Seawater acidification increases copper toxicity: A multi-biomarker approach with a key marine invertebrate, the Pacific Oyster Crassostrea gigas

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ABSTRACT

Ocean acidification (OA) has been found to increase the release of free Cu²⁺ in seawater. However, only a handful of studies have investigated the influence of OA on Cu accumulation and cellular toxicity in bivalve species. In this study, Pacific oysters, Crassostrea gigas, were exposed to 25 μg/L Cu²⁺ at three pH levels (8.1, 7.8 and 7.6) for 14 and 28 days. Physiological and histopathological parameters [(clearance rate (CR), respiration rate (RR), histopathological damage and condition index (CI)), oxidative stress and neurotoxicity biomarkers (superoxide dismutase (SOD) and glutathione transferase (GST) activities, lipid peroxidation (LPO) and acetylcholinesterase (AChE) activity), combined with glycolytic enzyme activities (pyruvate kinase (PK) and hexokinase (HK))] were investigated in C. gigas. The bioconcentration of Cu was increased in soft tissues of Cu-exposed oysters under OA. Our results suggest that both OA and Cu could lead to physiological disturbance, oxidative stress, cellular damage, disturbance in energy metabolism and neurotoxicity in oysters. The inhibited CR, increased glycolytic enzymes activities and decreased CI suggested that the energy metabolism strategy adopted by oysters was not sustainable in the long term. Furthermore, integrated biomarker response (IBR) results found that OA and Cu exposure lead to severe stress to oysters, and co-exposure was the most stressful condition. Results from this study highlight the need to include OA in future environmental assessments of pollutants and hazardous materials to better elucidate the risks of those environmental perturbations.

1. Introduction

Over the last two centuries, anthropogenic activities such as fossil fuel combustion and deforestation have released significant amounts of anthropogenic carbon dioxide (CO₂) into the atmosphere (Forster et al., 2007). Atmospheric CO₂ concentrations have increased at a rapid rate from 280 ppm in preindustrial times to 390 ppm in 2011, and the average concentrations of CO₂ in the atmosphere has recently surpassed 400 ppm (Intergovernmental Panel on Climate Change, 2014). A third of all anthropogenic CO₂ emissions have been absorbed by the oceans, leading to ocean acidification (OA) (Sabine et al., 2004). Seawater pH has decreased by 0.1 units since the Industrial Revolution and could decrease by a further 0.3-0.4 units by the end of this century based on current and projected future CO₂ emissions (Caldeira and Wickett, 2003). OA is projected to become more rapid and sustained as atmospheric CO₂ continues to rise (Caldeira and Wickett, 2005). Furthermore, reduced availability of carbonate ions (CO₃²⁻) and cellular acid-base homeostasis disturbance caused by OA could have negative effects on marine invertebrates, especially calcified bivalves (Gazeau et al., 2007, 2013; Dupont and Pörtner, 2013). In recent studies, OA has proven to have negative effects on calcification, respiration rate, clearance rate, acid-base regulation, immune response and overall survival of shelled mollusks (Pörtner et al., 2004; Dupont and Thornryde, 2009; Gazeau et al., 2013; Sui et al., 2015; Liu et al., 2016; Zhao et al., 2017; Castillo et al., 2017).

In addition to OA, estuaries and coastal areas are constantly influenced by trace metal pollution (Riba et al., 2004; Gao et al., 2014). As a common metal pollutant in coastal and estuaries, dissolved Cu levels in...
offshore marine waters of China range from 0.1 μg/L to 43.2 μg/L (Jin et al., 2015). Copper contamination in aquatic systems usually results from anthropogenic activities such as smelting, mining, domestic waste, and industrial processes (plating, steelworks and refineries). Although copper is an essential metal in organisms, toxicity will occur if the accumulation exceeds levels required for physiological functions (Flemming and Trevors, 1989). In particular, marine bivalves accumulate and concentrate high levels of trace metal pollutants within their tissues due to their filter-feeding lifestyle, which makes them susceptible to toxicity caused by Cu pollution. Cu has been demonstrated to induce biochemical alterations in bivalves, such as oxidative stress (Jing et al., 2006; Zhang et al., 2010; Gomes et al., 2012; Feng et al., 2015). It thus poses deleterious impacts on growth, reproduction, immune functions, behavior, and overall survival (Myint and Tyler, 2015). It thus poses deleterious impacts on growth, reproduction, immune functions, behavior, and overall survival (Myint and Tyler, 2015).

