while dead larvae were the least prevalent (Fig. 2A). At 100 μg As. L^{-1}, larvae presenting protruded mantle were most important at 32 °C (salinities 24–32), while kidney shaped larvae were more prevalent at 24 and 28 °C (excluding pre-D) (Fig. 2B). At 300 μg As. L^{-1}, embryo-larvae were mostly retarded in pre-D stages (Fig. 2C), a pattern also observed for 600, 1200 and 2400 μg As. L^{-1} (not presented).

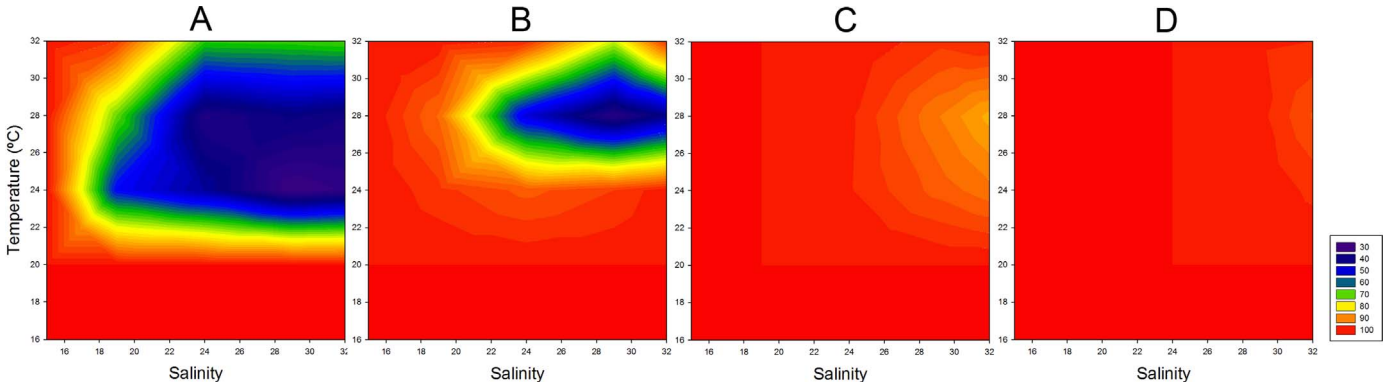

Fig. 1. Multiple stressor effects on *C. gigas* embryo-larval development after 24 h represented by contour plots for fixed As concentrations (μg L^{-1}): A = 0; B = 100; C = 300; D = 600. Percentage of abnormal larvae from 30% to 100% are represented in colours grading from blue to red, respectively; effects at 1200 and 2400 μg As L^{-1} were not displayed being equal to D.

