Seawater acidification aggravated cadmium toxicity in the oyster *Crassostrea gigas*: Metal bioaccumulation, subcellular distribution and multiple physiological responses

Ruiwen Cao a,b,c, Yongliang Liu a, Qing Wang a,b, Zhijun Dong a,b, Dinglong Yang a,b, Hui Liu a,b, Wen Ran a,b,c, Yi Qu a,b,c, Jianmin Zhao a,b,⁎

a Muping Coastal Environmental Research Station, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, Shandong 264117, PRC China
b Key Laboratory of Coastal Biology and Biological Resources Utilization, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, Shandong 264003, PR China
c University of Chinese Academy of Sciences, Beijing 100049, PR China

HIGHLIGHTS

• Ocean acidification aggravated the toxicity of environmental relevant concentration of Cd on *C. gigas*.
• OA exacerbated the oxidative stresses, histopathological damage, and apoptosis of Cd-exposed oysters.
• Apoptosis-pathway was generally stimulated in Cd-OA exposed oysters.
• Increased toxicity perhaps associated with the increased accumulation and altered subcellular distribution of Cd.

GRAPHICAL ABSTRACT

ABSTRACT

Mounting evidence has demonstrated the combined effects of ocean acidification (OA) and other environmental stressors on marine organisms. Although metal pollution is widely distributed in coasts and estuaries, the combined effects of OA and metal pollution have received little attention until recent years. In this study, the accumulation and subcellular distribution of cadmium (Cd) and the physiological responses of the oyster *Crassostrea gigas* were investigated after 31 days of exposure to OA and Cd, either alone or in combination. Increased Cd accumulation was found both in gills (about 57% increase at pH 7.8, 22% increase at pH 7.6) and digestive glands (about 38% increase at pH 7.8, 22% increase at pH 7.6) of *C. gigas* under elevated pCO2 exposure. Although a similar total Cd accumulation pattern was seen in oyster gills and digestive glands, a higher partition of Cd in the BIM (biologically inactive metal) fractions of gills (about 60%) was found in Cd-exposed treatments compared to the digestive glands (about 45%), which might correspond to the generally lower toxicity in gills. Moreover, synergistic effects of Cd and OA on the oxidative stresses, histopathological damage, and apoptosis of exposed oysters were observed in this study, which might be explained by significant interactions of these two factors on increased generation of ROS. These findings demonstrated that OA could aggravate the toxicity of metals in marine organisms, with significant implications for coastal benthic ecosystems regarding the widespread metal contamination and the concurrent increase of acidified seawater.

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1. Introduction

Increasing atmospheric CO₂ concentration and the subsequent rise of pCO₂ in seawater lead to decreases in seawater pH, carbonate ion concentration, and saturation states of biologically important calcium carbonate minerals. This net flux process is known as ocean acidification (OA) (Intergovernmental Panel on Climate Change, 2014). According to the “business-as-usual” IS92a scenario (Caldeira and Wickett, 2005), surface ocean pH is expected to decrease by 0.3–0.4 units by the end of this century. In estuaries and coastal regions, further input of acidic freshwater and eutrophication influenced by the nutrient-laden rivers contribute to the high pH variability of these sites (Melzner et al., 2012; Hu and Cai, 2013; Cai et al., 2011). Previous studies have suggested that seawater in coastal ecosystems could reach extremes in pH, sometimes daily, that are often considered to only occur in open ocean systems well into the future (Caldeira and Wickett, 2003; Hofmann et al., 2011; Duarte et al., 2013).

Ocean acidification is becoming a growing concern due to its deleterious effects on marine organisms through both direct (e.g., the disturbance of physiological processes) and indirect (e.g., changes in ecological interactions) effects (Gazeau et al., 2013; Ivanina and Sokolova, 2015; Parker et al., 2013; Kroecker et al., 2010; Wang et al., 2015; Brothers et al., 2016; Schram et al., 2016; Liu et al., 2016; Sui et al., 2015, 2016, 2017; Duquette et al., 2017; Xingguo Zhao et al., 2017). Additionally, OA does not occur in isolation but usually occurs in concert with other environmental stressors, such as global warming, ocean deoxygenation, and metal contamination in coastal areas.

Generally, coastal areas of China face great challenges due to trace metal contamination caused by rapid urbanization and industrialization (Gao et al., 2014; Pan and Wang, 2012; Wang et al., 2013). As very typical environmental stressors occur in estuaries and coastal areas, the combined effects of trace metals and OA exposure on marine organisms have seldom been investigated so far (Ivanina and Sokolova, 2015; Gunderson et al., 2016). Previous studies have suggested that OA could increase the bioaccumulation in marine organisms of certain metals such as copper and cadmium (Götze et al., 2014; Siddiqui and Bielmyer-Fraser, 2015; Ivanina et al., 2016; Shi et al., 2016). Metals that form strong complexes with chloride or are mainly in the free form will not be strongly influenced by a change in pH, but metals that form strong complexes with hydroxide and carbonate will undergo significant changes in speciation as the pH of seawater decreases (Millero et al., 2009). Thus, lowered pH levels have been found to affect the speciation of certain trace metals such as copper by favoring the metal ion, which has been shown to be the most bioavailable and toxic form (Adamu et al., 2013; Tatare et al., 1997; Millero et al., 2009). However, studies have suggested that Cd speciation is independent of pH and pCO₂ (Millero et al., 2009; Stockdale et al., 2016), as Cd forms strong complexes with chloride and decreasing the pH will not change the chloride concentration. Although very little research exists on the combined impacts of OA and Cd, those that do highlight increased Cd bioaccumulation in marine bivalves under OA exposure (Götze et al., 2014; Shi et al., 2016). Shi et al. (2016) suggested that the OA-induced increase in Cd accumulation may have occurred due to the ocean acidification increased the Cd²⁺/Ca²⁺ in the seawater, which in turn increased the Cd influx through Ca channel. The authors also suggested that easier Cd penetration brought by OA induced epithelial damage and hampered Cd exclusion under OA could attribute to the increased Cd accumulation in marine bivalves. Considering the increased bioaccumulation in marine organisms of certain metals such as copper and cadmium at lower pH levels, the combined exposure of marine organisms to OA and trace metals probably exert substantial influences on their physiological functions and further impact population distributions and perhaps even ecosystem services (Nikinmaa, 2013; Roberts et al., 2013; Lewis et al., 2013; Pascal et al., 2010; Zeng et al., 2015; Sampaio et al., 2018). Previous studies have suggested that key physiological functions involved in acid-base regulation, protein turnover and mitochondrial bioenergetics could be affected by both the OA and trace metal exposures (Ivanina and Sokolova, 2015). Besides, OA may also affect the marine food webs by altering predator-prey interactions and the trophic transfer of metals in the food chain. However, due to the scarcity of the published research and its bias towards certain taxonomic groups, our understanding of the degree to which these effects can impact the function and integrity of marine ecosystems is limited (Ivanina and Sokolova, 2015).