Estuaries and coastal areas are highly stressful environments. Multiple environmental stressors including increased temperature, ocean acidification, hypoxia and trace metal pollution occur in coastal and estuarine waters. Despite this, most of the studies focus on the action of a single stressor on organisms, while the combined effects of multiple stressors are less studied (Gunderson et al., 2016). Decrease in concentration of OH– and CO32− ions can affect the solubility, adsorption, toxicity, and rates of redox processes of metals in seawater (Byrne et al., 1988; Millero et al., 2009; Wilde et al., 2006). Future studies are needed to examine how pH affects the interactions of metals complexed to organic ligands and with marine organisms (Millero et al., 2009). Previous studies have found increased cadmium accumulation when pH decreases in Mytilus edulis, Tegillarca graminea, Meretrix meretrix and Crassostrea gigas (Shi et al., 2016; Cao et al., 2018). Furthermore, a previous study has found that further acidification of seawater will change the fate of Cu in aquatic environments, resulting in elevated free Cu2+ in seawater (Millero et al., 2009). It is predicted that the toxic free-ion concentration of copper (Cu2+) will increase by 115% in coastal waters over the next 100 years due to OA (Pascal et al., 2010; Richards et al., 2011). As Cu2+ is the most bioavailable form of copper (Flemming and Trevors, 1989), enhanced release of Cu2+ under conditions of OA might enhance copper toxicity to aquatic organisms. Correspondingly, previous research has observed increased copper toxicity under OA conditions at a pH of 7.71 in two key economic species, mussels (Mytilus edulis) and purple sea urchins (Paracentrotus lividus) (Lewis et al., 2016). In addition, synergistic negative effects of Cu (50 μg/L) and low pH (7.7) on oyster hemocytes were observed by (Huang et al., 2018). Besides, OA has been found to increase copper toxicity to the early life history stages of the polychaete Arenicola marina (Campbell et al., 2014) and to marine bivalves Crassostrea virginica and Mercenaria mercenaria (Götzte et al., 2014). Dorey et al. (2018) has found that although the development of the sea urchin Heliocidaris crassispina from Hong Kong is robust to ocean acidification and copper contamination, sublethal effects such as reduced body size were observed as a result of both lowered pH and added copper. Even though some studies investigated the combined effects that Cu and OA posed on marine organisms, they mainly focus on single or several biomarker results. Due to the variation in mechanisms involved in the organism, when they are exposed to the pollutants, the interpretation of findings could be made more comprehensive if results from multiple biomarkers from different levels were combined to estimate the overall impact of environmental perturbation on the selected organism (Beliaeff and Burgeot, 2002). Thus, the integrated biomarker response (IBR), as an indicator of environmental stress, was adopted in this study for better assessment of the ecological risk posed by Cu and/or OA.

As economic and ecological important bivalve species in coastal and estuaries areas, the Pacific oyster Crassostrea gigas (C. gigas) has been extensively used in ecotoxicological assessment of environmental perturbations (Behrens et al., 2016; Devos et al., 2015; Dineshram et al., 2016; Haberkorn et al., 2014; Luna-Acosta et al., 2017; Moreira et al., 2016; Xie et al., 2016). Therefore, the main goal of this study was to determine whether OA aggravates the toxicity of Cu on the C. gigas. In the present study, we mainly focused on the physiological responses, biochemical performance and bioconcentration capacity of C. gigas, under combined exposure to Cu and decreased pH. Thus, physiological and histopathological parameters including clearance rate (CR), respiration rate (RR), condition index (CI) and histopathological damage were tested in oysters. CI was widely used as an indicator of the physiological status of the mussels (Luna-Acosta et al., 2017; Tejeda-Vera et al., 2007). In addition, CR and RR represent key parameters in the physiology of suspension-feeding bivalves and are mostly likely to reflect toxic effects caused by environmental perturbations (Li et al., 2002; Echevarría et al., 2012; Chandurvelan et al., 2012; Gilroy et al., 2016; Hu et al., 2017; Sui et al., 2016; Xu et al., 2016; Basti et al., 2016; Ong et al., 2017). Reactive oxygen species (ROS) could be generated in marine organism under stressful environmental conditions, and excessive ROS production could lead to oxidative stress, causing damage to tissues and cellular components in marine organisms (V Valavanidis et al., 2006). Thus, the role of antioxidant systems and their sensitivity can be of great importance in environmental toxicology studies. The measurement of the oxidative stress-related biomarkers has been widely used in ecotoxicological assessment of environmental stressors (Canesi et al., 2010; Matozzo et al., 2013; Ferreira et al., 2015a; 2015b; Duarte et al., 2017; Nardi et al., 2017; Oliveira et al., 2017; Valerio-Garcia et al., 2017; Nardi et al., 2018). Therefore, oxidative stress biomarkers combined with glycolytic enzyme including pyruvate kinase (PK) and hexokinase (HK) activity were measured in oysters. Meanwhile, the accumulation of Cu in the soft tissues of oysters was also investigated. Additionally, the biological responses in oysters were assessed based on the integrated biomarker response (IBR) to obtain an overview of the impact of OA and/or Cu on selected organisms.