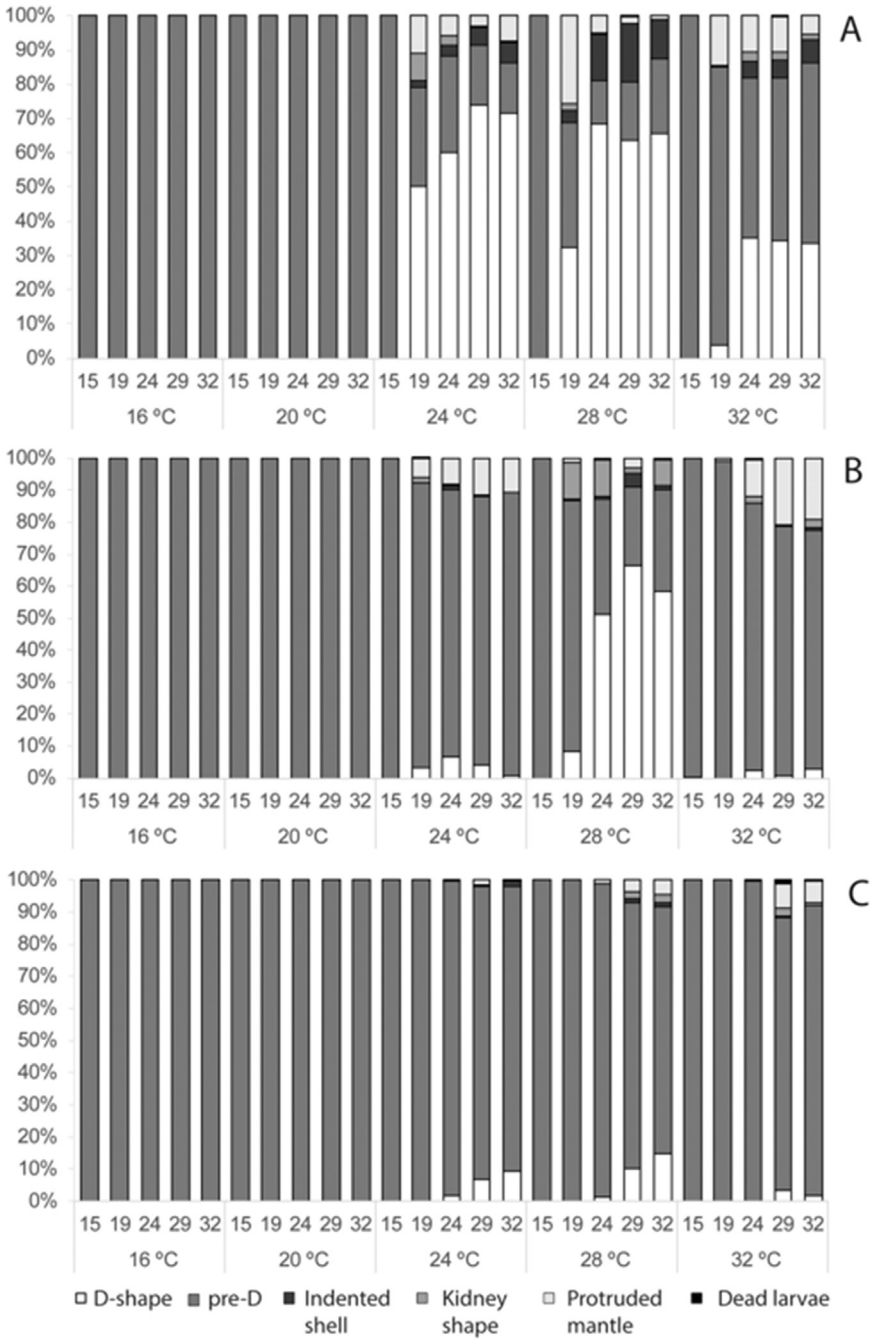

Fig. 2. Relative frequency of normal (D-shape) and different abnormalities in *C. gigas* embryo-larval development after 24 at changing salinity (15, 19, 24, 29, and 32) and temperature (16, 20, 24, 28, and 32 °C) and three As concentrations (μg L^{-1}) (A = 0; B = 100; C: 300); D-shape and abnormalities are represented in each bar according to the legend (from left to right) from the bottom to the top.

3.4. Specific effects of salinity and temperature

The specific effect of varying salinity at a fixed temperature of 24 °C, is presented in Fig. 2A. At the lowest salinity tested (15), 100% abnormal developed larvae were observed, all of which identified as pre-D larvae. The relative percentage of normal developed larvae increased with the increase of salinity from 19 to 28 (from 50 to over 70%) and remained similar at the highest salinities (29 and 32). Pre-D stages accounted for most abnormal development, followed by larvae presenting protruded mantle, indented shell and kidney shape, and the relative frequency of pre-D stages tended to decrease with the increase of salinity (Fig. 2A).

The specific effect of temperature at a fixed salinity of 32, is presented in Fig. 2B. At the lowest tested temperatures (16 and 20 °C) all embryos were identified as pre-D. At 24 °C, the percentage of normally developed larvae were the highest reaching over 70%, while pre-D stages presented the lowest percentage among different temperatures. Percentage of normal D-shape larvae tended to decrease with the increase of temperature to 28 and 32 °C in respect to control (24 °C), while the relative frequency of pre-D stages tended to increase (Fig. 2B).