Geological or anthropogenic activities such as metal mining, smelting, and fossil fuel combustion have released large amounts of cadmium (Cd) into the environment. Previous investigation reveals that typical Cd concentrations in coastal areas around the Bohai Sea are >0.1 μg/L, with a maximum value of 5 μg/L in the waters of Jinzhou Bay (Gao et al., 2014). Cadmium is not an essential trace element and is highly toxic to living systems (Das et al., 2014). Excessive Cd accumulation could result in the over production of reactive oxygen species (ROS) creating an imbalance that leads to oxidative stress. If the antioxidant system is overwhelmed, the irreversible damage of cells caused by excessive ROS may initiate programmed cell death (apoptosis) and lead to pathological damage of tissues (Maças-Mayorga et al., 2015; Rocha et al., 2016; Wang et al., 2012; Xu et al., 2016). Also, exacerbated immunosuppression and oxidative stress of Cd under decreased pH were observed in marine bivalves (Ivanina et al., 2014; Liu et al., 2016; Nardi et al., 2018).

After the uptake of metals by bivalves, some metals were excreted, and the residual metals were distributed into different subcellular parts of the cells. Generally, subcellular distribution of metals has been classified into five components (Wallace, 1998): metal-rich granules (MRG), nuclei and cellular debris (CD), organelles and particulate fraction (ORG), metallothionein-like protein supernatant (MTLP) fractions, and heat-denaturable fraction (HDF). The BAM-BIM model has been widely used in the assessment of toxicity caused by metal metabolism (Redeker and Blust, 2004; Wallace et al., 2003; Campana et al., 2015; Eckmanns et al., 2012). Here, the biologically active metal (BAM) was the trace metal Cd distributed in ORG and the HDF fraction. In contrast, the Cd in MRG and MTLP fraction was expected to be a biologically inactive metal (BIM), since Cd was sequestered and therefore not able to cause toxicity (Redeker and Blust, 2004).

_Crassostrea gigas_ is a benthic suspension feeder but a successful colonizer of intertidal estuaries, which plays important roles in community structure and ecosystem functioning (Dumbauld et al., 2009), and has been widely used in ecotoxicology assessment of environmental stressors (Haberkorn et al., 2014; Devos et al., 2015; Dineshram et al., 2016; Behrens et al., 2016; Moreira et al., 2016; Xie et al., 2016; Luna-Acosta et al., 2011, 2017). In this study, Pacific oysters _C. gigas_ were exposed to Cd and OA, alone and in combination, to assess the influence of OA on the toxicity of Cd exposure. Physiological parameters including antioxidant enzyme responses, histopathological changes and apoptosis were examined in both gills and digestive glands. The underlying mechanisms of elevated pCO₂ on the toxicity of Cd were also investigated through quantification of the accumulation and subcellular distribution of Cd in both gills and digestive glands.

2. Materials and methods

2.1. Experimental design

Pacific oysters _Crassostrea gigas_ were obtained from a local oyster farm from Yantai, Shandong Provinces of China (37°38′N and 121°59′E). Previous research suggested that this place is with low background levels of metals and other pollutants (Gao et al., 2014; Xie et al., 2016). Animals were acclimated in tanks with natural seawater collected from Muping Coastal Environmental Research Station, Shandong Provinces of China for 2 weeks before commencement of the experiment (salinity at 31.2 ± 0.5, pH at 8.12 ± 0.03 and temperature at 17.6 ± 0.2 °C). The combined effects of pCO₂ and metal Cd exposure
on the C. gigas were investigated by using three levels of pH (pH 8.10, pH 7.80 and pH 7.60) and either no metal addition or 10 μg/L Cd. The concentration of Cd (10 μg/L) was set according to the environmental concentrations of the coastal areas of Bohai Sea (Gao et al., 2014). Seawater Cd concentration was measured by using ICP-MS (ICP-MS, NexION 300X, Perkin Elmer). The starting seawater Cd concentration was about in average at 0.32 ± 0.02 μg/L. About 30 organisms were put in each 120 L glass aquaria containing 100 L of naturally filtered seawater. Three replicate aquaria were used in each treatment. The oysters were exposed in six treatments for 31 days. During the exposure experiment, seawater bubbled with atmospheric air was used as the control, while elevated pCO2 seawater treatments were bubbled with air-CO2 mixtures, which were adjusted through an air and CO2 gas flow adjustment system. The stimulation 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). A commercial algal blend containing Chlorella vulgaris Beij and Phaeodactylum tricornutum was provided as food at a concentration of 1 × 10^4 cells mL^-1. Seawater was renewed every other day using pre-equilibrated seawater during the exposure period. After each water change the tanks were re-dosed with Cd to bring the total concentration back to 10 μg/L. The observed mortality of oysters was lower than 5% throughout the experiment.

The pH was monitored daily with a pH electrode (pH meter PB-10, Sartorius Instruments, Germany) calibrated with NBS standard pH solution. An YSI meter (YSI® model 85, Yellow Springs, OH, USA) was used to measure salinity, temperature and dissolved oxygen (DO) daily. Water samples were collected from each tank every week to determine the total alkalinity (TA). TA was measured with the method of potentiometric titration (Grain, 1952) by an automatic potentiometric titrator (798MPT Titrino, Metrohm, Switzerland). Certified reference material (BW3162) from the National Certified Reference Material Center (Beijing, China) was used for calibration and accuracy assessments in the TA measurements. Other related parameters of the carbonate chemistry were calculated according to known values of pH and TA levels using the software CO2SYS. TA values and measured parameters (temperature, salinity and pH values) were plotted in CO2SYS software with the concentrations of the coastal areas of Bohai Sea (Gao et al., 2014). Sea-water Cd concentration was measured by using ICP-MS (ICP-MS, NexION 300X, Perkin Elmer). The validity of the analytical method was checked periodically by means of standard biological reference material (TORT-2 and DORM-2; CNRC, Ottawa, Canada). The recovery of the metal Cd from the standard reference material was consistently within 10% deviation from the certified values. Concentrations of metal Cd in gills and digestive glands were expressed as μg g^-1 dry weight.