2. Materials and methods

2.1. Experimental conditions

The selected bivalve species, oysters C. gigas (7–9 cm shell length) were collected from a local oyster farm from Yantai, Shandong Province of China (37°38’N and 121°59’E; pH 8.10; salinity 31.5%). The collection site had an average water temperature of about 17 °C. Previous research suggested that this place has low background levels of metals and other pollutants (Xie et al., 2016). Organisms were acclimatized in aerated seawater (salinity 31.2 ± 0.2) for 2 weeks at a temperature of 17.3 ± 0.2 °C and a pH of 8.14 ± 0.02. During acclimation, bivalves were fed with commercial algal blend (Anyuan marine technology, Yantai, China) at a concentration of 1 × 10⁴ cells ml⁻¹ and seawater was renewed daily.

After acclimation for two weeks, oysters were exposed to three levels of OA (pH 8.1, pH 7.8 and pH 7.6) and two levels of Cu (no metal addition and 25 μg/L Cu2+) for 28 days. Seawater pH levels were representative of the present-day condition (pH 8.1) and those predicted by the moderate scenarios of IPCC 2007 for the year 2100 (pH 7.8) and the year 2250 (pH 7.6). Trace metal contamination in coastal areas of China are prevalent mainly caused by rapid urbanization and industrialization (Gao et al., 2014; Pan and Wang, 2012; Wang et al., 2013). The concentration of Cu2+ was selected according the environmental concentrations of the coastal areas of Bohai Sea (maximum value of 25.5 μg/L in the waters of Northern Liaodong Bay) (Gao et al., 2014) and achieved by adding CuSO4. The exposure experiment was carried out in 120 L aquaria containing 100 L of sand-filtered and UV-treated seawater. There were approximately 30 individuals in each of the aquaria. Three replicate aquaria were used in each treatment (90 individuals in total for each experimental condition). Seawater bubbled with the atmospheric air was set as control, and OA treatments were bubbled with air-CO2 mixtures that were adjusted through an air and
CO₂ gas flow adjustment system. The simulation of the acidified scenario was achieved by bubbling dry air or a mixture of carbon dioxide and dry air with different but constant percentages to set the pH to the desired value and to maintain the dissolved oxygen (DO) concentration to near saturation (Burrell et al., 2016). After each water change, the tanks were re-dosed with Cu²⁺ to bring the total concentration back to 25 μg/L. Throughout the experiment, bivalves were fed with commercial algal blend (Anyuan marine technology, Yantai, China) at a concentration of 1 × 10⁴ cells mL⁻¹. Seawater was renewed daily using pre-equilibrated seawater throughout the exposure period.

A pH electrode (pH meter PB-10, Sartorius Instruments, Germany) calibrated with NBS standard pH solution was used to monitor the pH daily. Salinity, temperature, and DO were measured every day by using an YSI meter (YSI model 85, Yellow Springs, OH, USA). Total alkalinity (TA) was determined every week by using potentiometric titration (Gran, 1952). The pH and TA was used to calculate other related parameters of carbonate chemistry using the software package CO₂SYS (Lewis et al., 1998). Seawater carbonate chemistry parameters of all treatments are exhibited in Table S1.

At each sampling period (14 days and 28 days), gills and digestive glands of oysters (n = 8) were carefully excised and immediately frozen in liquid nitrogen and stored at -80 °C for subsequent biomarker analysis. In addition, soft tissues of oysters were carefully excised for subsequent copper quantification.

### 2.2. Metal concentration measurements

Seawater samples were filtered using a 0.45 μm nylon filter (Fisher Scientific, Pittsburg, PA), acidified with trace metal grade nitric acid (Fisher Scientific), diluted with 18 mΩ Milli-Q water to minimize salt interference, and analyzed for copper using inductively coupled plasma-mass spectroscopy (ICP-MS, NexION 300X, PerkinElmer, USA). Two blanks were digested simultaneously during each run, which showed negligible contamination. The soft tissues of oysters from each time point were dried to a constant weight. Six individuals from each treatment were sampled at each time point. Afterward, approximately 0.05-0.10 g of dry tissue was digested in concentrated 68% nitric acid and microwave digested at 80 °C for 12 h. MilliQ water was then used to dilute the completely digested solutions, and Cu²⁺ concentration was measured by inductively coupled plasma-mass spectroscopy (ICP-MS, NexION 300X, PerkinElmer, USA). The metal concentration in soft tissues was expressed as μg g⁻¹ dry weight. For quality control of metal analysis, bivalve standard SRM2976 (National Institute of Standards and Technology, USA) and oyster standard 1566b (National Institute of Standards and Technology, USA) were used as reference materials.

