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Sub-lethal and lethal toxicities of elevated CO_2 on embryonic, juvenile, and adult stages of marine medaka *Oryzias melastigma*

Changkeun Lee [a, 1], Bong-Oh Kwon [a, 1], Seongjin Hong [b], Junsung Noh [a], Junghyun Lee [a], Jongseong Ryu [c], Seong-Gil Kang [d], Jong Seong Khim [a, *]

[a] School of Earth and Environmental Sciences & Research Institute of Oceanography, Seoul National University, Seoul, Republic of Korea
[b] Department of Ocean Environmental Sciences, Chungnam National University, Daejeon, Republic of Korea
[c] Department of Marine Biotechnology, Anyang University, Ganghwagun, Incheon, Republic of Korea
[d] Korea Research Institute of Ships and Ocean Engineering (KRISO), Daejeon, Republic of Korea

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ABSTRACT

The potential leakage from marine CO_2 storage sites is of increasing concern, but few studies have evaluated the probable adverse effects on marine organisms. Fish, one of the top predators in marine environments, should be an essential representative species used for water column toxicity testing in response to waterborne CO_2 exposure. In the present study, we conducted fish life cycle toxicity tests to fully elucidate CO_2 toxicity mechanism effects. We tested sub-lethal and lethal toxicities of elevated CO_2 concentrations on marine medaka (*Oryzias melastigma*) at different developmental stages. At each developmental stage, the test species was exposed to varying concentrations of gaseous CO_2 (control air, 5%, 10%, 20%, and 30%), with 96 h of exposure at 0–4 d (early stage), 4–8 d (middle stage), and 8–12 d (late stage). Sub-lethal and lethal effects, including early developmental delays, cardiac edema, tail abnormalities, abnormal pigmentation, and mortality were monitored daily during the 14 d exposure period. At the embryonic stage, significant sub-lethal and lethal effects were observed at pH < 6.30. Hypercapnia can cause long-term and/or delayed developmental embryonic problems, even after transfer back to clean seawater. At fish juvenile and adult stages, significant mortality was observed at pH < 5.70, indicating elevated CO_2 exposure might cause various adverse effects, even during short-term exposure periods. It should be noted the early embryonic stage was found more sensitive to CO_2 exposure than other developmental stages of the fish life cycle. Overall, the present study provided baseline information for potential adverse effects of high CO_2 concentration exposure on fish developmental processes at different life cycle stages in marine ecosystems.

1. Introduction

Carbon dioxide capture and storage (CCS) is an alternative technology used to control increasing anthropogenic carbon dioxide (CO_2) in the atmosphere (Pachauri et al., 2014). This technique captures atmospheric CO_2 compresses, and transports it to subseabed storage sites for (semi)-permanent long-term isolation. However, it is possible leakage from maritime CCS sites can elevate pCO_2 concentrations in the water column or in sediment layers (Carroll et al., 2014; De Vries et al., 2013). Consequently, marine organisms in accidental sites or even remote coastal areas might be at extreme risk for the adverse effects from CO_2 leakage.

The primary environmental impact of CO_2 leakage is reportedly a change in seawater chemistry associated with changing waterborne pH (Blackford et al., 2008). Phelps et al. (2015) suggested the change might be slow or negligible in the open ocean due to carbonate buffering and presumed a short-term event (over the course of a day). However, local acidification under pH as low as 5, might occur given certain environmental conditions (e.g. when seawater circulation is limited), which can lead to possible acute adverse effects on aquatic organisms (Auerbach et al., 1997; Caulfield et al., 1997; Payán et al., 2012). Indeed, several studies reported extreme elevated CO_2 concentrations on various marine animals, including fish, bivalves, and polychaetes (Basallote et al., 2012; Lee et al., 2003, 2016). O_2/CO_2 imbalance caused by a rapid increase of

* Corresponding author.
E-mail address: jskocean@snu.ac.kr (J.S. Khim).
[1] These authors contributed equally to this work.

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hydrogen ions in the organism is primarily associated with these physiological effects. This change in body chemistry can disturb acid-base regulation, blood circulation, respiration, and the nervous system of marine organisms, further leading to long-term impacts, including but not limited to reduced growth rates and reproductive problems (Frommel et al., 2013).

The relationship of fish to other organisms is complex viewed through the marine food web, i.e., decimation of fish populations also impacts the entire marine community consequently, it is vital to elucidate acute or chronic effects of elevated CO_2 concentrations on fish. High CO_2 tolerance of adult fish was extensively examined for decades (Basallote et al., 2012; Mu et al., 2015) and results suggested no measureable effects on mortality, even at pH < 6.0 (Lee et al., 2016). Furthermore, Baumann et al. (2012) did not sufficiently demonstrate fish susceptibility to elevated CO_2 concentrations during early life stages. Finally, studies reported fish have various ion exchangers, were more CO_2-tolerant than marine invertebrates which was inconsistent with research demonstrating fish were the most sensitive to elevated waterborne CO_2 concentrations during early developmental stages (Forsgren et al., 2013; Kikkawa et al., 2003).

Fish embryos and larvae are small and exhibit low locomotive capacity. Therefore, early life stages lack the ability to avoid CO_2 plumes. Mu et al. (2015) suggested this developmental period was more CO_2-sensitive than later developmental stages and also more suitable for study than other organisms; therefore fish represent viable test organisms. Furthermore, in fish early life stages, elevated CO_2 concentrations were shown to effect skeletal calcification due to a drop in carbonate availability (Munday et al., 2011).