Subcellular fractionation of oyster tissues was conducted following the procedure adapted from Wallace et al. (2003) and Taylor and Maher (2012). Soft tissues of gills and digestive glands were homogenized in cold Tris buffer (20 mM, pH 7.6) and then centrifuged at 1000g for 10 min at 4 °C. The pellet was re-suspended in 1 N NaOH, heated at 60 °C for 1 h, and then centrifuged at 10,000g for 10 min. The resulting pellet from this step comprised the metal-rich granules (MRG), and the supernatant consisted of the nuclei and cellular debris (CD). After that, the supernatant of the first spin was centrifuged at 100,000g for 60 min at 4 °C to obtain the organelle and particulate fraction pellet (ORG). The supernatant obtained from this spin was heated at 80 °C followed by cooling and centrifugation at 50,000g for 10 min at 4 °C to produce the heat-denaturable protein pellet (HDP) and heat-stable proteins or metallothionein-like protein supernatant (MTLP) fractions. These procedures eventually yielded five fractions: MRG, CD, MTLP, HDP and ORG. Each fraction was dried, digested in concentrated (68%) nitric acid, and brought to a constant volume with Milli-Q water. Concentrations of Cd in aliquots were analyzed by ICP-MS as described previously.

### 2.2. Sampling procedure

At the end of the exposure experiment, eight replicates of digestive glands and gills were sampled for each treatment. Digestive glands and gills were carefully excised, thoroughly washed with phosphate buffer (50 mM, pH 7.4), surface dried with tissue paper, and then placed in tubes on ice. About two to three individuals were pooled into each replicate, and aliquots of each pooled tissue were immediately frozen in tubes on ice. About two to three individuals were pooled into each replicate, and aliquots of each pooled tissue were immediately frozen in tubes on ice. After homogenization, aliquots of each pooled tissue were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent biochemical analysis. Approximately 50 mg of digestive glands and gills were carefully excised and stored in RNAlater solution until RNA extraction.

### 2.3. Metal concentration measurements and subcellular fractionation

Six individual samples (gills and digestive glands) were dissected and freeze-dried to constant weight. Afterward, approximately 50 mg of dry tissue was digested in 2 mL of concentrated (68%) nitric acid in a microwave system at 80 °C for 12 h. Milli-Q water was then used to dilute the completely digested solutions. After serial dilution, the final volume was 10 mL for subsequent concentration measurements of Cd by inductively coupled plasma-mass spectrometry (ICP-MS, NexION 300X, Perkin Elmer). The validity of the analytical method was checked periodically by means of standard biological reference material (TORT-2 and DORM-2; CNRC, Ottawa, Canada). The recovery of the metal Cd from the standard reference material was consistently within 10% deviation from the certified values. Concentrations of metal Cd in gills and digestive glands were expressed as μg g^-1 dry weight.

Gills and digestive glands were homogenized with Ultra Turrax IKA T10 basic, Staufen, Germany). Tissues were homogenized (1:2 w:v for gills and 1:4 w:v for digestive glands) in phosphate buffer (50 mM potassium dihydrogen phosphate; 50 mM potassium phosphate dibasic; 1 mM EDTA; pH 7.0). Supernatants were harvested by centrifuging homogenates at 10,000g for 20 min at 4 °C. The resultant supernatants were then subjected to antioxidant enzymes assays and lipid peroxidation (LPO) determination. The manufacturer’s protocols (Jiancheng, Nanjing, China) were followed to perform enzyme activity (CAT, SOD, GST and GPX) assays and LPO determination.

CAT activity was determined according to Aebi (1984) by measuring the decrease in absorbance at 240 nm due to H2O2 decomposition. One unit of CAT activity was defined as the amount of decomposed H2O2 per second per milligram of protein. According to Beauchamp and Fridovich (1971), SOD activity was determined spectrophotometrically at 550 nm. One unit (U) of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the nitroblue tetrazolium chloride

### Table 1

| Parameters | pH 8.1 | pH 7.8 | pH 7.6 | pH 8.1 + Cd | pH 7.8 + Cd | pH 7.6 + Cd |
|------------|-------|-------|-------|------------|------------|------------|
| Temperature (°C) | 17.6 ± 0.4 | 17.3 ± 0.6 | 17.3 ± 0.6 | 17.9 ± 0.5 | 17.8 ± 0.4 | 17.3 ± 0.4 |
| Salinity (%) | 31.2 ± 0.5 | 31.2 ± 0.5 | 31.3 ± 0.5 | 31.2 ± 0.5 | 31.0 ± 0.5 | 31.3 ± 0.5 |
| pH (NBS scale) | 8.15 ± 0.02 | 7.81 ± 0.04 | 7.54 ± 0.04 | 8.17 ± 0.02 | 7.78 ± 0.03 | 7.57 ± 0.02 |
| TA (μmol/kg) | 2257.13 ± 41.14 | 2300.17 ± 45.33 | 2279.39 ± 25.16 | 2275.07 ± 55.08 | 2258.65 ± 33.26 | 2297.44 ± 28.95 |
| DIC (μmol/kg) | 2056.59 ± 9.15 | 2225.05 ± 12.97 | 2291.28 ± 12.29 | 2067.06 ± 4.68 | 2198.27 ± 7.83 | 2300.28 ± 6.12 |
| pCO2 (ppm) | 435.19 ± 23.26 | 1064.74 ± 105.58 | 2038.22 ± 195.56 | 427.460 ± 11.47 | 1143.67 ± 70.70 | 2136.70 ± 139.95 |

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(NBT) photoreduction rate. Glutathione S-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to Habig et al. (1974). Absorbance was measured at 340 nm and the enzymatic activity determined using the extinction coefficient of 9.6 mM cm\(^{-1}\) for 1-chloro-2, 4-dinitrobenzene (CDNB). GPx activity was measured by analyzing the NADPH oxidation at 340 nm (linked to GSSG reduction by excess glutathione reductase) using t-butyl hydroperoxide as substrate, as described by Sies et al. (1979).

All the enzyme activities were expressed in term of units per mg of protein (U/mg), where one unit represented the change in absorbance spectra per milligram protein in 1 min. The LPO level was determined by measuring MDA concentrations spectrophotometrically at OD 532 nm, and the results were expressed as nmol MDA mg\(^{-1}\) of protein. The protein concentration was determined using the Bradford method (Bradford, 1976), using bovine \(\gamma\)-globulin as a standard. All measured parameters in this experiment were normalized for the total protein concentration of each sample.