All EC50 values were summarised in Table 2. At control condition (24 °C, 32 salinity), it was not possible to accurately determine EC50, because at the first As concentration (100 μg L^{-1}) all larvae presented arrested development (pre-D), not allowing to fit an adequate concentration-response curve. Nonetheless, results showed that 24 h EC50 at standard control conditions would be situated between 0 and 100 μg L^{-1}. Previous studies reported higher EC_{50} values for As in *C. gigas*: 326 μg L^{-1} in Martin et al. (1981) and 920 μg L^{-1} in Mamindy-Pajany et al. (2013). It is not likely that these differences were related to specific sensitivity of the oyster batch itself, since negative and positive controls were within the acceptable range proposed for this species (ASTM, 2004; His et al., 1999). These differentiated results may rather

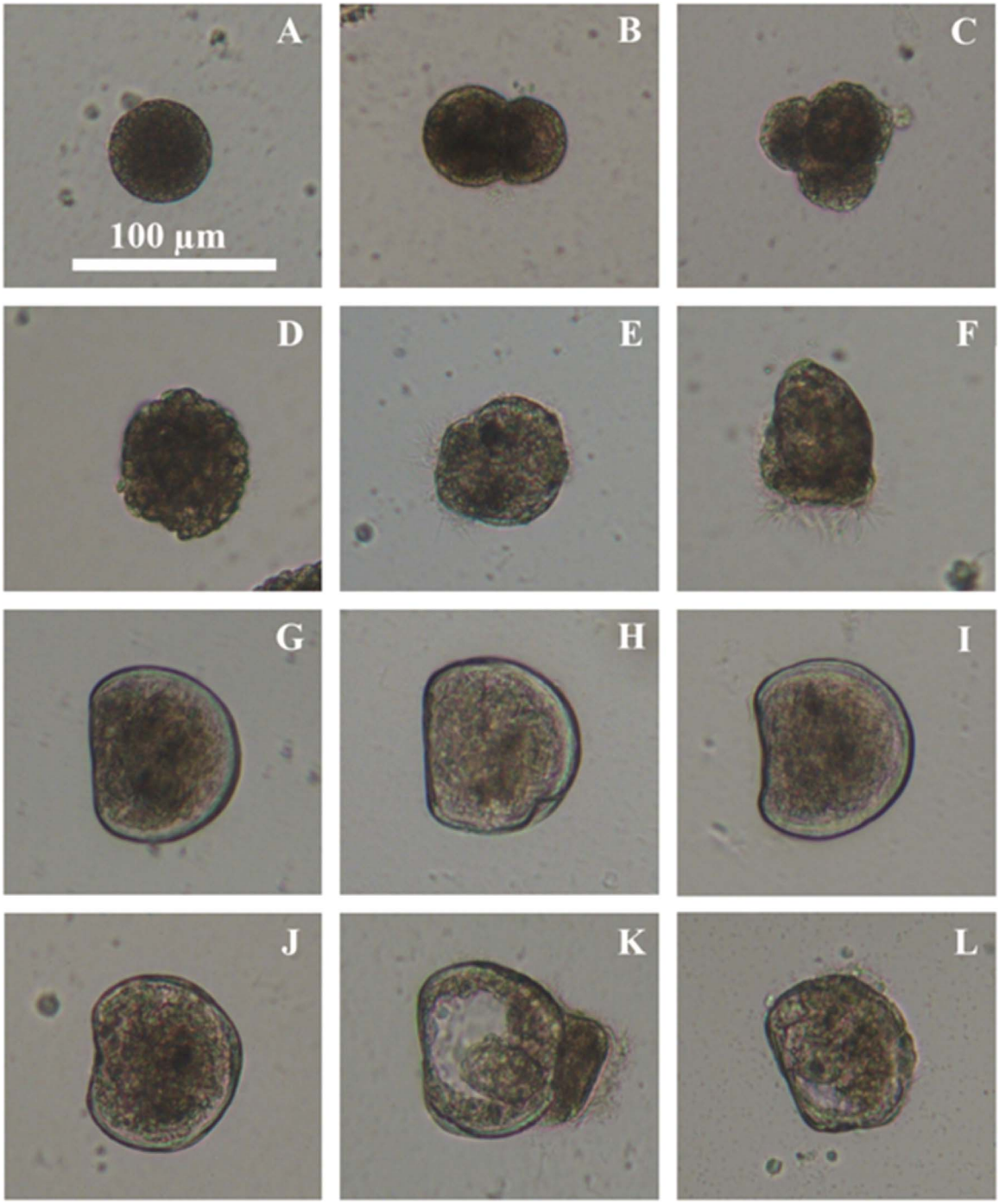

Fig. 3. *C. gigas* embryo-larval development stages after Leverett and Thain (2013) (A-G) and abnormally developed embryo-larvae in *C. gigas* after His et al. (1997) (H-L). A: fertilized egg; B: 2-cell embryo; C: 3-cell embryo; D: 32-cell embryo; E and F: trochophore larvae; G: normal D-shape larvae; H: indented shell; I and J: convex hinge or kidney shaped larvae; K: protruded mantle; I: dead larvae.