### 2.3. Physiological measurements

Clearance rate (CR) of the oysters was measured according to Coughlan (Coughlan, 1969), adapted by Wang et al. (Wang et al., 2015). Three replicates were tested in each treatment, and three oysters were tested in each replicate (n = 9). Briefly, when measuring the clearance rate of the oysters, the flow system was stopped and the water in the tank was static. Before measuring the CR, the oysters were starved for 12 h to empty their guts. After acclimation in the chamber for 30 min, to allow the oysters to open and resume pumping, the microalgae were added to achieve an initial concentration of 2.5 × 10⁴ cells mL⁻¹. Seawater was renewed daily using inductively coupled plasma-mass spectroscopy (ICP-MS, NexION 300X, PerkinElmer, USA). The metal concentration in soft tissues was expressed as μg g⁻¹ dry weight. For quality control of metal analysis, bivalve standard SRM2976 (National Institute of Standards and Technology, USA) and oyster standard 1566b (National Institute of Standards and Technology, USA) were used as reference materials.

### 2.4. Histopathological assay

Oyster gills were processed for histological examination using standard protocols. Gills were fixed in Bouin fixative for 24 h, embedded in paraffin and sections sectioned as 5–7-μm-thick slides using a microtome (Benor, China). Five slides of each sample were deparaffinized in xylene, rehydrated in a graded alcohol series and stained with hematoxylin-eosin (HE) and cover-slipped. Three individuals were sampled in each treatment. For each individual tissue, at least four slides were examined using light microscope equipped with a CCD camera (Olympus X61, Japan). Histopathological alterations are ranked according to the severity of lesions (grades 0 (−), 0.5 (+/−), 1 (+), 2 (++) and 3 (+++) as described in previous studies (Riba et al., 2004). Comparisons of histopathological responses between treatments are expressed as the index of damage, which is obtained from the average of the original semi-quantitative assessment of the lesions.

### 2.5. Oxidative stress markers, AChE activity and glycolytic enzyme activities

For the oxidative stress biomarker measurement, the gills and digestive glands were homogenized in phosphate buffer (50 mM potassium dihydrogen phosphate; 50 mM potassium phosphate dibasic; 1 mM EDTA; pH 7.0) and centrifuged at 10,000 g for 20 min at 4 °C to
obtain the supernatants. Meanwhile, for the measurement of AChE activity, supernatants were obtained by homogenizing the samples in 1:2 (w:v) Tris-HCl buffer (0.1 M Tris-HCl containing 0.1% Triton X-100, pH 7.0) and centrifuged at 10,000 g for 20 min at 4 °C.

The SOD and GST activities were determined as described by Beauchamp and Fridovich (1971) and Habig et al. (1974), respectively. Lipid peroxidation (LPO) level was assayed by measurement of the malondialdehyde (MDA) content, according to Ohkawa et al. (1979). The AChE activity was determined by using the colorimetric method described by Ellman et al. (1961). All enzymatic activities measured in this experiment were related to the total protein concentration of each sample. The protein concentration was determined according to the Bradford method (Bradford, 1976), using bovine γ-globuline as a standard. The activities of HK and PK were determined as described by Greenway and Storey (1999) and the results were expressed as U g⁻¹ of protein.

2.6. Integrated biomarker response

To integrate all measured biomarker responses into one general "stress index", the integrated biomarker response version 2 (IBRv2) was calculated according to Sanchez et al. (2013) as adapted from the integrated biomarker response previously described by Beliaeff and Burgeot (2002). In this study, the IBR was applied to assess the potential toxicity of the different exposure protocols of Cu, OA and their combined effects on C. gigas. The procedure of IBR calculation is briefly described here: individual biomarker data (Xi) were compared to the reference data (X0) and log transformed to reduce variance: Yi = log Xi/X0. The general mean (μ) and standard deviation (s) of each biomarker Yi were computed for all treatments, and Yi was standardized as Zi = (Yi - μ)/s. The biomarker deviation index (A) was calculated by using the mean of the standardized biomarker response (Zi) and the mean of reference biomarker data (Z0): Ai = Zi - Z0. To obtain IBRv2, the absolute values of the A parameters calculated for each biomarker were summed as IBRv2 = Σ[Ai]. Finally, the biomarker deviation index (A) was depicted in a star plot to represent the deviation of each investigated biomarker in OA and/or Cu exposure treatments compared to the control treatment. The area above 0 reflects biomarker induction, and the area below 0 indicates biomarker inhibition.