### 2.5. Histopathology and TUNEL assay

Histopathological specimen examination was performed using standard protocols. Gills and digestive glands were fixed in Bouin's solution for 24 h. The fixed tissues were dehydrated in ascending concentrations of alcohol, embedded in paraffin and then sectioned as 5- to 7-μm-thick slices using a microtome (Benor, China). The sections were deparaffinized in xylene, rehydrated through a series of graded ethanol and stained with hematoxylin and eosin (H&E). Digital images of 10 non-overlapping fields were captured randomly from each section using light microscopy equipped with CCD (Olympus, Tokyo, Japan). Three individual oysters were sampled in each treatment, and at least four slides were examined for each individual.

Detection of DNA fragments in situ using the terminal deoxynucleotidyl transferase DUTP nick end labeling (TUNEL) assay is commonly applied to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis. The method is based on the ability of TdT to label blunt ends of double-stranded DNA to the kit instructions and then treated with 3,3-diaminobenzidine (DAB). The nuclei were counterstained with hematoxylin, and the slides were dehydrated and mounted. Nuclei of apoptosis cells were stained by transparent DAB and negative nuclei were counterstained with hematoxylin. Three sections were analyzed from each sample. Ten non-overlapping fields were randomly selected for each section, and images were captured by a 40 × objective.

### 2.6. RNA extraction and qRT-PCR

Stress-related (HSPT0, HSP90 and MT) and apoptosis-related (p53, Caspase-3, Caspase-2, Caspase-1, Bax1, FADD, Bcl-2, and IAP) genes were selected in this study for qRT-PCR assay. Total RNA from the gills and digestive glands was isolated using the Trizol reagent (Invitrogen, Gaithersburg, MD, USA) following the manufacturer’s protocol. The first-strand cDNA was synthesized according to the M-MLV RT Usage information (Promega, Madison, WI, USA) following the ABI 7500 Real-Time Detection System (Applied Biosystems, Foster City, CA, USA). The comparative Ct method \((2^{-ΔΔCt}\text{method})\) was used to analyze the relative expression level of the candidate genes (Livak and Schmittgen, 2001). The primer sequences (Table S2) is provided in the supporting materials.

### 2.7. Statistical analysis

Raw data were assessed for normality and homogeneity of variances using the Shapiro-Wilk test and Bartlett’s test, respectively. Two-way ANOVA followed by post hoc tests (Fisher’s least significant difference) were used to test the effect of metal Cd and elevated pCO\(_2\) exposure. All factors were treated as fixed and had three levels for pCO\(_2\) (pH 8.10, pH 7.80, and pH 7.60) and two levels for metal exposure (control and Cd-exposed). The analyses were carried out by SPSS (Version 23.0). ANOVA results for all studied traits are given in Supplementary Tables S3 and S4. Unless otherwise indicated, data are represented as the means ± deviation (SD). The differences were considered significant if P < 0.05. Principal component analysis (PCA) was performed using CANOCO 5.0 software (Microcomputer Power Inc., NY, USA) to assess the variability associated with antioxidant biomarkers (GPx, SOD, GST, LPO and CAT).

### 3. Results

#### 3.1. Accumulation and subcellular partitioning of cadmium in oysters

The total concentration and subcellular distribution of Cd in the gills and digestive glands are presented in Fig. 1. In this experiment, the total...
concentration of Cd both in gills and digestive glands was similar at all three levels of pCO2 exposure without Cd exposure (Table S1). As a result, non-Cd-exposed treatment at pH 8.10 was set as the control group, and the subcellular distributions of Cd in non-Cd-exposed treatments under pH 7.80 and pH 7.60 were not investigated in this research. There was little variation in the total concentration of Cd between gills and digestive glands, with the exception of a significantly higher Cd concentration in the digestive glands of the control group than in the gills. Compared to the control group, the total concentrations of Cd in both tissues were significantly higher at all three pH levels, with the highest concentration (150 μg/gdw) in Cd-exposed treatment at pH 7.8. Additionally, in Cd-exposed treatments, there was a slight, but non-significant decreased Cd concentration in both tissues at pH 7.60 compared to the treatment at pH 7.80 (Fig. 1a).

After 31 days of exposure, approximately 60% of Cd was partitioned in the BIM fraction of gill tissues. Accumulated Cd was primarily distributed in the MTLP, CD and MRG fractions in the gills of control (Fig. 2a) and Cd-exposed groups under all levels of pCO2 exposure (Fig. 2b, Fig. 2c and Fig. 2d). At the same time, decreased compartment of Cd in the BIM fraction was seen in the gills of Cd-exposed oysters at pH 7.80 (Fig. 1b). In the digestive glands, the subcellular distribution of Cd appeared somewhat different from the distribution pattern in gills (Fig. 1c and Fig. 2). Most of the accumulated Cd was present in the MTLP and ORG fractions of the digestive glands (Fig. 2e, Fig. 2f, Fig. 2g and Fig. 2h). The almost equal partitioning of Cd between BIM and BAM (~47%) was reflected in an almost equal partitioning of Cd between the MTLP and ORG fractions in Cd-exposed digestive glands at pH 7.6. In addition, the partitioning of Cd in BAM was significantly increased in the digestive glands of Cd-exposed oysters at pH 7.60 (Fig. 1c). In contrast, the partitioning of Cd in BIM was significantly increased in Cd-exposed digestive glands at pH 8.10 (Fig. 1c). As expected, significant Cd-induced expression of MT was observed both in digestive glands and gills (Fig. 7c, Fig. 8c).

3.2. Antioxidant responses and principal component analysis

Generally, basal antioxidant enzyme activities in oyster gills were higher than those in the digestive glands, and OA modulated the responses of antioxidant enzymes to Cd exposure in both tissues (Fig. 3). Acclimation to elevated pCO2 in the absence of Cd did not affect the tested antioxidant activities in digestive glands. However, SOD activities in gills were significantly decreased following 31 days of OA exposure (Fig. 3g), and CAT activities were apparently increased at pH 7.80 compared to other pCO2 treatments (Fig. 3i). Two-way ANOVA analysis results suggested that there is significant interaction between Cd and OA on the enzyme activities of GST, GPx and CAT in oyster gills (Table 3).