be related to background levels of other contaminants present in the used seawater (Table S2). Indeed, it has been shown for sea urchin species (*Paracentrotus lividus* and *Arbacia lixula*) that sea water source can influence the output of embryotoxicity assay (Beiras and Albentosa, 2004; Bošnjak et al., 2011; Rosen et al., 2005).

Contaminants do not occur isolated in nature, and may interact with each other in an additive, synergistic or antagonistic manner (His et al., 1999). Previous studies have shown that exposure to mixtures of metals can result in additive toxic effects on embryogenesis of different invertebrate species (e.g. *Ruditapes decussatus* and *Mytillus galloprovincialis* exposed to Cu and Zn (Beiras and Albentosa, 2004)); *P. lividus* exposed to Hg and Pb (Fernández and Beiras, 2001); *Strongylocentrotus intermedius* exposed to Cu, Pb, Cd and Zn (Xu et al., 2011). It is therefore likely that our results, indicating lower As EC_{50} than previously reported values, were related to background contamination levels in seawater (i.e. Cu, Ni, Cr), and possible additive effects with As resulting in higher toxicity of the media. The point is that a full characterisation of natural filtered seawater in scientific papers is not available like as the complete list of constituents of artificial saltwater thus limiting the possibility to understand potential changes in the sensitivity of species against the same toxicant.

Relatively high background Cu levels determined in the used

Table 2
Median effect concentrations (EC50s) for *C. gigas* embryotoxicity considering a range of salinities (15, 19, 24, 28 and 32) and temperatures (16, 20, 24, 28 and 32 °C); data are in $\mu g\ L^{-1}$; n.c. = not calculable (the EC50 cannot be determined because 100% effect was detected in all tested concentrations).

Temperature	Salinity				
	15	19	24	29	32
16	n.c.	n.c.	n.c.	n.c.	n.c.
20	n.c.	n.c.	n.c.	n.c.	n.c.
24	n.c.	78.0	35.6	2.0	0.002
28	n.c.	96.7	152.3	219.3	215.2
32	n.c.	85.8	89.7	149.3	4.7

seawater (Table S1) could have influenced As toxicity due to the above stated reasons, even though dissolved organic matter can significantly affect its bioavailability like as the bioavailability of other contaminants. Although Cu toxicity was beyond the scope of the present study, it is important to reflect on the mechanisms involved in Cu toxicity, since some interaction might have occurred with As. Speciation analysis (Table S2) showed that only ~21% of Cu was present as Cu^{2+} at 24 °C 32 salinity being the most bioavailable and toxic Cu form. It is known that Cu complexates with organic and inorganic ligands (Hirose, 2006), and that these complexes are generally less bioavailable for uptake by organisms. Several studies have demonstrated the protective effects of organic compounds (e.g. humic acids, dissolved organic matter) on Cu toxicity to early life stages of marine invertebrate species (Lorenzo et al., 2002; Nadella et al., 2009; Rosen et al., 2005, 2008). However, labile copper (Cu^{2+}) concentrations in exposure media cannot exclusively explain toxicity exerted by this element as demonstrated by Brooks et al. (2007), that further suggested that dissolved organic matter (DOM) could have a fundamental role in protecting oyster embryos by competitive inhibition of binding sites for Cu at the cells surface thus reducing its bioavailability. On this basis, it is possible to explain why relatively high Cu concentrations in seawater did not cause significant effects at 0 $\mu g\ L^{-1}$ of As concentration, also consistent with high available DOM in solution (Table S2 i.e. 57% DOM dissolved in solution).