2.7. Statistical analysis

All response data to OA and Cu treatments were tested using a two-factor nested analysis of variance (ANOVA) with OA and Cu as fixed factors and tanks as replicates. Specimens were nested within tanks. However, because the tank factor was nonsignificant (Table S2), tanks were pooled in subsequent analyses and specimens were used as replicates, hence increasing the power of the analysis. Data were presented as the mean ± standard deviation. Normality and homogeneity of variance among groups was analyzed by performing the Shapiro-Wilk test and Bartlett’s test, respectively. Intergroup differences were assessed through two-way ANOVA with an LSD test. Data were statistically analyzed using SPSS 23.0 (SPSS, Chicago, IL, USA). Differences were considered statistically significant at a p value < 0.05.

3. Results

3.1. Accumulation of Cu in oysters

In this experiment, the accumulated Cu content in oyster soft tissues was exceedingly high after 14 and 28 days of exposure to Cu regardless of pH level (Fig. 1). Meanwhile, the Cu concentration in soft tissues of Cu-exposed oysters greatly increased in response to OA at pH 7.6 at both day 14 and 28. At day 28, Cu accumulation increased significantly in Cu-exposed oysters at pH 7.6 compared with that in individuals at pH 8.1 and pH 7.8.

![Fig. 1. Cu accumulation in soft tissues (μg g⁻¹ dry weight) of C. gigas after exposure to Cu and/or decreased pH for 14 days and 28 days (n = 6). Different letters indicate significant differences among different CO₂ treatments at the same metal exposure conditions (p < 0.05). Asterisks indicate significant difference between non-Cu exposure and Cu exposure at the same pH level (* p < 0.05, ** p < 0.01).](image-url)

3.2. Physiological parameters

In this study, no significant changes in physiological parameters occurred in the control treatment (non-Cu exposure at pH 8.1) throughout the exposure period. Oysters generally showed low sensitivity to OA in the whole-organism physiological parameters at both time points (Fig. 2A–C), except that there is significant increase in CR of non-Cu exposed oysters at pH 7.6 compared with individuals at pH 7.8 at day 14.

A general inhibitory effect on CR, and decrease of CI was caused by a 28-day Cu and OA exposure (Fig. 2A–C). Also, inhibitory effect of Cu on RR was observed at pH 8.1 and pH 7.6 at day 28. A stimulatory effect was found in the CR of Cu-exposed oysters in response to OA at pH 7.8 at day 14 (Fig. 2A). Significant inhibitory effect in CR was observed in Cu-exposed oysters at pH 7.6 compared with non-exposed oysters at the same pH level at day 14. A significant decrease was also observed at pH 7.6 compared with other treatments at day 14 (Fig. 2C). At day 28, physiological parameters including CR and RR were significantly affected by Cu exposure (Table 2). CR decreased significantly under Cu exposure at pH 8.1 and pH 7.6 at day 28 (Fig. 2A). Also, there is a significant decrease in CR in Cu-exposed oysters at pH 7.6 compared with individuals at pH 7.8. Meanwhile, there is a significant decrease in CI in Cu-exposed oysters at pH 7.6 compared with other treatments at day 28 (Fig. 2C). Overall, OA and Cu exposure had interactive effects on the CR and CI in oysters at day 14 (Table 2).

3.3. Histological damage in gills

In the control group, oyster gills showed well-defined lamellae (Fig. 3A). Increased degrees of injury were found in the oyster gills (Fig. 3B–F) in response to Cu and OA, either alone or in combination. Damages including vacuolization, hypertrophy, erosion of cilia and intracytoplasmic inclusions of eosinophilic granules occurred in oyster gills in response to Cu and/or decreased pH. Based on the damage index (Table 1), we concluded that oyster gills generally showed increased damages in response to Cu and/or OA. Cu exposure caused much more severe damage in bivalve gills than OA, and OA severely increased the damage index of Cu-exposed oysters.