In digestive glands, all antioxidant enzyme activities (with the exception of CAT activity) and lipid peroxidation (LPO) levels were significantly elevated under Cd exposure at pH 7.60 compared with other treatments (Fig. 3a, Fig. 3b, Fig. 3d and Fig. 3e). Also, at pH 7.80, GST activity was significantly elevated with Cd-exposed treatment compared with non-Cd-exposed treatment (Fig. 3a). As opposed to the digestive glands, no significant changes in antioxidant responses were observed in the gills of Cd-exposed treatments at pH 8.10 and pH 7.60 (Fig. 3a, Fig. 3b, Fig. 3c and Fig. 3d). Nonetheless, significantly decreased GPx activity was observed in Cd-exposed gills at pH 7.60 (Fig. 3c). Meanwhile, CAT activity in gills was decreased at pH 7.8 with Cd exposure compared to non-Cd-exposure, while the activities of GST and GPx were increased in Cd-exposed gills at pH 7.8. Two-way ANOVA analysis results suggested that there is significant interaction between Cd and OA on the enzyme activities of SOD, LPO, GST and GPx in oyster digestive glands (Table 3).

Principal component analysis (PCA) of the digestive glands validated the discrepancy between the non- and Cd-exposed treatments (Fig. 4a). PC1 and PC2 represented 90.38% of the total variance (PC1, 69.99% and PC2, 20.39%). The analysis showed the cluster of non-Cd-exposed treatments and the separation of Cd-exposed oysters at pH 7.80 and pH 7.60 from the Cd-exposed oysters at pH 8.10. We also found that extreme OA (pH 7.60) and Cd-exposed digestive glands were further influenced by the LPO levels in the PCA analysis. PCA analysis of all antioxidant parameters measured in the gills (Fig. 4b) revealed 80.81% of the total variance (PC1, 54.44% and PC2, 26.37%). The analysis highlighted a cluster of non-Cd-exposed treatments with Cd exposure treatment at pH 8.10 and showed the separation at the PC1 coordinate between parameters measured in the gills of Cd-exposed oysters at pH 7.80 and pH 7.60.
Different antioxidant responses between gills and digestive glands were seen in the PCA of antioxidant parameters in both tissues (Fig. 4c), in which PC1 and PC2 accounted for approximately 79.6% of total variance (PC1, 54.06% and PC2, 25.54%). The first component clearly separated gills from digestive glands in all the treatments, which provided further validation for the discrepancy between the antioxidant responses in gills and digestive glands. Overall, the different LPO level and antioxidant responses implied that the digestive glands suffered more severe oxidative stress than gills under the combination of OA and Cd exposure.

3.3. Histopathological damage and apoptosis assay

In the control group, oyster gills showed well-defined lamellae (Fig. 5a), and digestive glands showed well-preserved digestive tubules (Fig. 5c). After exposure to Cd and OA, either alone or in combination, increased degrees of injury were found both in the gills (Fig. S1b–f) and the digestive glands (Fig. S2b–f). Vacuolization, hypertrophy and intracytoplasmic inclusions of eosinophilic granules were among the most observed damage of gills in Cd- and/or OA-exposed oysters. Digestive glands generally showed increased damage in response to Cd and/or OA exposure, those damage including lipofuscin aggregates, hemocyte infiltration in the connective tissues, necrotic connective tissues, degenerating tubules, disorganized epithelium and epithelium hypertrophy. In general, the histopathological injury was much more severe in digestive glands than in gills, as revealed by higher indexes of damage in digestive glands (Table 2). In both tissues, the indexes of damage measured in Cd-exposed treatment were higher than non-Cd-exposed treatment under each level of pCO2 exposure (Table 2). In addition, from data of Table 2, we observed that elevated pCO2 exposure could aggravate the Cd-induced tissue damage in both tissues, with the highest index of damage occurring in treatment of Cd exposure at pH 7.60.

The TUNEL assay was employed to evaluate the apoptosis induced by Cd and/or OA exposure in gills and digestive glands. After 31 days of exposure, a high number of apoptotic cells (stained in brown) were detected in the gills exposed to Cd at pH 7.80 and pH 7.60 (Fig. 6b and Fig. S3), as well as in digestive glands exposed to Cd at three levels of pCO2 (Fig. 6d and Fig. S4). In addition, highest percentage of apoptotic cells were detected in Cd-exposed oysters at pH 7.60 for both gills and digestive glands (Fig. 6b, Fig. 6d, Fig. S3 and Fig. S4).

3.4. Expression analysis of apoptosis-related genes

The expression profiles of several apoptosis-related genes were quantified to investigate the possible mechanisms of apoptosis caused by OA and/or Cd exposure (Fig. 7 and Fig. 8). In the absence of Cd exposure, elevated pCO2 exposure had no significant effect on the expression of apoptosis-related genes in both gills and digestive glands of C. gigas, with the exception of the p53 gene in gills at pH 7.60 (Fig. 7d). In addition, there were no apparent changes in the expression of FADD, IAP and
Bcl-2 transcripts in all the treatments of both tissues. Meanwhile, the genes (p53, Bax1, caspase-3, caspase-2, and caspase-1) involved in the intrinsic apoptosis pathway were generally over-expressed in Cd-exposed treatments, especially in Cd-exposed gills at pH 7.6 (Fig. 7). In addition, the expression of apoptosis-related genes in the digestive glands displayed a similar yet stronger pattern compared to that of the gills. In the digestive glands of Cd-exposed treatments, the expression of genes involved in the intrinsic apoptosis pathway (p53, Bax1, caspase-3, caspase-2, and caspase-1) was generally up-regulated at pH 7.80 and pH 7.60 (Fig. 8). Two-way ANOVA results were presented in Table 4, with the expression of most genes investigated were significantly affected by Cd in both gills and digestive glands. In addition, the expression of four genes (HSP 70 and p53) were significantly affected by OA in digestive glands, and the expression of two genes (HSP 70 and p53) were significantly affected by OA in gills. Significant interaction between Cd and OA were observed in the expression of gene HSP 70 and gene caspase-2 in oyster digestive glands and gene MT in gills (Table 4).

