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Impact of environmental hypercapnia on fertilization success rate and the early embryonic development of the clam *Limecola balthica* (Bivalvia, Tellinidae) from the southern Baltic Sea – A potential CO_2 leakage case study

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ABSTRACT

Carbon capture and storage technology was developed as a tool to mitigate the increased emissions of carbon dioxide by capture, transportation, injection and storage of CO_2 into subterranean reservoirs. There is, however, a risk of future CO_2 leakage from sub-seabed storage sites to the sea-floor sediments and overlying water, causing a pH decrease. The aim of this study was to assess effects of CO_2-induced seawater acidification on fertilization success and early embryonic development of the sediment-burrowing bivalve *Limecola balthica* L. from the Baltic Sea. Laboratory experiments using a CO_2 enrichment system involved three different pH variants (pH 7.7 as control, pH 7.0 and pH 6.3, both representing environmental hypercapnia). The results showed significant fertilization success reduction under pH 7.0 and 6.3 and development delays at 4 and 9 h post gamete encounter. Several morphological aberrations (cell breakage, cytoplasm leakages, blastomere deformations) in the early embryos at different cleavage stages were observed.

1. Introduction

Carbon dioxide (CO_2) concentration in the atmosphere has increased from the pre-industrial level of 280 ppm to 407 ppm observed nowadays (Tans and Keeling, 2017). According to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC) surface seawater pH decline resulting from increased atmospheric CO_2 concentration may reach as much as 0.1 to 0.3 units over the next century. In this context, Carbon Capture and Storage (CCS) offers an innovative technique aiming to reduce emissions of carbon dioxide from industrially combusted fuels to the atmosphere by interception, concentration and deposition of CO_2 into geological formations (IPCC, 2013). Potential reservoirs can be located in natural, either terrestrial or sub-seabed geological formations (Bouzalakos and Mercedes, 2010). The risk associated with implementation of such techniques includes the environmental safety during injection into the storage place and the risk of leakage within deposition period, due to the cap rocks breakage (Damen et al., 2006; Shaffer, 2010). In case of a leakage from the sub-seabed storage site, an excess of CO_2 will cause a decrease of seawater pH, and thus alter physical, chemical and biological processes in situ (Rastelli et al., 2015; Clements and Hunt, 2017). Recently, the interest in studying effects of high partial CO_2 has increased due to the rising awareness of consequences of potential leakage on benthic biota worldwide (Rodríguez-Romero et al., 2014; Basallote et al., 2015; Borrero-Santiago et al., 2017).

A potential CO_2 storage site in the Polish Exclusive Economic Zone (oil-carrying B3 field) at a water depth of 80 m has been proposed and offers a promising perspective of CCS implementation in the southern Baltic Sea region (for review see: Sliaupa et al., 2012; ECO2, 2014). With ongoing global ocean acidification, the Baltic Sea appears to be more susceptible to increasing levels of carbon dioxide due to its low salinity buffering capacity, especially in the highly productive coastal areas (Müller et al., 2016). The high biomineralization rates of organic matter deposited on the seafloor in this eutrophic water-basin often cause bottom sediment interstitial water pH to decrease below 7.0, particularly in organic-rich, stratified surface sediments in deep areas (Jansson et al., 2013). Formation of seasonal halocline, that reduces vertical water mixing, implies that deeper- and near-bottom waters should not immediately be affected by increased uptake of carbon dioxide from the atmosphere (Väli et al., 2012). On the other hand, a halocline may also act as a natural hindrance for vertical carbon dioxide dispersal, and hence dilution, in case of a potential CO_2 leakage from a

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sub-seabed storage site. Notably, the concentration of carbon dioxide close to a leakage point is estimated to increase up to 20,000 ppm, resulting in a considerable local decrease of the overlying bottom waters pH, in some cases below 5.8 (Herzog et al., 1996). Dispersal models assessing the range of leakage from the already existing CCS storage areas predict gradient decrease of seawater pH on a large scale (km; ECO2, 2014). So far, only few authors have focused their studies in the Baltic Sea area. For instance, Schade et al. (2016) reported altering effects of high $p\mathrm{CO_2}$ on benthic infaunal community composition in a mesocosm experiments. Additionally, Sokołowski et al. (2018) pointed out deleterious impact of potential carbon dioxide leakage from the sub-seabed storage site on behavior and physiology of the Baltic clam *Limecola balthica*. However, the effects of elevated CO_2 concentrations on reproduction of benthic species in the Baltic Sea have received little attention up to the present (Van Colen et al., 2012; Jansson et al., 2013). None of the studies have investigated the effect of seawater acidity within a range reflecting the potential CO_2 leakage from CCS sites, leaving space for a case study. In order to assess effects on reproduction of benthic species, The Baltic clam *Limecola balthica* (Linnaeus, 1758), formerly known as *Macoma balthica* (Sartori, 2016), was used a keystone species. The bivalve has external fertilization; gametes are released through siphons to the water few centimeters above the sediment where they encounter. A spawning female or male in a group stimulates release of gametes by the neighboring clams, inducing broadcast spawning (Caddy, 1967). Fertilization and early larval development of marine invertebrates are usually the most vulnerable life stages (Hamdoun and Epel, 2007; Pörtner and Farrell, 2008; Byrne et al., 2010) and there are numerous environmental stressors in line of disturbances to reproductive success of species with external fertilization (Pörtner and Langenbuch, 2005; Graham et al., 2015). It has been proven that acidic conditions cause depolarization of the cell membrane followed by a rise in intracellular concentrations of calcium [Ca^{2+}] (Buckler and Vaughan-Jones, 1994). Such depolarization affects polarity establishment before initiating the 1st cleavage by disturbing the polarity cues, which further influences interactions between cytoskeleton and cell-to-cell signaling (Ajduk and Zernicka-Goetz, 2016). Additionally, the increase of free Ca^{2+} levels in the cytoplasm was shown to cause a delay of the onset of mitosis in the fertilized eggs of two sea urchin species (*Strongylocentrotus purpuratus* and *Lytechinus variegatus*) preventing the mitotic proteins from activation, which could directly disturb the first cellular division (Wagenaar, 1983). In this context, we draw a hypothesis that in case of a CO_2 leakage, fertilization and early development of *L. balthica* may be impacted due to imposed acidic conditions. Therefore, the aim of this study was to evaluate the effects of seawater acidification on the fertilization success and early developmental stages of a common benthic species *L. balthica* within a pH range which simulates changes in seawater acidity following CO_2 leakage from the potential sub-seabed storage site. Results of the present study may contribute to environmental risk assessment regarding possible CCS implementation in the Southern Baltic Sea.