3.4. Oxidative stress markers, AChE activity and glycolytic enzyme activities

Independent of the treatment, oyster gills maintained constant AChE activity after 14 days of exposure (Fig. 4A). At the end of the experiment (28 days), the AChE activity was inhibited significantly in
gills at pH 7.6, regardless of Cu exposure, compared with individuals at pH 8.1. Additionally, under Cu exposure, significantly decreased AChE activity was observed in oyster gills at pH 7.8 compared to individuals at pH 8.1. However, Cu exposure led to significantly stimulated AChE activity in oyster gills at pH 8.1 and pH 7.6. From our two-way ANOVA results (Table 2), significant interaction between Cu and OA on AChE activity was observed at day 28. Significantly increased SOD activity in Cu-exposed oysters at pH 7.6 was observed at day 14 and day 28 (Fig. 4B). The SOD activity increased under decreased pH in oyster gills without Cu exposure at day 28. Additionally, Cu significantly stimulated SOD activity in oyster gills at pH 8.1 and pH 7.6 at day 14. Significantly increased GST activity was observed in oyster gills at pH 7.8 without Cu exposure and in Cu-exposed oyster gills at pH 7.8 compared to controls at day 28 (Fig. 4C). At day 14 and day 28, Cu-exposed oyster gills at pH 7.6 showed significantly elevated LPO levels compared to all other treatments (Fig. 4D).

The PK activity increased significantly in Cu-exposed oyster digestive glands at pH 7.8 and pH 7.6 compared to the control at day 14 (Fig. 4E). In addition, PK activity increased significantly in non-Cu exposed oyster digestive glands at pH 7.6 compared to the two other pH levels at day 14. Additionally, PK activity was significantly increased in oyster digestive glands in response to Cu at pH 8.1 and pH 7.8 after 14 days of exposure. There was no alteration of PK activity in oyster digestive glands after 28 days of exposure to Cu and/or decreased pH (Fig. 4E). After 14 days of exposure, non-Cu-exposed oyster digestive glands showed significantly higher HK activity at pH 7.6 compared to the control (Fig. 4F). Furthermore, in digestive glands at day 28, Cu-exposed oysters showed significantly higher HK activity at pH 7.6 compared to controls (Fig. 4F). Overall, significant interaction between OA and Cu was observed in PK activity at day 14 and AChE activity at day 28 (Table 2).

3.5. Integrated biomarker response (IBR)

The transformed data from all biomarkers in oysters after 14 and 28 days of exposure to Cu and/or OA are presented as star plots in Fig. 5A and B. The star plots revealed that generally the measured parameters showed higher responses under co-exposure to Cu and OA than after exposure to each individual stressor in oysters (Fig. 5C). According to the calculated IBR values, the degree of stress of each treatment can be ordered (Fig. 5C). The IBR index increased in response to Cu and/or OA throughout the entire exposure period (14 and 28 days). The co-exposure treatments led to the highest IBR values compared to either individual stressor at day 14 and 28 in oysters. In addition, the IBR index increased in oysters treated by OA and Cu at day 28 compared with day 14, suggesting a time-dependent increase in toxicity caused by OA and Cu on oysters. In addition, a decrease in IBR values was observed in OA-exposed (pH 7.8 and pH 7.6) oysters at day 28 compared with individuals at the same pH level at day 14.

4. Discussion

Previous studies have shown that OA can increase the free ion concentration of Cu in seawater, as Cu$^{2+}$ forms strong complexes with carbonate and will be most strongly affected by the change in pH (Millero et al., 2009). In this study, the accumulated Cu in soft tissues of oysters increased under OA at both day 14 and 28. Elevated Cu accumulation in bivalves under OA can transfer through the food chain, leading to a potential threat to human health (Cheung and Wang, 2008). Furthermore, the increases in bioaccumulation of Cu under OA might contribute to much severer toxic effects in Cu-exposed C. gigas in response to OA. In addition, increased damage index in gills of Cu-exposed oysters in response to decreased pH were observed in this study. This phenomenon is consistent with the increased copper toxicity (damage to DNA and lipids) in mussels (Mytilus edulis) and purple sea urchins (Paracentrotus lividus) (Lewis et al., 2016).

Generally, the CR was suppressed in oysters with OA and Cu exposure, which suggested that energy acquisition was inhibited. Bivalves have high filtering capacity (Au, 2004), and therefore, gill tissues are in continuous contact with seawater and susceptible to pollutants contained therein. We further focused on the histopathological changes in gills of oysters under OA and/or Cu exposure to explore the mechanism behind the inhibited CR. A previous study indicates increased gill damages under metal exposure, which correlates negatively with CR in invertebrates (Gregory et al., 2002). Correspondingly, in this study, the exacerbated CR inhibition of Cu-exposed oysters in response to OA could be attributed to the increased gill damages.