4. Discussion

4.1. Metal accumulation, subcellular distribution and oxidative stress

Recent studies have suggested that Cd speciation is independent of pH and pCO2 (Millero et al., 2009; Stockdale et al., 2016). Therefore, the interference of OA on the accumulation of Cd found in this study was perhaps due to the influence of OA on the cellular and systemic functions of oysters. Similarly, earlier studies indicated that OA would

| Parameter | pH 8.1 | pH 7.8 | pH 7.6 | pH 8.1 + Cd | pH 7.8 + Cd | pH 7.6 + Cd |
|-----------|--------|--------|--------|-------------|-------------|-------------|
| Gills      |        |        |        |             |             |             |
| Intracytoplasmic inclusions of eosinophilic granules | ± | + | + | + | +++ | +++ |
| Hypertrophy (Hp) | − | − | ± | ++ | +++ | +++ |
| Cilar erosion (CE) | − | − | ± | ± | ± | + |
| Connective alteration (CA) | − | − | − | − | − | + |
| Lamellae alteration (LA) | − | − | − | ± | + | + |
| Vacuolization | ± | + | +++ | + | + | +++ |
| Index of damage | 0.17 | 0.33 | 0.67 | 1.17 | 1.50 | 2.17 |
| Digestive glands |        |        |        |             |             |             |
| Hemocytic infiltration in the connective tissues (HI) | ± | ± | ++ | +++ | +++ | +++ |
| Disorganized epithelium (DE) | ± | ± | ++ | ++ | ++ | + |
| Degenerating tubules (DT) | ± | ± | ++ | ++ | ++ | ++ |
| Necrotic connective tissue (NCT) | ± | ± | ++ | + | + | + |
| Epithelium hypertrophy (EH) | ± | ± | +++ | ± | ± | + |
| Lipofuscin aggregates (LA) | − | − | − | ± | ± | + |
| Index of damage | 0.42 | 0.67 | 1.83 | 1.50 | 1.75 | 2.50 |

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.
increase Cd accumulation in bivalves through increased uptake and reduced exclusion (Shi et al., 2016). The authors suggested that increased uptake of Cd might be attributed to the OA-dependent epithelial damage and the increased Cd$^{2+}$/Ca$^{2+}$ in the seawater. Meanwhile, reduced Cd excretion by OA exposure could also account for the increased Cd accumulation in invertebrates (Logan, 2016). In the meantime, it has been found that OA could lead to the increased gaping rate of mussel *Mytilus edulis*, thus increasing the water filtration and taking on more waterborne pollutants (Logan, 2016). Consequently, we suppose the increased bioaccumulation of Cd in oyster gills at decreased pH could be attributed to the increased filtration rate of oysters under acidified seawater. However, the decreased bioaccumulation of Cd at pH 7.6 compared to pH 7.8 might be associated with histopathological damage (resulting in the low filtration efficiency) caused by the combined exposure to Cd and higher acidified seawater in both oyster gills and digestive glands.

Although similar total Cd accumulation was observed in the gills and digestive glands, OA exhibited significant but different effects on the subcellular partition of Cd in these two tissues. The generally high partition of Cd in the BIM fractions of gills was indeed more pronounced compared to the digestive glands, which correlated with the different toxic sensitivity of these two tissues to Cd exposure. Additionally, of particular interest was that the partitioning of Cd in the BIM fractions was reduced significantly in Cd-exposed gills at pH 7.8. In light of this, the change in subcellular distribution of Cd might ultimately explain the altered antioxidant responses in Cd-exposed gills at pH 7.8. Although SOD and CAT activities were decreased at pH 7.8 with Cd exposure, the increased GST and GPx activities might compensate for this antioxidant impairment, leading to the unchanged LPO level. However, the partitioning patterns of Cd in the BIM and BAM fractions of Cd-exposed gills at pH 8.10 and pH 7.60 were similar to that of non-Cd-exposed gills at pH 8.10. The unchanged subcellular distribution pattern in all the treatments might explain the absence of elevated LPO level in gills, with the exception of Cd-exposed treatment at pH 7.6. However, Hawkins and Sokolova (2017) has recently found that OA could increase Cd accumulation in organelles and potentially Cd toxicity in gills of Eastern oysters *Crassostrea virginica* and the hard shell clams *Mercenaria mercenaria*. We supposed that the difference between results obtained from our study and the previous study might be associated with the different Cd concentration used in different research. After all, the Cd exposure level is much higher in research done by Hawkins and Sokolova (50 μg/L) than that used in this study (10 μg/L).

In this study, the partitioning of Cd in the BAM fraction comprised approximately 40% of the total Cd present in the digestive glands, consistent with the increased oxidative stress in this tissue. Meanwhile, the increased partitioning of Cd in the BAM fraction of oyster digestive glands under OA at pH 7.6 might associated with the high oxidative stress in this treatment. Thus, digestive glands might reduce the toxic effects caused by the high oxidative stress through increased antioxidant enzymes activities and elevated expression of HSPs transcripts. Other mollusks, such as *Tellina deltoidalis* and *Crassostrea hongkongensis*, have also shown a significant correlation between toxicity sensitivity and metal distribution equilibrium in the BAM-BIM fraction (Campana et al., 2015; Liu et al., 2013). These results demonstrated that the difference in subcellular distribution of Cd between oyster gills and digestive glands strongly reflected their capacity to handle Cd overdose and explained their divergence in sensitivity to Cd toxicity.

Two-way ANOVA analysis showed that OA and Cd worked synergistically on most of the oxidative stress-related parameters including the antioxidant enzymes activities and LPO levels in both tissues (Table 3). These results suggested that OA could further aggravate the oxidative stress caused by Cd exposure. The synergistic interaction of OA and Cd on oxidative stress of oysters could be attributed to the above-mentioned altered subcellular distribution of Cd by OA. Besides, previous researches has suggested that OA may represent a prooxidant stressor, as a common consequence of the metabolic and acid–base disturbance in animals (Dean, 2010; Tomanek et al., 2011; Hu et al., 2015; Benedetti et al., 2016; Moreira et al., 2016; Ricevuto et al., 2016; Nardi [Image 117x452 to 487x741].}
et al., 2017, 2018). Since bivalves have a limited capability for pH regulation, thus OA could lead to intracellular acidosis (Tomanek et al., 2011). Such intracellular condition may affect the efficiency of mitochondria negatively by disturbing the electron transport chain, resulting in elevated rates of ROS production, which could be another reason for the synergistic effect of OA and Cd in both tissues. Furthermore, intracellular acidosis can also lead to oxidative stress through Fenton reactions and generation of hydroxyl radicals (Tomanek et al., 2011). Moreover, significant interactions of Cd and OA on increased generation of ROS help explain the synergistic effects of these two factors on the oxidative stresses, histopathological damage, and f.