2. Materials and methods

2.1. Test species collection and initial handling

Individuals of *L. balthica* were collected using a bottom dredge at one coastal site (MW) in the Gulf of Gdansk (southern Baltic Sea), at a water depth of 30 m, in January 2017 (Fig. 1). Surface sediment (top 6 cm) was sampled with a van Veen grab of the catch area 0.1 m^2. Additionally, samples of overlying bottom seawater (ca. 0.2 m above the sea floor) were taken with a 5-dm^3 GoFlo Niskin water sampler to record basic hydrological parameters (salinity, temperature, pH) with a portable WTW Multiset 340i meter ($S = 7.5$, $T = 4.5\,^{\circ}C$, pH = 7.8). Clams were transported in thermostable containers filled with sediment and aerated seawater taken in situ to the laboratory within 2 h after sampling, where individuals of shell size range between 15 and 17 mm (corresponding to individuals 3–4 years old, which is the dominant size class of the local population; Sokołowski, 1999; Jansen et al., 2007) were selected. The bivalves were kept in sediments with overlying seawater, and acclimated from 4.5 °C (bottom water temperature at the sampling site) to 10 °C by increasing the ambient water temperature by 1 ± 0.5 °C daily.

The clams were then packed in moist paper tissue and transported by car at constant temperature of 10 °C from Institute of Oceanography, University of Gdansk (Gdynia, Poland) to NTNU SeaLab (Trondheim, Norway) within 48 h. Upon arrival, 50 bivalves were put into a 10 dm^3 container filled with sediment and aerated water from the sampling site (S = 7.5) at 9.5 °C and fed daily with fresh cultured algae mix (BioTrix, Trondheim) (*Dunaliella tertiolecta* (Butcher, 1959); *Rhodomonas baltica* (Karsten, 1898); *Isochrysis galbana,* (Parke, 1949); in a volumetric proportion of 1:2:4) for a month. Randomly chosen 36 individuals were then taken for the assessment of gonadal development stage according to criteria described by Caddy (1967). During the first week, half of water from the container was exchanged every second day with brackish water (S = 7.2) produced by dilution of water from the nearby fjord (Trondheimsfjorden) (S = 32) with conditioned domestic water to acclimate organisms to laboratory conditions. During the next three weeks, 90% of the water volume was exchanged every third day just prior to feeding time.

2.2. Obtaining eggs and sperm

A thermal-shock-induced spawning method combined with shaking was used to collect eggs and sperm from the remaining *L. balthica* individuals (Stephen and Shetty, 1981; Le et al., 2016). Fourteen clams were taken out of sediment, put into a new container with seawater in darkness at 4 °C overnight. The day after, the bivalves were shaken vigorously by hand for 5 min and then transferred to large PVC troughs filled with 3 cm thick, 10 °C brackish water, allowing a 20 cm space between individuals. The troughs were left then at room temperature to warm up for 1 h. During this time the bivalves were observed carefully and water temperature was monitored continuously. As the water temperature reached 18 °C both males and females started spawning. In order to avoid random fertilization, the spawning females were isolated from the spawning males immediately. The aliquots of sperm and eggs were collected with a Pasteur pipette into separate 5 ml polyethylene collection tubes (one tube for one individual). Eggs from two first spawning females were pooled in a glass beaker. The sample was stirred to ensure homogenized distribution of oocytes and then acclimated back to 10 °C in a temperature-controlled room. Semen suspension was collected from the first three spawning males, homogenized separately and stored at 4 °C to prolong its viability. The sperm of each male was then examined under a light microscope (Nikon E200, magnification 300×) and the semen of two males which showed the highest viability was selected for fertilization bioassays.

2.3. In vitro fertilization and embryonic development bioassays under different pH treatments

A custom-built CO_2 injection system was used to set up three different pH treatments: 7.7 as control conditions as well as 7.0 and 6.3, simulating seawater acidification in a gradient of CO_2 dispersion above the sediment in case of potential leakage from the sub-seabed CCs storage site (Taylor et al., 2014; ECO2, 2014) (Fig. 2). CO_2 from a pressured flask was mixed with air in appropriate proportion in an automatic mixer and distributed to three separate containers which were filled with brackish seawater. Proportion of CO_2 and air in the mixture was adjusted based on calculations in CO2SYS (Pierrot et al., 2006) to ensure that a target seawater pH level in a given treatment was reached within 24 h. The CO_2 mixture pumping into the containers started one day before fertilization and embryonic development bioassays in order to reach the target seawater pH and sustain the

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