Previous research suggests that metabolic depression is an energy-saving strategy in response to environmental perturbation, but the cost
is reduced aerobic scope for cellular functional capacity, with ultimate consequences for population dynamics (Pörtner, 2002). Thus, the general decrease in RR in Cu-exposed oysters at all pCO2 levels might passively alleviate the stress caused by Cu exposure. By contrast, the increase in PK and HK activities in Cu-exposed oysters at increased pCO2 exposure suggested that oysters adopted an active glycolytic metabolism in response to Cu exposure under decreased pH. Additionally, because oyster RR decreased in response to Cu exposure, we propose that oysters might adopt anaerobic metabolism to partially compensate the energy required by stress response and detoxification. However, this strategy occurs at the expense of overall fitness of this species because energy ingestion decreased as indicated by the inhibited CR in response to the combined exposure to Cu and OA. Furthermore, in an energy metabolism model proposed by Sokolova et al. (2012), pessimum range of energy metabolism refers to where aerobic scope disappears and anaerobic metabolism is engaged to partially cover the energy costs of basal maintenance. In addition, transition to partial anaerobiosis and metabolic rate depression are characteristics of

**Table 1**  
Semi-quantitative results of lesions in gills of oysters exposed to OA and/or Cu for 28 days.

| Parameters                  | pH 8.1  | pH 7.8  | pH 7.6  | pH 8.1 + Cu | pH 7.8 + Cu | pH 7.6 + Cu |
|-----------------------------|---------|---------|---------|-------------|-------------|-------------|
| Gill damages in oysters     |         |         |         |             |             |             |
| Intracytoplasmic inclusions of eosinophilic granules | –       | +/-     | +       | +           | +           | +           |
| Hypertrophy                 | –       | +/-     | +/-     | +/-         | +           | +           |
| Cilia erosion               | –       | +       | +       | +           | +           | +           |
| Cavity enlargement          | –       | +/-     | +/-     | +/-         | +/-         | +/-         |
| Vacuolization               | –       | +/-     | +/-     | +/-         | +/-         | +/-         |
| Index of damages            | 0.500   | 0.600   | 1.000   | 0.900       | 1.000       | 1.400       |

Note: Histopathological alterations are ranked according to the severity of lesions [grades 0 (–), 0.5 (+/–), 1 (+), 2 (+ +) and 3 (+ + +)] as described in previous studies (Riba et al., 2004). Comparisons of histopathological responses between treatments are expressed as the index of damage, which is obtained from the average of the original semi-quantitative assessment of the lesions.
organisms on the pessimum range of energy metabolism under environmental perturbations (Sokolova et al., 2012). As the balance of ATP supply and demand is disrupted in the pessimum range of energy metabolism, populations are unable to survive. Furthermore, the decreased CI observed in Cu-exposed oysters at pH 7.6 could be attributed to the partial anaerobiosis and decreased CR in this treatment. Activation of anaerobic metabolism has also been found in the mussel Mytilus galloprovincialis (L.) during acclimation to elevated temperature (Anestis et al., 2010). Previous studies also suggest that the physiological disturbance and lesions caused by environmental stress can increase overall maintenance costs of organisms (Ferreira et al., 2010). In general, our results suggested that oysters excessively exploited energy to counteract the stress caused by OA and Cu exposure. The decrease in CI of oysters exposed to OA and/or Cu might be associated with inefficiency of the energy budget maintenance. The efficiency of the energy budget is crucial for the health, growth, reproduction, and survival of these organisms because detoxification processes, stress responses and damage repair processes are all energy consuming. In the meantime, the CI reflects organism health status, which can provide a direct assessment of environmental stressors (Luna-Acosta et al., 2017; Soto et al., 2000; Tejeda-Vera et al., 2007). The decrease in CI indicated the health status of oysters was low in response to Cu exposure in OA, which could have ecological repercussions.

AChE is commonly used as a biomarker of neurotoxicity for aquatic organisms in response to pollutants (Rickwood and Galloway, 2004; Frasco et al., 2005; Matozzo et al., 2005). In this study, AChE activity was generally inhibited by OA, regardless of Cu. Previous research observed that CO2 could disrupt basal inhibitory potential on the GABA-A receptor, generating shifts in ionic concentrations of Cl− and HCO3− (Nilsson et al., 2012). This leads to increased GABA release, which can provide a direct assessment of environmental stressors (Anestis et al., 2010). Furthermore, in accordance with our research results, Le Moullac et al. (2016) and Lannig et al. (2010) have found that OA could affect energy metabolism in oysters. In addition, the simultaneous exposure to Cu and moderate hypercapnia (800 μatm) led to energy deficiency in clams (Götze et al., 2014).