4.2. Histopathology and apoptosis

In aquatic organisms, gills assume the principal roles in respiration, osmoregulation, and nutrient absorption, while digestive glands bear the crucial biological functions of digestion, metabolic homeostasis regulation and immune defenses. The inflammatory changes observed in oysters under OA and/or Cd exposure could have ecological repercussions because damage to gills and digestive glands could cause malfunction of their biological processes (Au, 2004; Nicholson and Lam, 2005; Krishnakumar et al., 1990). In the present study, interacted impact of OA and Cd on histopathological damage and cell apoptosis of gills and...
digestive glands of oyster *C. gigas* could be attributed to the synergetic effects of these two factors on the oyster oxidative stresses.

In addition, the difference in the degree of damage between gills and digestive glands could be linked to their disparity in toxicity caused by OA and/or Cd exposure. The generally high tissue damage index and percentage of apoptotic cells in digestive glands corresponds to the higher oxidative stress caused by OA and/or Cd exposure. In contrast, the generally lower tissue damage index and less severe apoptosis in gill cells indicated much lower toxic effects of OA and/or Cd on this tissue.

### 4.3. Apoptosis pathway response

Generally, Cd is considered to be a weak genotoxicant in mammals (Hartwig, 1995). However, Cd contamination could induce DNA strand breakage caused by oxidative stress and impair the ability of cells to recover by interfering with DNA repair systems (Pruski and Dixon, 2002). Significantly, the ratio of apoptosis cells in Cd-exposed oysters increased when exposed to elevated pCO2, which could be attributed to the aforementioned synergistic effect of OA and Cd on the elevated production of

![Graphs showing mRNA expression profiles of stress- and apoptosis-related genes in the digestive glands of oysters after elevated pCO2 and Cd exposure.](image-url)

*Fig. 8. The mRNA expression profiles of stress- and apoptosis-related genes in the digestive glands of oysters after elevated pCO2 and Cd exposure. a–HSP70, b–HSP90, c–MT, d–p53, e–Caspase-3, f–Caspase-2, g–Caspase-1, h–Bax1, i–FADD, j–Bcl-2, and k–IAP. Each bar represents the mean ± SD (n = 8). Different letters indicate significant differences among treatments at the same concentration of Cd exposure (P < 0.05). Asterisks indicate significant difference between non-Cd exposure and Cd exposure at the same pH level (*P < 0.05, **P < 0.01).*
Table 3
Two-way ANOVA: effects of elevated pCO2 and Cd exposure on the antioxidant enzymes activities in C. gigas. Significant effects are highlighted in bold.

| Factors/interaction | CO2 | Cd | CO2 × Cd |
|---------------------|-----|----|----------|
| SOD                 | F (2, 42) = 5.80 | F (1, 42) = 1.07 | F (2, 42) = 0.52 |
|                     | P = 0.007        | P = 0.308        | P = 0.599      |
| MDA                 | F (2, 42) = 0.17 | F (1, 42) = 3.46 | F (2, 42) = 1.18 |
|                     | P = 0.841        | P = 0.071        | P = 0.191      |
| GST                 | F (2, 42) = 1.15 | F (1, 42) = 0.01 | F (2, 42) = 4.48 |
|                     | P = 0.328        | P = 0.94         | P = 0.019      |
| GPx                 | F (2, 42) = 3.13 | F (1, 42) = 0.26 | F (2, 42) = 12.47 |
|                     | P = 0.056        | P = 0.611        | P < 0.0001     |
| CAT                 | F (2, 42) = 0.13 | F (1, 42) = 0.01 | F (2, 42) = 5.76 |
|                     | P = 0.882        | P = 0.057        | P = 0.007      |
|                     | F (2, 42) = 1.86 | F (1, 42) = 3.43 | F (2, 42) = 3.09 |
|                     | P = 0.171        | P = 0.072        | P = 0.058      |

Table 4
Two-way ANOVA: effects of elevated pCO2 and Cd exposure on the expression of apoptosis- and stress-related genes in C. gigas. Significant effects are highlighted in bold.

| Factors/interaction | CO2 | Cd | CO2 × Cd |
|---------------------|-----|----|----------|
| HSP70               | F (2, 42) = 5.20 | F (1, 42) = 13.29 | F (2, 42) = 8.42 |
|                     | P = 0.01         | P = 0.001        | P = 0.001      |
| HSP90               | F (2, 42) = 1.15 | F (1, 42) = 11.89 | F (2, 42) = 1.44 |
|                     | P = 0.326        | P = 0.001        | P = 0.249      |
| MT                  | F (2, 42) = 2.24 | F (1, 42) = 56.95 | F (2, 42) = 1.75 |
|                     | P = 0.12         | P = 0.0001       | P = 0.188      |
| p53                 | F (2, 42) = 3.77 | F (1, 42) = 6.14 | F (2, 42) = 0.19 |
|                     | P = 0.033        | P = 0.018        | P = 0.826      |
| Caspase-3           | F (2, 42) = 0.12 | F (1, 42) = 6.64 | F (2, 42) = 0.29 |
|                     | P = 0.891        | P = 0.015        | P = 0.754      |
| Caspase-2           | F (2, 42) = 8.36 | F (1, 42) = 30.01| F (2, 42) = 5.11 |
|                     | P = 0.001        | P = 0.0001       | P = 0.012      |
| Caspase-1           | F (2, 42) = 0.48 | F (1, 42) = 15.39| F (2, 42) = 2.27 |
|                     | P = 0.622        | P = 0.00004      | P = 0.118      |
| Bax1                | F (2, 42) = 4.55 | F (1, 42) = 20.18| F (2, 42) = 1.31 |
|                     | P = 0.019        | P = 0.0001       | P = 0.285      |
| FADD                | F (2, 42) = 1.32 | F (1, 42) = 1.36 | F (2, 42) = 0.13 |
|                     | P = 0.281        | P = 0.352        | P = 0.877      |
| Bcl-2               | F (2, 42) = 1.55 | F (1, 42) = 1.11 | F (2, 42) = 0.16 |
|                     | P = 0.226        | P = 0.299        | P = 0.851      |
| IAP                 | F (2, 42) = 0.05 | F (1, 42) = 0.25 | F (2, 42) = 0.85 |
|                     | P = 0.954        | P = 0.622        | P = 0.435      |
|                     | F (2, 42) = 2.39 | F (1, 42) = 0.83 | F (2, 42) = 2.90 |
|                     | P = 0.107        | P = 0.367        | P = 0.069      |
|                     | F (2, 42) = 2.45 | F (1, 42) = 5.50 | F (2, 42) = 1.32 |
|                     | P = 0.101        | P = 0.025        | P = 0.279      |
| MT                  | F (2, 42) = 4.19 | F (1, 42) = 198.10| F (2, 42) = 3.92 |
|                     | P = 0.022        | P = 0.0001       | P = 0.028      |
| p53                 | F (2, 42) = 6.17 | F (1, 42) = 13.58| F (2, 42) = 1.20 |
|                     | P = 0.005        | P = 0.001        | P = 0.313      |
| Caspase-3           | F (2, 42) = 1.11 | F (1, 42) = 10.41| F (2, 42) = 0.49 |
|                     | P = 0.343        | P = 0.003        | P = 0.617      |
| Caspase-2           | F (2, 42) = 0.75 | F (1, 42) = 0.19 | F (2, 42) = 1.48 |
|                     | P = 0.48         | P = 0.663        | P = 0.244      |
| Caspase-1           | F (2, 42) = 2.48 | F (1, 42) = 6.61 | F (2, 42) = 1.09 |
|                     | P = 0.097        | P = 0.014        | P = 0.346      |
| Bax1                | F (2, 42) = 1.19 | F (1, 42) = 28.15| F (2, 42) = 0.48 |
|                     | P = 0.315        | P = 0.0001       | P = 0.622      |
| FADD                | F (2, 42) = 1.22 | F (1, 42) = 2.78 | F (2, 42) = 0.19 |
|                     | P = 0.306        | P = 0.104        | P = 0.827      |
| Bcl-2               | F (2, 42) = 1.36 | F (1, 42) = 7.38 | F (2, 42) = 0.76 |
|                     | P = 0.271        | P = 0.067        | P = 0.473      |
| IAP                 | F (2, 42) = 0.494| F (1, 42) = 4.47 | F (2, 42) = 0.02 |
|                     | P = 0.614        | P = 0.042        | P = 0.983      |
ROS. Consistent with our results, previous studies have shown that Cd induced apoptosis in the gill cells of Mytilus edulis (Pruski and Dixon, 2002), in oyster hemocytes (Sokolova, 2004) and in hemocytes of blood clams (Shi et al., 2018). Also, OA has been found to lead to apoptosis in hemocytes of Pacific oyster Crassostrea gigas (Wang et al., 2016, 2017) and crustacean Nephrops norvegicus (Hernroth et al., 2012). Wang et al. (2017) revealed that OA could activate the mitochondria-CgsAC pathway of apoptosis in oyster hemocytes. Apoptosis is the primary mode of mollusks to eliminate damaged cells from tissues when exposed to environmental stresses such as trace metals (Romero et al., 2015).