The effect of As on oyster embryos was characterized by an arrest of embryonic development, with the majority of malformations identified as pre-D stages after 24 h embryogenesis. To the best of our knowledge, few studies have assessed the mechanisms involved in As toxicity during marine invertebrates ontogenesis. Early studies by Litchfield and Whiteley (1959) showed that As competitively inhibits phosphate uptake in sea urchin embryos of *S. purpuratus* due to the structural similarity of both molecules, resulting in a negative effect in larval development. These authors also showed that As retarded embryonic development in a concentration dependent manner, and phosphate uptake inhibition led to the development arrest at the blastula stage. More recently, Gaion et al. (2013) investigated the effects of As on several embryonic development stages of *P. lividus* showing that the toxic effects of As^{5+} (100 $\mu g\ L^{-1}$) were more significant at particular stages, namely during development up to the morula stage. Blockage of embryonic development was also described in *C. gigas,* yet at an earlier stage (fertilized egg), but at higher concentrations (10 $mg\ L^{-1}$) (Mamindy-Pajani et al., 2010). Accordingly, results obtained in the present study also showed retarded development of oyster embryos exposed to As, which was further characterized by high frequency of embryos presenting arrested development at the 32-cell stage in embryos exposed to As (personal observations) in line with the above mentioned studies.

The effects of salinity and temperature on embryonic development of bivalve and of other marine invertebrate species have been studied, mostly to define optimum levels for aquaculture rearing purposes. Salinity outside the optimum tolerance window of a given species generally induces lower percentage of normally developed larvae. In *C. gigas* embryos, the optimum salinity range has been proposed to be within salinities 23 and 33 (Coglianese, 1982). The upper salinity threshold has been proposed to be between 30 and 36 by Robert and His (1985), that observed higher percentage of abnormal developed larvae at high salinity (36) compared to that observed between salinities 20–30. While the lower limit of salinity for high rates of embryonic development has been proposed to be at 24 (Gamain et al., 2016). Similarly, our results showed highest percentage of well-developed larvae (D-shape) at highest salinities (28 and 32), and decreasing yields accompanying the decrease of salinity (19 and 24), and no completely developed D-shape larvae at salinity 15. These results illustrate the influence of salinity on the rate of embryonic development, however this pattern showed some variation depending on temperature of exposure (28 and 32 °C).

Temperature as well as salinity can reduce embryonic development speed at suboptimal conditions as shown in *Pinctada imbricata* oysters (O'Connor and Lawler, 2004). Our data showed that embryonic development was not completed at low temperatures (16 and 20 °C) regardless of salinity, and higher rates of well-developed larvae were observed at 24 and 28 °C. Accordingly, Parker et al. (2010) observed similar percentages of D-shape *C. gigas* embryos reared at temperatures ranging between 22 and 30 °C after 48 h incubation, but lower percentages of well-developed D-shape larvae were observed at 18 °C. On the other hand, we observed lower embryonic development at the highest temperature (32 °C) at all salinities tested indicating the upper thermal tolerance limit was surpassed. Generally, higher temperatures increase the rate of development of oyster larvae (His et al., 1989; Dove and O'Conner, 2007; Thiyagarajan et al., 2013). However, our results showed higher frequencies of under developed larvae at 32 °C (pre-D stages) at all tested salinities, indicating that development rates were not enhanced at the highest temperature tested. These results could be related to an interactive effect of background contamination and higher temperature resulting in retarded development.

The bioavailability of contaminants can be influenced by factors other than total concentration. Factors such as salinity, temperature, pH and organic matter content, can alter the toxicity of the media (Knezovich et al., 1981; Fernández and Beiras, 2001). Speciation analysis allowed to infer As predominant chemical forms and bioavailability with varying salinity and temperature, showing only slight changes in As chemical equilibrium with changing physico-chemical conditions (Table S2). Most As was present in all solutions as free hydrogen arsenate ion ($HAsO_4^{2}$) (> 96%), and therefore should be readily available for uptake by oyster embryos. Hence, the effects of varying salinity and temperature on As toxicity were more likely related to environmental factors other than its chemical form.