Marine medaka (*Oryzias melastigma*) have been increasingly used as a model fish species for marine environmental risk assessments (Bo et al., 2011). Mu et al. (2015) was one of only a few studies that addressed sub-lethal and/or lethal effects of elevated CO_2 concentrations on marine medaka. Previously, we demonstrated marine medaka adults were unusually susceptible to relatively high CO_2 concentrations (Lee et al., 2016). Of note, in our previous study, marine medaka were exposed to very high CO_2 concentrations in a late developmental stage following organogenesis, including heart formation, considered a major developmental step (Lee et al., 2016). Since marine medaka take a considerable time to hatch (10–12 d), it has been difficult to determine the short-term effects of ocean acidification.

The objective of our study was to explore the data gaps in the current toxicological profiles related to marine medaka CO_2 exposure during various developmental stages under varying concentrations of exposed CO_2. Specific aims were to: 1) assess acute lethal and sub-lethal effects of elevated CO_2 on marine medaka at different life stages, i.e. embryo, juvenile, and adult; 2) scrutinize acute lethal effects at different embryonic stages of marine medaka, i.e. early–cleavage, segmentation, primary organogenesis, middle–blood circulation, and/or heart development, and late stages–hatching periods by a 4 d exposure to varying CO_2 concentrations under a short-term CO_2 exposure scenario; 3) build a toxicological database on pH levels (control: just seawater; treatments: 5%, 10%, 20%, and 30% CO_2 exposure) that effect various toxicities in marine fish species by compiling previous and present results, as part of a mini-review.

2. Materials and methods

2.1. Rearing of test organisms

Marine medaka (*O. melastigma*) was reared in the Laboratory of Marine Benthic Ecology at Seoul National University (Seoul, Republic of Korea) for over 12 generations, which were initially donated from NeoEnBiz Inc. (Bucheon, Republic of Korea). Continuously cultivated marine medaka were used for the CO_2 exposure tests. The organisms were placed in a glass tank at 26 °C with a light/dark photoperiod of 14 h/10 h, respectively and 35 psu of salinity. The fish were fed brine shrimp and dry flakes once a day until satiation. Collecting all eggs within 3 h following initiation of spawning and fertilization ensured developmental synchronization of embryos. Viable eggs were selected under a dissecting microscope and used for the series of experiments.

2.2. Experimental settings of CO_2 exposure

CO_2 exposure systems applied in the present study were developed in our previous work (Lee et al., 2016). The CO_2 exposure systems, particularly those targeting waterborne pH maintenance during CO_2 exposure with minimal physical disturbance to the test fish species were performed under strict quality assurance and control guidelines (Fig. S1 of Supplementary Materials (S)). The following two systems were used: i) an air-tight box system (indirect exposure) and ii) a glass chamber system (direct exposure), chosen based on the experimental design (Table 1). Under both systems, the target pH in the water column was successfully maintained with minimal variation (< ± 0.1) during CO_2 gas exposure. Dissolved oxygen (DO) was controlled > 6.0 mg L^{-1} and > 80% (ASTM, 2007, 2008) using O_2-balanced air, ensuring standard water quality throughout the exposure period.

The air-tight box system was designed for small-sized test organisms and used for fish embryo toxicity testing. This system employs indirect CO_2 exposure, which prevents direct bubbling of CO_2 gas in the 6-well plate (Fig. S1a). The CO_2 concentration in the air-tight box system was maintained by a continuous supply of air (control) or CO_2 gas mixture (5%, 10%, 20%, and 30% CO_2 balanced with 20% O_2). Two large beakers filled with seawater were placed inside the air-tight box. One beaker was bubbled with the direct injection of air or CO_2 gas mixture. The other beaker (without bubbling gas) was used to monitor pH and DO in the system. Preliminary tests confirmed pH in the test beaker was stabilized by continuously dissolving the gas, reaching targeted CO_2 concentrations within 48 h.

The glass chamber system was used for toxicity testing of marine medaka at juvenile and adult life stages. This spacious chamber system represents direct exposure conditions, which were more suitable for exposure of large-sized fish and the individuals did not appear effected by the vigorous gas flow (Fig. S1b). In this system, the CO_2 gas mixture (5%, 10%, 20%, and 30% CO_2 balanced with 20% O_2) was directly injected into each test chamber containing 2.5 L of filtered seawater. Of note, the preliminary tests confirmed CO_2 and DO in the glass chamber system (i.e. direct exposure) were saturated faster (< 3 h) than the same gases in the air-tight box system (i.e. indirect exposure). The exposure experiments were initiated with a decreasing gas flow to maintain saturation and to minimize possible physical stress on the fish.

Water quality was monitored daily for pH and DO using a pH meter (Orion Star, Thermo Scientific, Waltham, MA) and a YSI multi-parameter meter (Yellow Springs, OH), respectively. Total alkalinity was measured applying the five-pH point titration method at the same time daily (Moosbrugger et al., 1993). Other parameters (total inorganic carbon content, HCO_3^-, CO_3^{2-}, CO_2, pCO_2, saturation state of calcite, and saturation state of aragonite) were calculated using the CO2SYS program (Pierrot et al., 2006) with the dissociation constant reported by Mehrbach et al. (1973), refit following Dickson and Millero (1987), and KSO_4 as described by Dickson (1990) (data refer to Table S1).

The experimental design might influence results, e.g. tank effect on pH values. Therefore, a two-way analysis of variance (2-way