Fish & Shellfish Immunology 49 (2016) 24–33

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

# Effects of ocean acidification on immune responses of the Pacific oyster *Crassostrea gigas*

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ARTICLE INFO

*Article history:*
Received 27 May 2015
Received in revised form
11 December 2015
Accepted 14 December 2015
Available online 17 December 2015

*Keywords:*
Ocean acidification
$pCO_2$
Oyster
Immune response
Antioxidant enzyme

ABSTRACT

Ocean acidification (OA), caused by anthropogenic $CO_2$emissions, has been proposed as one of the greatest threats in marine ecosystems. A growing body of evidence shows that ocean acidification can impact development, survival, growth and physiology of marine calcifiers. In this study, the immune responses of the Pacific oyster *Crassostrea gigas* were investigated after elevated $pCO_2$ exposure for 28 days. The results demonstrated that OA caused an increase of apoptosis and reactive oxygen species (ROS) production in hemocytes. Moreover, elevated $pCO_2$ had an inhibitory effect on some antioxidant enzyme activities and decreased the GSH level in digestive gland. However, the mRNA expression pattern of several immune related genes varied depending on the exposure time and tissues. After exposure to $pCO_2$ at ~2000 ppm for 28 days, the mRNA expressions of almost all tested genes were significantly suppressed in gills and stimulated in hemocytes. Above all, our study demonstrated that elevated $pCO_2$ have a significant impact on the immune systems of the Pacific oyster, which may constitute as a potential threat to increased susceptibility of bivalves to diseases.



## 1. Introduction

Rising atmospheric $CO_2$ has become one of the most critical environmental problems with the development of industries. About 30% of the atmospheric carbon dioxide has diffused into the ocean through the direct chemical exchange [1]. The absorption of $CO_2$ by the seawater results in a net increase in protons ($H^+$) and a reduction in pH, which finally leads to ocean acidification (OA). Presently, the atmospheric global average level of $CO_2$ has reached 400 ppm, and is predicted to increase to 500–1000 ppm by the end of this century [2,3]. It is estimated that the pH of oceanic surface waters will decrease by 0.14–0.35 pH units by the year 2100 [2,4]. OA may have profound and diverse consequences for marine biodiversity and ecosystem functioning [5,6], and poses a serious threat to marine organisms, especially calcifying organisms such as coral, sea urchin and mollusk [7].

Over the past 10 years, accumulating evidence suggests that OA could result in delayed embryonic development [8,9], decreased larval growth [10,11] and increased mortality [12] of many marine mollusks. Moreover, OA stress has also been found to affect many physiological processes, such as calcification [13], energy metabolism [14] and behavior [15,16] of calcifying organisms. However, there is a lack of studies on the effects of OA on other vital processes of marine organisms such as immune and stress responses [17]. To date, only few studies have investigated the impacts of OA stress on immune systems in bivalve, echinoderm and crustacean [18–21], which have highlighted the potential negative effect of reduced seawater pH on the host defense responses of marine invertebrates.

The Pacific oyster *Crassostrea gigas* (Thunberg, 1793) is one of the most important fishery and aquaculture species, which is widely used as animal model for studying the adaptive mechanism to

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http://dx.doi.org/10.1016/j.fsi.2015.12.025
1050-4648/

coastal environment changes [22]. Previous studies have found that OA stress impaired the energy metabolic pathways in oysters, and also retarded the normal growth and development [23,24]. However, the impact of OA on the immune responses of oysters has received little attention so far. In order to understand how the oyster modulates its immune capacity to cope with OA stress, the immune and defensive responses of oyster *C. gigas* from the Yellow Sea (China) were investigated after short-term exposure (4 weeks) to $CO_2$-acidified seawater, hopefully shedding light on the understanding of the potential implications of OA for invertebrate immunological function.

## 2. Materials and methods

### 2.1. Animals and experimental design

Adult Pacific oysters, *C. gigas*, with a shell length of 5–7 cm, were purchased from a local shellfish farm, and acclimated for 2 weeks at 17 °C and 31‰ salinity. During the acclimation period, the oysters were fed three times daily with a commercial algal blend containing the *Chlorella vulgaris* Beij and *Phaeodactylum tricornutum* at a concentration of $1 \times 10^4$ cells $ml^{-1}$.

After the acclimation period, oysters were cultured in seawater continuously bubbled with the ambient air or air–$CO_2$ mixture. Three $CO_2$ levels were used that were representative of the present-day condition and atmospheric $CO_2$ concentrations predicted for the years 2100 (~$pCO_2$ 1000 μatm) and 2250 (~$pCO_2$ 2000 μatm). A total of 180 oysters were randomly divided into nine assigned tanks (30L) with three replicates per treatment. For elevated $pCO_2$ treatments, the target $pCO_2$ levels were achieved through air and $CO_2$ gas flow adjustment system (Qingdao AKB Instrument & Equipment Co. Ltd). For the control group, the ambient air was bubbled into experimental containers. Seawater was changed every other day using pre-bubbled seawater which had been equilibrated previously following the methodology described above.

pH was measured daily with a pH electrode (pH meter PB-10, Sartorius Instruments, Germany) calibrated with National Institute of Standards and Technology standard pH solutions (NIST, USA). Other water parameters (temperature, salinity, total dissolved inorganic carbon) were determined every other day. Total alkalinity (TA) were measured weekly with the method of Haraldsson et al. [25]. Seawater carbonate chemistry parameters (Table 1) were estimated according to the known values of pH and TA levels using the software CO2SYS [26]. For CO2SYS settings, seawater pH constants used the NIST scale from Millero et al. [27], and the $KSO_4^-$ constant was set from Dickson et al. [28] and refitted by Lewis and Wallace [26]. The information of the relevant water chemistry parameters is summarized in Table 1.