Salinity is one of the most important factors affecting the toxicity of aquatic media to early stages of marine invertebrates and has been subject of extensive research (Boukadida et al., 2016; Coglianese, 1982; Gamain et al., 2016; MacInnes and Calabrese, 1979). Generally, varying salinity influences the toxicity of aqueous solutions contaminated with metal elements (i.e. Cd, Cr, Cu, Hg, Ni, and Zn) by increasing its toxicity with the decrease salinity, in what is generally considered to be related to greater bioavailability of free metal ions at lower salinities (Hall and Anderson, 1995). However, speciation analysis showed no alterations in As availability among different salinity conditions, and therefore no differences existed on free As in solution, but still higher toxicity of As at lower salinities was observed. In line with these results, studies have shown that the toxicity of elements such as Cu, Ni and Ag with varying salinity do not depend exclusively on their availability as free ions, but also depends on organisms' osmoregulation status as been shown in crustaceans (Leonard et al., 2011; Pinho and Bianchini, 2010). At lower salinities osmoregulation involves increased ion flux between the organism and the surrounding media (Connell, 1989), derived by enhanced active transport processes that can induce higher uptake of labile contaminants, thus influencing organisms' susceptibility (Grosell

et al., 2007; Leonard et al., 2011). Hence, our results showing higher As toxicity at lower salinities were more likely related to embryos physiological status rather than As availability.

Results on the combined effects of temperature on As toxicity showed that embryo development was less affected at 28 °C, while at the remaining temperatures embryonic development in the presence of As was impaired. Few studies have assessed the influence of temperature on pollutant toxicity to bivalve embryonic stages. Early studies Hrs-Brenko et al. (1977) observed an increased toxicity of Pb in *M. galloprovincialis* larvae at higher temperatures (15–20 °C). These results could be related to higher uptake due to increasing rates of physicochemical reactions and diffusion rates through membranes with increasing temperatures (Hochachka and Somero, 1980; Hazel, 1997). MacInnes and Calabrese (1979) studied *C. virginica* embryos exposed to copper in a range of different temperatures, and observed that Cu toxicity was higher at 20 °C than at 30 °C, and justified their results with the fact that closer to suboptimal temperature (20 °C) induced higher toxicity than 30 °C. Results obtained in the present study also indicate that As was less toxic at a relatively high temperature (28 °C). Hypothetically, the retarding effect of As on embryo development could have been buffered at 28 °C due to higher rates of metabolism and chemical reactions occurring at a higher temperature, but such interaction require further investigation.

The combined effects of varying salinity, temperature and contaminants have been studied to a least extent. In *C. virginica* embryos, salinity interacted with copper by increasing abnormal developed larvae percentages with the decrease of salinity only at high temperature (30 °C) (Machles and Calabrese, 1979). The same authors observed that oysters presented an optimum combination of salinity (26) and temperature (25 °C) where resistance to Cu was higher. Similarly, we observed that *C. gigas* presented increased tolerance to As at a given temperature (28 °C), and at a narrower salinity range.

The presence of As in seawater reduced the range of optimum salinity and temperature at which embryonic development occurred. Implications of such findings are broad, and include impacts at species and ecosystem levels. The retarding effects observed directly influence the duration of planktonic life stage, which further challenges these organisms' life history (i.e. dispersal range, risk of predation, contact with pollutants, food availability, and competition for settlement) (Byrne, 2009, 2012; Pechenik, 1999), and can ultimately lead to carry over effects at population and ecosystem levels. The question on the outcome of complete embryonic development in the range of environmental factors studied remains to be investigated, since most abnormally developed larvae in the present study were identified as pre-D's after 24 h incubation. If As induced complete embryonic development arrest or a retardation yet reversible effect, may be of interest for future research.

4. Conclusions

This study provides new insights on the interactive effects of varying salinity and temperature on the embryotoxicity of As to *C. gigas*. We provide new data on As toxicity, namely in respect to EC_{50} values that were lower than those previously described in literature, and likely resulted from interaction of As with other elements present in the natural seawater used. In this respect, it seems that As can be more toxic when present in coastal waters with background levels of contamination relatively higher than those encountered in open ocean waters. The presence of As ($\leq$ 100 $\mu g\ L^{-1}$) in seawater influenced the range of embryos tolerance to both salinity and temperature, resulting in a reduction of embryonic development to a range of salinities between 24 and 32 and to a temperature of 28 °C. These results reflected the interaction of the retardation effects on embryo development induced by As exposure, and also with different development rates varying with salinity and temperature. These findings have ecological implications because retardation effects of As on early life stage development can lead to carry over effects at the population level, since extending the time of planktonic life stage can impair recruitment output, with further implications on ecosystem functioning. Our results show that in the presence of As, climate change related stressors such as temperature and salinity shifts further constrain *C. gigas* embryonic development to a narrower range of both these physicochemical parameters.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.08.043.

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