At present, two major apoptosis pathways have been identified clearly in mollusks: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Sokolova, 2009; Kiss, 2010; Terahara and Takahashi, 2008). In the present study, the expression of several genes from both intrinsic and extrinsic apoptosis pathways was evaluated to better understand whether or not the apoptosis pathway was activated in oysters gills and digestive glands following exposure to OA and/or Cd (Fig. 7, Fig. 8 and Fig. S5). FADD works in the extrinsic apoptosis pathway through interaction with the death domain of Fas and initiates the apoptotic program (Eichler et al., 2006). As an initiator caspase, Caspase-2 does not directly activate executioner caspsases but acts via mitochondrial outer membrane permeabilization (MOMP) to induce apoptosis (Guo et al., 2002). It is well known that IAPs typically inhibit apoptosis through interaction with caspases, and Bcl-2 is also an apoptosis inhibitor working on mitochondrial membrane (Budihardjo et al., 1999). Previous studies have shown that the p53 could induce the up-regulation of the pro-apoptotic Bax gene and repress the transcription of anti-apoptotic genes Bcl-2 and Bcl-1 in mammals and invertebrates (Mihara et al., 2003). Caspase-3 and Caspase-1 are two executioner caspases, which play central roles in the execution phase of cell apoptosis (Budihardjo et al., 1999).

In oyster gills and digestive glands, the expression of genes involved in the apoptosis pathway, including p53, Bax1, and Caspase-3, Caspase-2 and Caspase-1, was generally up-regulated. In contrast, no significant change was observed in the expression of the extrinsic pathway-related gene FADD and anti-apoptosis related genes (IAP and Bcl-2). These results indicated that the apoptosis pathway including both extrinsic and intrinsic pathway was activated under Cd exposure, either alone or combined with OA. Numerous studies have suggested that some trace metals could trigger the activation of apoptosis pathway through elevated ROS production (Simon et al., 2000; Wang et al., 2012; Takeyama et al., 2002). Therefore, we assumed that the ROS generated by OA and Cd exposure caused apoptosis in both tissues through the activation of apoptosis pathway.

In general, synergic effects of Cd and OA on the oxidative stresses, histopathological damage, and apoptosis of exposed oysters were observed in this study. A review summarized by Ivanina and Sokolova (2015) suggested that physiological interactions between OA and metals may impact the organisms’ capacity to maintain acid-base homeostasis and reduce the amount of energy available for fitness-related functions such as growth, development and reproduction thereby affecting survival and performance of estuarine populations. Thus, the aggravated Cd toxicity on C. gigas under OA exposure found in this study might make the long-term survival of the population impossible, as more energy was required to compensate for the antitoxic response and damage repair processes at the expenses of decreased energy supplement for fitness-related functions.

4.4. Conclusion

To summarize, our results strongly demonstrate that ocean acidification aggravated the toxicity of environmental relevant concentrations of Cd on C. gigas, as revealed by the synergistic effect of these two factors on antioxidant responses, lipid peroxidation, histopathological damage and apoptosis. Generally, increased toxicity of Cd exposure at elevated pCO₂ could be explained by the increased ROS production, probably due to indirect act of OA on the increased accumulation and changed subcellular distribution of Cd in both tissues and direct act of OA serving as a prooxidant stressor. In addition, our results also suggested that apoptosis pathway in gills and digestive glands might be activated by overwhelmed ROS level in these tissues. The present study shows that adult oysters experienced low health status caused by severe physiological stress in response to OA and/or Cd exposure, although they were able to survive for the duration of this experiment. Our results suggested that the habit of bivalve aquaculture may shrink significantly, considering some of the serious metal pollution of the already turbulent and acidified coastal environment. However, due to the scarcity of the published research on the interacted impact of OA and trace metal on bivalves and its bias towards short-term experiments (notably, four weeks), presently, it is difficult to assess to what degree these interactions are likely to affect the organisms’ fitness and thus survival and performance of marine populations. Thus, further laboratory studies of interacted impacts of metals and OA should focus on long period of exposure or field studies to investigate the trace metal accumulation level, damage and survival rates of bivalves.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.06.126.

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