In contrast, Cu exposure led to elevated AChE activity in oysters at day 28. The AChE activation observed in this study could be attributed to the accumulation of the substrate acetethylcholine in oyster tissues, leading to receptor overstimulation. A similar phenomenon has been observed in Rhamdia quelen exposed to clomazone, quinclorac and metsulfuronmethyl (dos Santos Miron et al., 2005). Based on the AChE...
activity results, we conclude that Cu and OA could lead to neurotoxic effects in oysters.

Oxidative stress caused by over-production of reactive oxygen species (ROS) is a common pathway of toxicity induced by many classes of chemical pollutants. Copper could interact with hydrogen peroxide through the Harber-Weiss and Fenton-like metal-catalyzed reactions to produce highly toxic hydroxyl radicals, thereby causing oxidative stress (Liu et al., 2011). Meanwhile, OA has been demonstrated to cause oxidative stress by increasing the production of ROS either indirectly, by enhancing the Fenton reaction caused by decreased internal pH, and/or directly, by forming additional free radicals through interactions of CO2 with other ROS (Tomanek et al., 2011). Excessive ROS can be highly toxic to aquatic organisms, causing damage to crucial cellular components including proteins, DNA and membranes lipids (Davies, 2005; Hemnani and Parihar, 1998; Winterbourn, 2008).

Protection against the toxicity of oxyradicals on cellular targets is provided by a complex defense system consisting of low molecular weight scavengers and antioxidant enzymes. Measurements of enzymatic activities for the detoxification of ROS and the degree of LPO have been proposed as biomarkers of oxidative stress in bivalves exposed to different types of environmental stressors (Coppola et al., 2017; Hu et al., 2015; Teixeira et al., 2017; Valerio-García et al., 2017; Velez et al., 2016). SOD catalyzes the conversion of superoxide anions to hydrogen peroxide and oxygen (Halliwell, 1974). GSTs are a major group of phase II detoxification enzymes, which play an antioxidant role by acting as glutathione peroxidase or simply as binding proteins that sequester hydrophobic molecules (Hayes and Strange, 1995). Lipid peroxidation (LPO) is commonly used as an indicator of oxidative damage. In the present study, GSTs and SODs were activated in response to Cu and/or OA in oyster gills. However, the detoxification mechanism

![Fig. 4. AChE activity, antioxidant enzyme activities, LPO level in gills and glycolytic enzyme activities in digestive glands of C. gigas in response to Cu and/or decreased pH. A–AChE; B–SOD; C–GST; D–LPO; E–PK; F–HK. Each bar represents mean ± SD (n = 8). Different letters indicate significant differences among different CO2 treatments at the same metal exposure conditions (p < 0.05). Asterisks indicate significant difference between non-Cu exposure and Cu exposure at the same pH level ("p < 0.05, **p < 0.01).](image-url)
was not sufficient to prevent cellular damage to the gills of oysters exposed to Cu and/or decreased pH, leading to higher LPO levels. In general, our results suggested that the inefficient detoxification system of oyster gills under Cu and acidification exposure, either alone or jointly, led to cellular damage in this tissue. Furthermore, increased cellular damage as suggested by elevated LPO level was observed in Cu-exposed oysters under OA. Similarly, previous studies have also reported increased toxic effects of trace metals on marine organisms, exposed oysters under OA. Similarly, previous studies have also reported increased toxic effects of trace metals on marine organisms, exposed oysters under OA. Moreover, Cu exposure combined with OA was associated with the decreased pH. The results of this study indicate that OA could exacerbate the toxicity caused by Cu in C. gigas, as indicated by increased oxidative stress, cellular damage, neurotoxicity and histopathological damage of gills, combined with disturbed physiological functions and decreased condition index. In addition, the IBR results indicate that co-exposure was the most stressful condition at both time points and co-exposure posed more severe stress to oysters with prolonged exposure. Consider that co-exposure poses the severest stress on oysters under sa hot exposure time in this experiment, we suppose that the toxic effects posed by OA and Cu might have long-term (e.g. years long) ecological repercussions. Thus, long-term experiments on the combined effects of OA and Cu need to be conducted in the future. Furthermore, to assess the environmental risks comprehensively and better preserve biodiversity and environmental health, our results suggest that OA should be considered in future research on the ecotoxicology of existing and emerging hazardous materials on marine species.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2019.03.002.

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