After exposure of 7, 14 and 28 days, hemolymph of 6 individuals in each treatment was withdrawn using a 2 ml syringe equipped with a 22G needle from the pericardial cavity. 1.5 ml of hemolymph was collected from each individual and filtered through an 80 μm mesh to eliminate debris. After that, the hemolymph samples were maintained on ice to prevent spontaneous aggregation, and then subjected to the measurement of phagocytic activity and reactive oxygen species (ROS) production. Meanwhile, additional 0.5 ml of hemolymph was withdrawn and centrifuged to collect hemocytes which were used for RNA extraction. In addition, oysters of both control and OA-treated groups were immediately dissected for gills and hepatopancreas, respectively. All the tissues were flash frozen in liquid nitrogen, and stored at −80 °C prior to RNA extraction and antioxidant enzymes assay.

**Table 1**
Measured and calculated seawater chemistry parameters during the experimental exposures. The pH was monitored daily with a pH electrode calibrated with NBS Standard pH solutions, and total alkalinity (TA) was determined weekly. $pCO_2$, total dissolved inorganic carbon (DIC) and $\Omega_{calcite}$ were calculated using CO2SYS software.

| Parameters | Control | Elevated $pCO_2$ exposure | |
|---|---|---|---|
| | | ~1000 ppm | ~2000 ppm |
| Temperature (°C) | 17.4 ± 0.2 | 17.5 ± 0.5 | 17.4 ± 0.3 |
| Salinity (‰) | 31.2 ± 0.5 | 31.3 ± 0.5 | 31.2 ± 0.5 |
| pH (NBS scale) | 8.17 ± 0.02 | 7.80 ± 0.02 | 7.55 ± 0.02 |
| TA (μmol/kg) | 2385.4 ± 33.88 | 2367.2 ± 49.07 | 2369.2 ± 13.09 |
| $pCO_2$ (ppm) | 438.80 ± 28.11 | 1126.66 ± 43.79 | 2062.06 ± 94.69 |
| DIC (μmol/kg) | 2170.54 ± 12.19 | 2298.82 ± 5.09 | 2379.21 ± 5.96 |
| $\Omega_{calcite}$ | 3.98 ± 0.19 | 1.86 ± 0.06 | 1.09 ± 0.04 |

### 2.2. Phagocytic capacities

Phagocytosis was measured by ingestion of fluorescent beads through an *in vitro* assay. Briefly, 500 μL of hemolymph were incubated with 50 μl of a 1/10 dilution of fluorescent latex beads (2.0 μm in diameter, Polysciences Inc.) for 1 h in the dark at ambient temperature. Then the hemocytes were analyzed on the FL1 detector of the flow cytometry (FACSCalibur, BD Biosciences), and phagocytic activity was estimated by the percentage of hemocytes that had ingested three or more beads.

### 2.3. Reactive oxygen species (ROS) production

The non-induced reactive oxygen species (ROS) production was measured using 2′7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Invitrogen). Oxidation of non-fluorescent DCFH-DA to fluorescent product DCFH was used to quantify the production of reactive oxygen species (ROS) by hemocytes.5 μL of DCFH-DA working solution was added to 500 μl of the hemolymph to yield a final concentration of 10 μM. The mixtures were then incubated in the dark at ambient temperature for 15 min prior to flow cytometry analysis.

### 2.4. Apoptosis detection

The apoptosis of hemocytes was detected using the Annexin V–FITC Apoptosis Detection Kit (BioVision, USA) according to the manufacturer's recommendations. Briefly, the hemocytes were washed twice with PBS and re-suspended using 1× Annexin V Binding Buffer at $2–3 \times 10^6$ cells/mL. Then, 5 μl of Annexin V–FITC and 10 μl of Propidium Iodide (PI) Buffer were added to each tube with 100 μl of hemocyte suspension. Next, 400 μl 1× Annexin-V Binding Buffer was added to each tube. Finally, the hemocytes were incubated at room temperature for 15 min in the dark and then analyzed by flow cytometry for fluorescence in the FL-1 (Annexin V) and FL-2 (PI) channels [29].

### 2.5. Quantitative real-time PCR analysis

Total RNA from the tissues of hemocytes, gills and hepatopancreas was extracted using Trizol reagent following the manufacturer's instructions (Invitrogen), respectively. The first-strand cDNA was synthesized according to M-MLV RT Usage information (Promega) using oligo (dT)-adaptor as primer and the DNase I-treated total RNA as template. Expression of selected immune responsive genes was measured in an Applied Biosystems 7500 Real-Time PCR System. Gene-specific primers for heat shock protein 70 (Hsp70, AAD31042.1), heat shock protein 90 (Hsp90, ABS18268.1), extracellular Cu–Zn superoxide dismutase (SOD, EKC39002.1), glutathione peroxidase (GPx, selenium-dependent